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# Glucose and glycation in birds: phylogenetic and ecological factors, physiological mechanisms and individual fitness links

Adrián Moreno-Borrallo

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UNIVERSITÉ DE STRASBOURG



École Doctorale  
des Sciences de la Vie  
et de la Santé  
STRASBOURG

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**Glucose and glycation in birds: phylogenetic and ecological factors, physiological mechanisms and individual fitness links**

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## Résumé français

# **Glucose et glycation chez les oiseaux : facteurs explicatifs phylogénétiques et écologiques, mécanismes de régulation et liens avec l'aptitude individuelle**

Parmi les nombreux facteurs qui influencent le processus complexe du vieillissement, on trouve la concentration de glucose dans le sang/plasma. En ce sens, des niveaux de glucose sanguin supérieurs à la normale physiologique, comme ceux enregistrés dans le diabète sucré, favorisent la glycation non enzymatique des protéines (connue sous le nom de réaction de Maillard), laquelle participe ainsi à un vieillissement accéléré dû à la formation de produits finaux de glycation avancée (AGEs). Ceux-ci peuvent conduire à l'athérosclérose ou des dysfonctionnements rénaux chroniques, causés par leur accumulation dans les tissus et leurs effets pro-oxydants et pro-inflammatoires. Cet aspect du processus de vieillissement a cependant été largement négligé du point de vue éco-évolutif, par rapport à d'autres caractéristiques moléculaires telles que l'état oxydatif et la dynamique des télomères. À cet égard, les oiseaux sont de parfaits candidats pour être au centre d'une telle perspective, car ils semblent gérer avec succès des niveaux de glucose sanguin qui sont, en moyenne, deux fois plus élevés que ceux des mammifères, et qui conduiraient au diabète chez ces derniers. À cela s'ajoutent un taux métabolique ajusté à la masse et une température corporelle plus élevées, autant d'éléments qui laisseraient présager une durée de vie plus courte. Cependant, les oiseaux affichent au contraire une durée de vie ajustée à la masse plus longue que celle des mammifères. Bien que les études sur le métabolisme du glucose des oiseaux soient de plus en plus nombreuses, la plupart de nos connaissances sur la glycation des protéines sont basées sur peu de données qui, dans certains cas, ne sont que descriptives et se limitent à un faible nombre d'espèces. En outre, les études sur les effets de la glycation sur la valeur sélective (fitness) des oiseaux sont très rares et les mécanismes moléculaires et cellulaires qui pourraient lier la glycation et la valeur sélective sont absents.



Un autre problème lié à l'étude de la glycation des protéines chez les oiseaux est que la plupart des études réalisées à ce jour ont utilisé des techniques non spécifiques, parfois basées sur des kits développés à l'origine pour les humains. Pour remédier à ce problème, nous avons utilisé une méthode basée sur la chromatographie liquide couplée à la spectrométrie de masse (LC-MS), considérée comme une méthode de référence pour les mesures de glycation. Celle-ci a été précédemment développée par notre équipe sur des échantillons de plasma d'oiseaux. Nous avons également mis en place une stratégie de métabolomique ciblée pour mesurer les AGEs dans le plasma et plusieurs évaluations moléculaires, cellulaires et à l'échelle de l'organisme entier afin de mieux comprendre les mécanismes sous-jacents et les conséquences des niveaux élevés de glucose circulant chez les oiseaux. Ce travail s'inscrit dans une perspective d'écophysiologie évolutive.

Par conséquent, ma thèse vise à générer de nouvelles connaissances sur le glucose et la glycation chez les oiseaux, à travers (i) une approche comparative évaluant les niveaux de glucose et de glycation plasmatiques le long de l'arbre phylogénétique des oiseaux en relation avec la diète et les traits d'histoire de vie, (ii) une étude exploratoire sur son lien avec le valeur sélective dans une population sauvage et (iii) une approche expérimentale en captivité explorant les effets néfastes du glucose, de la glycation et du niveau des AGEs sur le processus de vieillissement des individus.

### **Premier axe**

Afin d'évaluer la variation des niveaux de glucose plasmatique et des taux de glycation des protéines chez les oiseaux, en collaboration avec plusieurs collègues et institutions, nous avons échantillonné de nombreuses espèces, provenant de populations captives et sauvages, dans de nombreux ordres d'oiseaux, rapportant une variation considérable entre les taxons. Au total, nous avons rapporté des valeurs sur 88 espèces réparties dans 22 ordres, incluant divers modes de vie. Cette étude visait à décrypter, par une approche comparative, les liens entre ces paramètres physiologiques le cycle de vie des espèces, ainsi que leur régime alimentaire. Cette étude est discutée dans le contexte de l'hypothèse des syndromes du rythme de vie (POLs), qui suggère que les traits des organismes, principalement le cycle de vie, la physiologie et le comportement, co-évoluent en formant des ensembles de

phénotypes associés appelés syndromes, qui peuvent être classés selon des axes de rythme de vie, l'exemple principal étant le continuum rapide- lent. Ainsi, les organismes ayant une durée de vie plus courte et un rythme de développement plus rapide, par exemple, auraient des métabolismes et des concentrations de métabolites circulants plus élevés (comme le glucose), montrant également moins de résistance à l'accumulation de dommages (comme la glycation). Nous avons également voulu explorer comment les facteurs écologiques auraient pu affecter l'évolution de ces paramètres physiologiques, l'un des plus pertinents à considérer étant la spécialisation alimentaire des espèces, qui devrait être associée à certaines adaptations physiologiques liées à la régulation de métabolites tels que le glucose. Par conséquent, nous avons réalisé des modèles linéaires mixtes bayésiens en contrôlant pour la phylogénie pour déterminer les effets de la diète et des traits d'histoire de vie (masse corporelle, masse de la couvée, durée de vie maximale et temps de développement) sur les niveaux de glycation de l'albumine et du glucose plasmatique. Nous avons également pris en compte les effets des niveaux de glucose pour évaluer la variation du taux de glycation.

Dans cette étude, nous avons confirmé l'association négative précédemment rapportée entre le glucose plasmatique et la masse corporelle, et montré une relation quadratique négative inattendue avec la durée de vie maximale, avec une augmentation de la longévité lorsque la concentration en glucose augmente, ceci jusqu'à un certain niveau. En ce qui concerne la glycation, nous avons constaté que les carnivores terrestres présentaient des niveaux de glycation de l'albumine plus élevés que les omnivores, bien qu'aucune différence significative dans les niveaux de glucose n'ait été constatée entre les groupes de différentes diètes. Ceci est intéressant, car le même schéma n'était pas présent chez les prédateurs aquatiques, ce qui suggère la possibilité d'effets qui pourraient être médiés par les différences de composition en nutriments dans les écosystèmes terrestres par rapport aux écosystèmes aquatiques.

De plus, les taux de glycation de l'albumine et les niveaux de glucose plasmatique ont une relation positive à travers des espèces qui suivent une allométrie, ce qui suggère une plus grande résistance à la glycation pour les espèces ayant un taux de glucose plus élevé.

## Deuxième axe

Un autre point important de la thèse est de déterminer quels sont les effets de la glycation sur la valeur sélective dans les populations d'oiseaux sauvages. Pour cela, nous avons échantillonné des individus dans une population suisse de martinets alpins (*Tachymarptis melba*), un oiseau migrateur à longue vie. Cette population fait l'objet d'une étude à long terme, ce qui nous permet d'avoir accès à l'âge et au sexe des animaux, et de suivre la reproduction et la survie d'une année sur l'autre. Nous avons ainsi mesuré le glucose plasmatique et le taux de glycation de l'albumine avant et après la saison de reproduction, afin d'étudier (i) l'impact de ces marqueurs physiologiques sur la reproduction, (ii) s'il existe des coûts de reproduction qui peuvent être observés à travers les changements de ces valeurs et (iii) comment les niveaux de glucose et de glycation après la reproduction prédisent la survie jusqu'à l'année suivante, aidant à les identifier comme les coûts susmentionnés, et donc comme des médiateurs potentiels d'un compromis entre la reproduction et la survie. La variation du glucose plasmatique et de la glycation avec l'âge, avec une étude transversale, a également été évaluée, en essayant d'explorer les profils démographiques associés. La glycation de l'albumine s'est avérée être associée à l'âge selon une relation en forme de U, avec un seuil d'âge similaire à celui d'autres paramètres précédemment étudiés chez cette espèce (comme des traits reproductifs tels que la taille de la couvée ou des traits morphologiques comme la taille des ailes). Il s'agit de la première tentative d'étude de la glycation de l'albumine en tant qu'indicateur potentiel du vieillissement chez les oiseaux sauvages.

Nous avons constaté une augmentation significative de la concentration en glucose et une diminution du taux de glycation pendant la période de reproduction, ainsi qu'une tendance du glucose à influencer le résultat de la reproduction en augmentant le succès à l'envol, au moins jusqu'à un certain niveau de glucose. Les valeurs post-reproductives étaient toutes les deux liées à la survie à la saison suivante, positivement pour le glucose et négativement pour la glycation, ce qui pourrait représenter un effet du glucose constituant une pléiotropie antagoniste, contribuant à façonner le processus de vieillissement chez cette espèce.

### Troisième axe

Comme axe final de la thèse, une expérience de supplémentation sur une population captive de diamants mandarins (*Taeniopygia guttata*) a été réalisée afin d'établir les effets du glucose, de la glycation et des AGEs sur le vieillissement chez les oiseaux. Pour cela, un ensemble de 90 oiseaux a été maintenu dans des volières extérieures à Strasbourg et trois groupes ont été constitués : un groupe supplémenté avec 50 g/L de glucose dans l'eau de boisson, un autre supplémenté avec du méthylglyoxal, un composé dicarboxylique réactif qui favorise la formation d'AGEs (dilué dans l'eau de boisson à un dosage équivalent à 500 mg/Kg\*jour), et un groupe témoin avec de l'eau plate. Ces oiseaux ont été suivis pendant un an, en enregistrant les événements de mortalité et en faisant l'objet d'un suivi régulier de différents paramètres de vieillissement évalués tous les 3 à 6 mois (en fonction de la mesure). Ces paramètres comprenaient la glycémie, la glycation de l'albumine, divers AGEs et d'autres caractéristiques connues pour varier avec l'âge ou susceptibles de le faire, comme la masse corporelle, le métabolisme, la coloration du bec, le stress oxydant, la longueur des télomères ou les performances de vol. Certains oiseaux ont également été sacrifiés à la fin de l'étude afin d'effectuer des analyses métabolomiques et protéomiques dans les mois à venir pour tenter d'explorer les mécanismes liés aux résultats observés.

Les principaux résultats de cette étude sont les suivants i) un risque de mortalité plus élevé dans le groupe glucose, qui a également montré des taux d'albumine glyquée plus élevés à la fin du traitement, bien que la glycémie n'ait pas été modifiée, ii) une augmentation par les deux traitements de supplémentation du glyoxal et de la CML (carboxyméthyl-lysine, un AGE) plasmatiques, qui montre également une tendance à l'augmentation avec l'âge, iii) une association positive entre le CEL plasmatique (carboxyéthyl-lysine, un autre AGE) et le glyoxal plasmatique et avec le risque de mortalité, iv) une relation positive entre le glucose plasmatique et les d-ROM, un marqueur de dommages oxydatifs et iv) la confirmation de la variation liée à l'âge de certains des paramètres évalués, comme une diminution de la consommation d'O<sub>2</sub> avec l'âge.

## **Conclusions**

Dans l'ensemble, les résultats obtenus dans cette thèse nous aident à approfondir les maigres connaissances actuelles sur l'influence du glucose et de ses produits dérivés, comme les protéines glyquées et les AGEs, sur la valeur sélective et le vieillissement des oiseaux. Une grande variation des quantités de glucose plasmatique et d'albumine glyquée a été observée entre des groupes phylogénétiques jusqu'ici inexplorés. Mon travail identifie ainsi les espèces qui pourraient constituer des cibles prometteuses pour de futures recherches sur les liens glucose - santé. Mes résultats montrent également que les niveaux de glucose et de glycation peuvent être des médiateurs de la valeur sélective individuelle dans la nature, ce qui suggère qu'il s'agit d'indicateurs intéressants à explorer davantage dans les études d'écologie évolutive. Enfin, bien que la glycémie se soit révélée une variable très bien régulée même face à une manipulation expérimentale de la diète chez notre espèce captive (diamant mandarin), nos résultats nous donnent un aperçu des mécanismes sous-jacents potentiels impliqués dans la médiation des effets du glucose sur les taux de survie, les mécanismes liés au vieillissement et la valeur sélective chez les oiseaux.

# Introduction

## Ageing, glucose and birds





## Introduction

### 1. Ageing: a still unresolved problem in biology... and beyond.

Ageing is undoubtedly one of the major problems not only in biology, but in our general life experience. It can be defined as a progressive decline in function of living organisms leading ultimately to death (see e.g. Rose 1991; Kirkwood and Austad 2000). The reason for its existence, as for any other biological trait, can be explored from different philosophical perspectives. As it was laid out by Niko Tinbergen (Tinbergen 1955), in his *“Aims and methods of ethology.”*, one can answer differently the question of “why” depending on what it is meant by “why”. He famously proposed two main “classification systems” for the ways of answering those “why”: the one based on temporal range, i.e. current versus historical reasons, and the one based on the *kind of cause*: proximate versus ultimate, i.e. the *how* and the *why*. Here, the proximate causes are the physicochemical and cellular or tissue processes that directly (here and now, as in regular physiological processes) or indirectly (by the means of developmental processes in the wider context of ontogeny) produce an outcome, while the ultimate causes are the reasons why the trait itself came to be as a “conceptual thing”, either directly (what is the current function of the trait, if there is any, that keeps it into existence by the means of natural selection or other biological processes) or indirectly (evolutionary history of the trait, that brought it into its current state, potentially not linked to its current *raison d’être*).

This thesis aims to somehow bridge these two (proximal and ultimate) explanatory forms for the process of ageing, in the context of a very specific trait: glycaemia and its consequences (mainly glycation), in birds. For this, I will sequentially discuss here the following topics: firstly, I will lay out the main theories for the evolution of ageing, followed by a general description of the underlying mechanisms that provoke it; secondly, I will explain why birds constitute an interesting group to study ageing, notably by comparing them with other organisms, in particular with mammals; thirdly, I will dive into the reasons why sugars are relevant into the ageing process: how sugars’ concentration is regulated, and what happens when it is not (adequately), and therefore pathology ensues. In this part, I will make a special emphasis on protein glycation and Advanced Glycation End-products (AGE) as mediators of ageing and ageing-related pathologies. Finally, an introduction on previous studies about them on birds and a justification of the methods employed here to address this issue will be deployed.

#### 1.1. Really, why do we age?

*“Nothing in biology makes sense except in the light of evolution.”*

Theodosius Dobzhansky. 1973\*

\*Initially stated as “[...] nothing makes sense in biology except in the light of evolution, *sub specie evolutionis*.” in Dobzhansky 1964. Note that Dobzhansky meant to defend with this sentence a theistic evolution argument (which is not the view supported here) used in his more known 1973 publication as an answer to the many creationists that intended to eliminate evolution from education programs in the USA (see Giaimo 2023).



### 1.1.1. First hints into the evolution of ageing

Ageing is an issue that has worried humanity since antiquity, with Aristotle explaining it as a consequence of the progressive loss of the innate heat and moisture of the body, ideas later associated with the Hippocratic theories of the humours, that were later developed by Galen and medieval Arab thinkers as Avicenna (Gilleard 2015). Attempts to answer this question in the framework of the current understanding of biology, i.e. Darwinian evolution, have existed since at least more than a century. Several theories have emerged, some non-exclusive among them, but sometimes with very different perspectives. We can divide them into two main categories: those that propose that ageing is adaptive, normally considering the issue from some sort of group selection perspective, and those that suggest it is maladaptive, but natural selection cannot eliminate it. The ones proposing it is adaptive are today not well accepted, at least in cases for which the theory intended to be general, but this approach may work in some cases, being only applicable to some particular species or conditions.

The first of these “modern” theories of ageing was proposed late in the XIX<sup>th</sup> century (somewhen between 1865 and 1870) by Alfred Russel Wallace (mentioned in an editor footnote in page 23 of Weismann 1889), and further developed by August Weismann, as an adaptive mechanism directed to eliminate old individuals from the population, leaving more resources available for younger descendants (Weismann 1882, 1892, the first translated to English and collected with other essays in Weismann 1889, second edition in 1891). This theory was somehow based on a particular kind of group selection arguments that were strongly criticized and became very controversial later on the 60s and 70s of the XX<sup>th</sup> century, and was therefore doomed to rejection.

### 1.1.2. Current evolutionary theories of ageing

We had to wait until the mid XX<sup>th</sup> century to see the first evolutionary theories of ageing that are still valid today. During the first half of the century, it became apparent that the strength of natural selection would decline with age. For example, Bidder (1932) already hinted that selection would favour patterns of growth and development such that after a certain age, once reproduction had been assured, any decay in functioning would not be of importance. A version of this idea was further developed and popularized by Peter Medawar (1946), known as the mutation accumulation theory, based on the supposition that organisms in the wild, due to extrinsic causes of mortality, as predation or inclement weather, would not normally live enough to show the age-related decline in performance that we observe in humans or captive animals, and thus natural selection would not be able to eliminate such late-onset pathologies linked to senescent state, leading to the accumulation of deleterious outcomes only becoming relevant late in life. The second biggest contribution to the evolutionary understanding of ageing was done by George C. Williams (Williams 1957), with the later contribution in developing the mathematical structure of this theory by William D. Hamilton (Hamilton 1966). Williams developed the antagonistic pleiotropy theory, based on the idea that some genes with multiple effects (i.e. pleiotropic) would have differential impacts on fitness at different ages, which could potentially be opposite (antagonistic), such that genes causing beneficial effects during one period of life, but pernicious in another period of life, would be favoured by natural selection as far as these beneficial effects overcame the deleterious ones, and made those individuals fitter than others in the population. This would normally be the case when the benefits of those genes were manifested early in life, while the pernicious effects appeared later in life, when the decrease in fitness produced by these would be lower, as the individual would already have potentially reproduced. This theory is more complete than the mutation accumulation idea, as

the latter assumed that almost no senescent animals would be seen in the wild, which has turned out not to be true, after data has been accumulated in the last half a century of research on ageing in the wild (Nussey et al. 2013). Also, as William pointed out himself, the absence of very old and senile individuals is not the same as the absence of senescent individuals, that may be young enough to be abundantly present, but still see their performance reduced over time (Williams 1957). Thus, with William's theory, we can expect to observe wild organisms that do show senescent states and processes, even if they are not yet totally senile. These states would be by-products of something else which is indeed favoured by natural selection, as mechanisms contributing to high growth rates or investment into reproduction early in life, which would have an important positive contribution to fitness. Nevertheless, antagonistic pleiotropy is not incompatible with mutation accumulation, in such both are founded on the ideas of a lowered strength on natural selection over an organism's age, and an age-dependent manifestation of certain traits, with older ages presenting therefore a higher manifestation of deleterious traits or states, just differing in the mechanism that would cause that trait to appear: either the mere lack of capacity of selection to eliminate them, or their indirect promotion by natural selection. William's theory could be thus considered a refining of Medawar ideas, who indeed already hinted the possible mediation of antagonistic pleiotropy (Medawar 1952), including the possibility of senescence to occur in natural conditions. However, both are ultimately based on the idea that there is some way in which the age of expression of a trait can be somehow determined or regulated. This could only be possible if there is either a) an ontogenetic program based on external or internal signals that guide the onset and potentially the end of stages as growth, maturation, reproduction or senescence, or b) the unintended accumulation of molecules or changes that can act as a "clock of aging", regulating the apparition of deleterious states (see Kirkwood 1977; Kirkwood and Holliday 1979; Kirkwood and Melov 2011 for discussion). The former option would be feasible if we assume that ageing has indeed evolved as a regulated process by natural selection, and it is therefore adaptive (but see below for discussion on developmental constraints), for which we would lack an explanation within these theories, and the latter would assume that there is something that is being unintendedly accumulated. If such accumulation is to cause the differential expression of pleiotropic genes or their *somatic environment* (in Williams terminology) causing differential outcomes, we would still need to explain such previous accumulation that cannot be adaptive, following the previous reasoning.

### 1.1.3. Kirkwood and the disposable soma theory: does it really add novelty to the discussion?

Following the abovementioned reasoning, the primary cause of ageing would be the mere accumulation of damage. This is pointed out by Kirkwood (1977) and Kirkwood and Holliday (1979) when developing their "disposable soma" theory. This theory, initially developed as an extension on Orgel's error catastrophe idea of the unavoidability of accumulation of errors during molecular replication (Orgel 1963; 1970), further combined with the idea of the existence of trade-offs between germ-line (reproductive cells lineage) and soma (the rest of the body) maintenance, poses that the reparation of the accumulated errors implies the utilization of limiting resources that cannot otherwise be directed towards reproduction or at least germ-line maintenance. Therefore, natural selection will favour the maintenance of the soma just up to a point in which it is necessary for reproduction, but with the decrease in the strength of natural selection over age, such contingent and "expensive" task of maintaining the soma would turn out progressively less favoured. Nevertheless, the difference of this theory with William's one is subtle and they are not mutually exclusive (antagonistic pleiotropy can still occur within

Kirkwood's theory), so the confusion between them and their predictions at the moment of experimentally proving them is likely to happen. Indeed, it could be considered that if, following Kirkwood's idea, ageing appears due to optimization of the trade-off between soma and germ-line, such optimization has to be somehow under genetic control (otherwise it could not have evolved by natural selection and therefore such a "optimization" would be difficult to explain), so that the genes that control these resources' allocation can be considered pleiotropic genes, as their expression leads to different implications for different systems (i.e. higher reproductive output and lower soma reparation efficiency). Thus, if these genes have been selected because they increase fitness through reproduction despite reducing it through reparation of the soma, we would ultimately have a classical antagonistic pleiotropy mechanism underlying the explanation, with the only possible difference of damage not being *directly* caused by the genes in question, but by some unavoidable damage, which is subsequently less repaired. In any case, the ultimate outcome of this gene that reduces repairing efficiency, in comparison with other alleles present in the population, is the presence of higher damage accumulation in the soma of the individuals who bear it, and the intermediary mechanism by which the damage was originally produced seems relatively irrelevant. Indeed, Kirkwood and Holliday (1979) initially recognised that: "The disposable soma theory is, in a sense, a special case of Williams's [sic] (1957) pleiotropic gene hypothesis, the gene in question controlling the switch to reduced accuracy in somatic cells. The good effects of the gene is the reduced investment of resources in the soma, while the bad effect is ultimate somatic disintegration, or ageing."

One could nevertheless still argue that the theory is introducing the idea of damage already being present before the mechanism proposed to cause ageing takes place, so that ageing is due to the inability to correct such damage. In this sense, disposable soma theory is more explicit about the *mechanisms* causing ageing. However, Williams does not explicitly exclude the possibility of damage to occur without antagonistic pleiotropy taking place. Indeed, he argues that the reason why this "wear" (mere entropy increase following second thermodynamics' law) does not cause ageing in living beings as in inanimate objects is intrinsic reparation of the former. This more physiological/mechanistic emphasis made by disposable soma theory probably led it to become, during the 1980's (Kirkwood, 1981; Kirkwood, 1985; Kirkwood, 1990; Kirkwood & Holliday, 1986), a more "physiological ecology" theory of ageing, with the use of the key concept of trade-off within the framework of life-history evolution, adopting optimization models (Kirkwood and Rose 1991) to reach the same conclusion to which Williams and Hamilton and later Charlesworth (1980) arrived with quantitative genetics logic and models: ageing evolves due to declining selective forces over age favouring higher investment into earlier fitness (mostly reproduction) than in later fitness (mostly survival) (Kirkwood and Rose 1991). Indeed, the authors end up considering: "Is the disposable soma theory a causal subset of the antagonistic pleiotropy theory? [...] The two theories are more accurately seen as complementary than overlapping" (Kirkwood and Rose 1991). Nevertheless, the confusion between both Williams and Kirkwood theories has become reasonably important, as Kirkwood and Rose (1991) themselves exposed: "During recent years [...] advocacy of the antagonistic pleiotropy theory has emphasized the trade-offs between survival and fecundity. With this shift in focus the antagonistic pleiotropy theory has become rather similar to the disposable soma theory and now also rests heavily on the idea of costs of maintenance and reproduction." This confusion has probably been extended with further reviews on the topic made by Kirkwood during the following decades (Kirkwood 2005; Kirkwood and Melov 2011), in which he seems to shift away from antagonistic pleiotropy theory, with statements like:

“[...] unless we suppose that the pleiotropic genes regulate maintenance and repair. This, however, would be to equate antagonistic pleiotropy with the disposable soma theory. Although it is possible to press the kinds of gene actions envisaged by the disposable soma theory into the mold of antagonistic pleiotropy (Kirkwood and Rose, 1991), the fit is awkward. This is because the genes in question are for the repair functions, which are uniformly beneficial. What one has to argue in order to make them fit antagonistic pleiotropy is that the early benefit comes from *not repairing too much*, which seems to stretch the concept that Williams (1957) originally had in mind for such genes. It is surely easier to talk simply of trade-offs.” (Kirkwood 2005).

Here, I believe, he makes a mistake at assuming that the “repairing genes” themselves should be the ones underlying the antagonistic pleiotropies, instead of, as he correctly suggested before, the genes controlling the level of repair: “[...] the gene in question controlling the switch to reduced accuracy in somatic cells.” (Kirkwood and Holliday 1979). He also later favours the idea of disposable soma being a distinct theory that has obtained more empirical support than antagonistic pleiotropy:

“[...] there are still relatively few confirmed instances of individual genes that have definitely been shown to have antagonistic pleiotropy of the kind proposed by Williams. On the other hand, there is considerable evidence that phenotypic trade-offs are important for the evolution of ageing. [...] while it is understandable that there are more examples of phenotypic trade-offs than specific cases of antagonistic pleiotropy that have so far been traced down to the genetic level, the term ‘antagonistic pleiotropy’ has a specific meaning in terms of gene action and should not be used as a blanket term to cover the more general (and succinct) concept of trade-off.” (Kirkwood and Melov 2011).

Here I consider that the error is that even if of course the concept of trade-off is wider than that of genetic pleiotropy, if the optimization of a phenotype affected by trade-offs (here, I assume, physiological trade-offs, cf. e.g. Stearns 1992) has evolved by natural selection, it must be underlined by a genetic basis regulating the allocation of resources between the “traded” traits. Therefore, in the context of the *evolution of trade-offs’ optimization by natural selection*, as in the evolution of ageing, pleiotropy of some kind is still taking place, regardless of the approach we use to arrive to our conclusions (more directly through quantitative genetics or indirectly through optimization models).

#### 1.1.4. Can ageing be adaptive?

Despite the demise of the initial ideas of Wallace and Weismann on a selective advantage (see section 1.1.1) of ageing (Weismann himself apparently changed his mind later during his life), some theories proposing the adaptive origins of ageing have emerged over the second half of the XX<sup>th</sup> century and the XXI<sup>st</sup> century, based on either some kind of vague idea of group selection (Skulachev et al. 2015), or on kin selection theory (Libertini 1988; Bourke 2007).

In general, the theories arguing that ageing is adaptive propose that it follows a programmed plan, same as what we would see with most ontogenetic processes, as growth or sexual maturation. This kind of theories looks *a priori* difficult to sustain within Darwinism, except maybe for some cases like semelparous organisms (e.g. annual or biennial plants) or some social animals (although some degree of “programming” could be argued in the perhaps too wide sense of emerging from a developmental constraint produced by antagonistic pleiotropy mechanisms). One of the main examples of such reasoning is that from Vladimir P. Skulachev and his colleagues (see e.g. Skulachev et al. 2015), which in 1997 proposed the concept of

phenoptosis (i.e. the programmed death of an individual organism; Skulachev 1997) purportedly coined by his friend Mikhail Leonovich Gasparov (cf. Skulachev 2012), and since then have published several articles on the issue, mostly in the journal *Biochemistry (Moscow)*.

They propose this phenomenon as a mechanism promoting evolvability. This could be considered as an interesting idea, but the way they express it presents logical circularity issues. They use as an example a population of hares that has to escape from foxes, so that they can use their speed based on muscular strength and running dexterity, or their intelligence. Basing their reasoning on a Russian proverb praying: “If you are strong, it is not necessary to be clever”, they continue arguing that ageing related sarcopenia would make intelligence relevant for the hares to escape from foxes, and therefore allow more intelligent hares to evolve. This reasoning contains an evident problem: they seem to be assuming that there is some intrinsic adaptive advantage on being more intelligent, when they precisely say that it is not needed if you are fast, so such advantage only emerges when ageing has already evolved, making the argument circular; this could work only if we assume that higher intelligence could be a trait being later exapted, so that ageing would increase exaptive pool, but the evident pernicious effects of ageing on individual fitness would likely be enough for avoiding such kind of long-term evolvability advantage to ever manifest, and it is also not clear if it would even exist outside of this very specific example. To justify all of these, they seem to hold it on some kind of group selection framework, but at the same time they argue that individuals can “switch off”, or at least slow down the “programme of aging” if certain circumstances in which their survival could be menaced ensue, giving as examples the lifespan extension effects of dietary restriction and other environmental conditions as cold exposure (whose mere existence, together with the knowledge of important signalling pathways mediating them, they also propose as an evidence of ageing having a programmable nature and therefore being adaptive). This strange logic can be observed in sentences like the following: “At a time when the survival of the population is at stake, the organism can slow the activity of this counterproductive program, an event reducing evolvability, but increasing chances of individual survival. It would be unsurprising if the aging program was slowed in individuals working for the benefit of the community.” (Skulachev et al. 2015). This looks really difficult to conceal into a consistent logical interpretation, as they seem to be proposing that ageing would evolve as a evolvability mechanism favouring populations, but also with a way for *individuals to favour their survival* by switching it off only when it would *favour the population* (or “community”, which is probably referring to a social group, as they are basing a considerable part of their argumentation on humans, and not to the ecological concept of community as the set of populations from different species sharing space and time).

Other sounder theories have nevertheless been developed, based on kin selection theory (Hamilton 1964a; 1964b) and the evolution of sociality (reviewed in Bourke 2007). This theoretical framework proposes that ageing may affect inclusive fitness, particularly in circumstances in which some kind of social organization exists. These theories can be classified (see Bourke 2007) depending on the relationship between effects on fitness of recipients and donors of the behaviour (in this case a phenomenon): if each of them is benefited or disadvantaged by the action. Thus, we can find cases in which parents’ longevity can positively affect offspring or grand-offspring viability, therefore making slowed ageing beneficial, particularly in cases in which fecundity is severely decreased or even totally null after a certain age. This is the case of menopausal females, something present in some social mammals, and explained partially by the considerable need for offspring care, being humans the most marked example (high need for care of the offspring and very long post-reproductive lifespan, especially nowadays; see e.g. Mace 2000; Shanley and Kirkwood 2001). On the other side, some

descendants may benefit from an increased ageing and earlier death of their progenitors in order to inherit resources as territory. The opposite outcome can be inferred for both cases, with either reduced lifespan of parents reducing offspring viability or increased longevity foregoing scarce resources inheritance. These examples are based on more direct influence of relatives through caregiving or transgenerational effects (e.g. Lee 2003), but there also exist other models based on effects of mortality and dispersion rates through density dependence mechanisms that can affect variation in selection pressures (e. g. Travis 2004; Mitteldorf and Pepper 2009; Martins 2011; Mitteldorf and Martins 2014; Werfel et al. 2015). Some of these models also point towards interdemic selection, although implicitly assuming possible kin selection effects at employing clonal reproduction in them (Longo et al. 2005).

There are also models that try to establish that shorter lifespans can be favourable as they reduce the time a beneficial gene would take to spread through a population or a deleterious one to disappear (Libertini 1983, 1988), trying also to use kin selection to explain how this gene reducing lifespan would spread in stable populations (with density dependence). It is not clear to me though how this argument may work as, first, kinship coefficient, however high as average in a population, does not predict high probability of sharing a *particular* gene, especially if this is rare, and second, the “good genes” have in principle the same probability (at least initially) to be present in individuals with or without the lifespan-affecting gene; even if they become more common after a certain time *within* the share of the population that has the lifespan reduction gene, those will still have to compete against the ones that have these beneficial genes but not the deleterious life-shortening mutation. The author also seems to think that non-adaptive ageing theories should predict the opposite that they do, i.e. that high extrinsic mortality leads to weaker selection force and therefore higher ageing, in saying that what we see is precisely less IMICAW (Increased Mortality with Increased Age in the Wild). In doing this he is missing the point of that these theories are actually not predicting that age-related mortality will be higher in the wild for those species, but simply that they will have lower lifespans and earlier onset of senescence, but the few individuals that indeed reach old ages will very likely suffer from ageing-related deaths (as it happens more commonly in captivity).

Recently, Kowald and Kirkwood (2016) discussed many of the models, both analytical and simulations, that were created in order to prove the evolution of ageing as a positively selected trait, demonstrating in most cases how they do not work if the assumptions are slightly modified to more realistic ones. Most of these models that lead to the evolution of reduced lifespans work by the same logic from Libertini (1983, 1988, 2019), with the reduction in maximum lifespan in closed populations with density dependence and relatively high average kinship coefficient benefiting the spread of positive mutations. Nevertheless, if sexual reproduction is introduced, which increases the variability, environmental change rate (i.e. strongly changing selective pressures) is reduced or movement of the agents (representing organisms) in the simulations is allowed, the advantage of shorter lifespan is rapidly lost (Kowald and Kirkwood 2016).

In conclusion, although some aspects of population dynamics and genetic relatedness may modulate optimum lifespan, ageing as an intrinsic mechanism reducing lifespan cannot originate favoured by selection unless very specific conditions, which are far from universal, are assumed.

#### 1.1.5. Theories versus data: comparative data, environmental correlates and experiments

Since these theories were proposed, many studies have been conducted to test their predictions and validity. Although the review of all studies performed up to date is out of the scope of this

section, I will make a general outline of the main sources of evidence for them, mostly mutation accumulation, antagonistic pleiotropy and disposable soma (insofar as the underlying genes involved have not been discovered and thus an antagonistic pleiotropy cannot technically be explicitly accounted for).

First of all, what classic non-adaptive theories of ageing have in common is the supposition of a decline in the strength of natural selection over age (but see Baudisch 2005 for a discussion on the validity of this assumption depending on the parameter used to estimate fitness, and Giaimo and Traulsen 2022 for a model proving the inversion of the logic: ageing causing the reduction on the selection strength), which in some cases would be leading to an increase in mortality rates with age. Such increase in mortality may be mediated either by direct deleterious mutation accumulation or indirect deleterious effects of pleiotropic genes with beneficial fitness effects early in life. Although both hypotheses are complementary, more evidence has accumulated in favour of the antagonistic pleiotropy theory than in the mutation accumulation theory. Most data supporting antagonistic pleiotropy unequivocally comes from selection experiments or mutations found to increase longevity at the cost of early life reproduction, growth rate or resistance to stresses like food deprivation in flies (mostly *Drosophila sp.*), nematodes (*Caenorhabditis elegans*), yeast (*Saccharomyces cerevisiae*) and house mice (*Mus musculus*) (reviewed in Austad and Hoffman 2018). In some cases, this adverse effect is not detectable in lab conditions unless the animals are subjected to food limitation or seminatural conditions, in which long-lived mutants or selected lines tend to be outcompeted and get extinct in a few generations (e.g. Jenkins et al. 2004; Voorhies et al. 2005; Delaney et al. 2011; Giorgio et al. 2012). Many studies have been performed assessing trade-offs potentially affecting the evolution of ageing, mostly those between reproduction and survival (e.g. Dobson and Jouventin 2010; Cox et al. 2010; Flatt 2011) and growth and long-term survival (reviewed in Metcalfe and Monaghan 2003). Many have also assessed possible indirect effects of early life conditions or levels of reproductive investment on survival or late-life reproduction by assessing molecular or physiological proxies of the ageing-related decline in functions, as telomere length or loss rate, oxidative stress/status, changes in metabolism, immune response, etc. (see e.g. Criscuolo et al. 2008; Geiger et al. 2012; Plot et al. 2012; Reichert et al. 2014; 2015; Guivier et al. 2018). In these cases, the background reasoning is usually related to disposable soma theory, as the specific genes involved in controlling the trade-offs assessed are very rarely known, especially in studies in wild animals.

It is of relevance to mention that of the 9 predictions explicitly made by Williams on his 1957 article, 6 can be considered proven, or at least not disproven (Gaillard and Lemaître 2017). Being the second one (the positive relationship between adult age-independent mortality rate and actuarial senescence rate) the most heavily scrutinized, many data has accumulated in favour of it (e.g. Promislow 1991; Ricklefs 1998), although in some cases other parameters may modulate its action, as condition-dependent mortality, that can act reversing the pattern: high levels of condition dependent mortality leads to higher longevity (Chen and Maklakov 2012). The predictions that are actually most contended are the synchronicity of ageing, the start of senescence right after the age of sexual maturity and the expectancy of finding a negative relationship between longevity and vigour early in life. Indeed, the onset and rate of senescence for different parameters is clearly different (Nussey et al. 2013) and senescence has been shown to start normally later than just after sexual maturity (e.g. Promislow 1991). This mismatch from the prediction and the data may come by errors in the way of estimating proportional age-related fitness weights, which depends on demographic factors not accounted for by Williams 1957 and Hamilton 1966 (see discussion in Gaillard and Lemaître 2017). Moreover, there is

generally a positive relationship between longevity and performance-related parameters early in life (Rose 1991), as the negative relationship due to a trade-off in the allocation of resources is only expected for individuals with the same level of resource acquisition (Noordwijk and Jong 1986), so that there will be no genetic trade-offs in this sense if fixation of the alleles responsible for these is accomplished. Nevertheless, in regard of the prediction on the synchronicity of ageing, it can be argued that at least what was probably one of the main underlying ideas of it, i.e. that: “Basic research in gerontology has proceeded [sic] with the assumption that the aging process will be ultimately explicated through the discovery of one or a few physiological processes. [...] the essential task of gerontologists to be that of distinguishing cause from effect among the multitude of observable changes in aging organisms, and eventually isolating the presumably few ultimate causes. *Any such small number of primary physiological factors is a logical impossibility if the assumptions made in the present study are valid.*” (Williams 1957) has actually come to be reinforced, ageing being widely recognized as a multifactorial phenomenon with primary and emergent causes interacting in complex ways, with retro-causation happening across them (see Cohen et al. 2022).

Apart from all that has previously been discussed, we may wonder as well what drives the observed differences in ageing patterns (both rates and shapes) across taxa (Jones et al. 2014; Roper et al. 2021). For this, the classical theories pose that differences in environmentally driven mortality levels determine the observed differences in expected lifespan across species, which in turn, and depending on specific niche characteristics and life-history trade-offs, will shape the patterns of fecundity and mortality of each population and species. Thus, considerable variation in senescence rates and shape can be observed across the tree of life (Jones et al. 2014; Roper et al. 2021). This can be somehow expressed partly through the classic concept of survival curves: type I (concave), II (straight line) and III (convex), attributed to different kinds of organisms (sensu Pearl and Miner 1935). Indeed, the exploration of the diversity of ageing patterns indicates that many species do not seem to show decline in survival probability and fecundity with age (negligent senescence) or even show an increase in such (negative senescence) (Roper et al. 2021). This is the case in some indeterminate growers, in which body mass, as a predictor of fecundity and survival, increases with age indefinitely and thus actuarial senescence patterns are reversed (Finch 1994; Charnov 2001, Vaupel et al., 2004; Baudisch 2008). Some of the reasons that have been argued to explain these differences in lifespan and ageing patterns are related to characteristics that lower the levels of extrinsic mortality, such as flight, mostly for birds and bats, subterranean life, like in mole-rats, arboricity, protections (spines, shells...) sociality or large brains (although these two may be related in many cases and also imply slower developmental processes affecting life-history in a more direct way) (Healy et al. 2014).

Sociality in itself is a parameter whose interplay with ageing evolution is complex and has largely been discussed (see e.g. (Møller 2006; Thorley 2018; Vágási et al. 2021; Kreider et al. 2022)). It is of particular interest to remark how social behaviour sometimes leads to high interindividual specialization, in manners that in the most extreme cases it can cause huge differences in longevity between members of the same families, as in bees or ant queens versus workers (Keller and Genoud 1997). These differences in lifespan may in part be modulated by extrinsic mortality rates, but it is also related with the differences in function of the individuals in a colony, especially in terms of reproductive division of labour, and therefore on the existence of different selective pressures leading to striking physiological and developmental divergences in genetically very close individuals (Michener 1969; Hölldobler and Wilson 1990; Quque et al. 2022).



## 1.2. Proximate causes: the Hallmarks of ageing

Once the evolutionary causes of ageing have been set out, one can consider nevertheless, that in the end, the primary causation of such decrease in general performance with age needs to be addressed. Worthily enough, it remains incomprehensible how such decline in function with age can be instantiated if, despite assuming this is not (at least directly) based on an adaptive program, age-dependent expression of traits is still predicted by the evolutionary theories, such that a mechanism to “distinguish” the age at which a trait is manifested, either by the expression of an “age-specific mutation” or by the apparition of deterioration by a gene that has been expressed continuously. The best way to explain this may indeed be the accumulation of damage, or any other compound that may alter the phenotype, either directly or by inducing a change in gene expression, as made explicit by the disposable soma theory (Kirkwood 1977; Kirkwood and Holliday 1979). Many of such damage sources have been proposed over the last century, ranging from a literal accumulation of altered molecules to mechanism of regulation of change in gene expression showing variation with age, also including theories about the sources of such damage or change (see below). In the recent decades, a big effort has been made both into improving the knowledge about each of these specific mechanisms and into how to integrate them into a more comprehensive model of understanding of ageing as a complex process. This shift, together with vast empirical data, has allowed us first to recognize the interconnectedness of many of such processes, and foremost it has allowed us to identify emerging phenomena that take place at different organisational level (molecular, cell, tissue, organ, organismic or demographic), and that may even retroactively affect lower organizational levels making it a complex process, and indirectly confirming the pleiotropic nature of ageing. As Williams stated himself within his fifth prediction: “Any such small number of primary physiological factors is a logical impossibility if the assumptions made in the present study are valid.” (Williams 1957).

### 1.2.1. A brief history of the mechanistic ageing theories: from single mechanisms to a bewildering complexity

Many independent theories trying to explain the mechanisms causing ageing have emerged during the XX<sup>th</sup> century. Some of them were very specific and tried to explain rather the degeneration of specific organs or tissues, so they should not be considered theories of ageing as such (see Medvedev 1990). Other more general theories also emerged during approximately the first half of the century, attributing ageing to different causes, as the mechanochemical deterioration of biological colloids (Růžická, 1924; 1926) autointoxication by bacterial derived toxins (Metchnikoff, 1904, 1907), cross-linking of molecules (Nikitin 1954; Verzar 1956; 1957; Bjorksten 1958; 1974; Sinex 1964; von Hahn 1966; 1970; Cutler 1976) and other based on the toxicity of certain metabolic waste products (Muhlmann, 1914; Korenchevsky, 1956) or in the loss in replication fidelity, with the consequent error accumulation (e.g. Orgel’s 1963 “error catastrophe”, which served as an inspiration for the development of disposable soma theory; Kirkwood 1977). Some of these purposed mechanisms continue to be relevant at some level, even if not as a general theory explaining ageing alone, like cross-linking, mainly in the context of AGE accumulation and general loss of proteostasis. Nevertheless, the one that probably became more successful and is still widely considered as one of the main primary damage mechanisms recognized to mediate in ageing is the Harman theory of “free radical and radiation” damage (Harman 1955, 1956), today mostly known for the “free radical” part,

considering reactive oxygen (and nowadays also nitrogen) species, or R(N)OS, and the role of mitochondria on its production (Harman 1981;1983) as one of the pillars of mechanistic ageing research. This is particularly interesting, as if we consider reactive species as an unavoidable consequence of metabolism, it will help explaining the predominance of ageing (at least in aerobic organisms). Still, it is worth mention that recently the role of mitochondria on ROS balance (Hickey et al. 2012; Sena and Chandel 2012) and the important functions of RNOS in cellular signalling (see e.g. Hurd and Murphy 2009; Weidinger and Kozlov 2015) have been reconsidered, with concepts as mitohormesis being upraised (see Tapia 2006 for the origin of the concept and Cheng et al. 2023 for a recent review).

This is in line with the so called “rate of living” theory, initially proposed by Raymond Pearl in 1928, inspired by the observations of Max Rubner in 1908 of that several species of very different longevities showed the same amount of energy expenditure *per gram* of mass along the whole lifespan, supported by later experimentations modifying energy consumption by changing ambient temperature in *Drosophila melanogaster* (Loeb and Northrop 1917). This theory proposed that organisms’ lifespan is determined by the waste of some kind of vital component proportionally to the metabolic rate. This way, each organism would have a genetically fixed amount of lifespan to live, which could be modified by changes in metabolic energy consumption (i.e. increases in metabolic rate would decrease lifespan and vice versa). This idea was also reinforced by some measurements which related other parameters like the total number of heart beats *per life* with the same concept of rate of living (MacArthur and Baillie 1929). Nevertheless, subsequent experimentations trying to assess such within species variation in metabolic potential depending on the conditions demonstrated that the exact value was variable (e.g. Miquel et al. 1976). These values were also found to be different for different taxonomic groups (reviewed in Sohal et al. 1989), and its value is still debated (e.g. Stark et al. 2020 against and Zhang et al. 2021 partially in favour). Mostly, the original theory as an universal governor of ageing process has been rejected, but a derivation of such ideas remains still alive on the basis of the abovementioned Harman’s theory of ageing. Thus, the understanding of this line of thought is that metabolic rate negatively correlates with lifespan, partly due to the production of ROS like superoxide ( $O_2^{\cdot-}$ ) and  $H_2O_2$  or  $OH^{\cdot}$  derived from it, as a by-product of respiration (Sohal et al. 2002), but that total “metabolic scope” can be different in different taxa depending on factors like the rate of superoxide produced by  $O_2$  consumed, the susceptibility of their components to get oxidized and the level of antioxidants or repairing enzymes they present (see e.g. Hulbert et al. 2007). However, even this position has been challenged (see e.g. Hou and Amunugama 2015), as if  $O_2$  consumption *per se* as a metabolic rate estimator is accounted alone, it can be found to be positively related to lifespan (Speakman et al. 2004). This is due to the antioxidant effect of mitochondrial uncoupling (i.e. oxygen consumption not linked to ATP production), which makes the relationship between  $O_2$  consumption levels and ROS production not so straightforward (Brand 2000). Also, it has been argued that high ROS production has been measured in mitochondrial resting state, which is rare in natural conditions (Hou et al. 2021).

From the cellular level perspective, the realization that cell division is not infinite dates as far back as Weismann himself, who proposed there to be a limited number of cell division taking place in animal bodies (Weismann 1982). Although he proposed that as a theoretical mechanism leading to the programmed death of the organism (see section 1.1.1.), and Alexis Carrel (Carrel 1912) and Albert Ebeling (Ebeling 1913) later criticized his proposal as apparently, they were able to cultivate chicken cells *in vitro* for what seemed to be an indefinite time (at least 34 years), later studies confirmed his hypothesis. That incapability of animal cells to proliferate indefinitely *in vitro* is what we nowadays know as the Hayflick limit (Hayflick and Moorhead 1961), although

the same conclusion was reached previously by H. Earle Swim (Swim 1959). This was initially thought to be caused by telomere shortening, which make this a very popular mechanism of ageing (see e.g. Goldstein 1990; Olovnikov 1996). Telomeres are (generally) non-coding repetitive DNA sequences at the end of linear eukaryotic chromosomes, that due to the so-called end-replication problem, get shorter each time DNA replicates, and consequentially when a cell divides. This is so because one of the DNA strands (the lagging strand) cannot get replicated to the end, as it needs an RNA primer to be inserted every few nucleotides. This way, there is always a 3' OH end which remains free after the last primer is removed, and cannot get completed as its new complementary strand ends in a 5' phosphate (and replication proceeds in a 5'→3' direction, needing a free 3'). This problem does not exist in prokaryotic circular chromosomes, but does in linear ones. Telomeres, thus, serve to maintain DNA stability avoiding them to lose coding sequences straight forward after cell replication, at least until cells age and telomeres become too short (see Chuaire 2006 for an history of telomeres). Although telomere shortening after each cell division cycle is still considered as relevant phenomenon, it is now recognized that other factors play a role in what is currently understood as cell senescence and stem cell exhaustion (Lopez-Otín et al. 2013; 2023), and also that DNA damage caused by factors as oxidative stress may actually be more relevant in telomere shortening (Metcalf and Olsson 2021) than the length diminution produced after each division by the terminal end-replication problem linked to linear eukaryotic chromosomes and telomerase repression in somatic cells (Wellinger 2014).

### 1.2.2. The Hallmarks of ageing

The concept of “The Hallmarks of aging”, as named by Lopez-Otín et al. (2013; 2023) constitutes a good example of an attempt to recognize the multifactorial, multilevel and interacting nature of ageing, which has brought to some authors to call for the employment of a complex systems approach into its study (A. A. Cohen et al. 2022). These hallmarks can be divided into primary, antagonistic and integrative (Lopez-Otín et al. 2023). The first case is mostly based on accumulation of damage, either in the DNA (including telomere shortening) or in proteins (so called “loss of proteostasis”, part of which is the basis of glycation, as we will see later), although other kinds of damage can happen, and molecules as lipids are increasingly being accounted for in the understanding of cell damage/resistance dynamics (e.g. Hulbert et al. 2007). The loss of ability to repair this damage, through DNA maintenance systems, including telomerase in the case of telomeres (Chakravarti et al. 2021), proteasome activity in the case of proteins (Yang et al. 2019; Sacramento et al. 2020), or macroautophagy in general for cellular or tissue structures (Levine and Kroemer 2019), is frequently the cause of such accumulation happening. For the integrative causes, they are based on the expansion of the dysfunction across levels of organization, with mainly alterations in the integration of information fluxes, as in disruption of cell signalling, including inflammation, and dysbiosis, i.e. alteration of microbiota function with negative organismic level consequences. Such alteration in cell signalling is caused by a differentiation in the tissues' cell profiles, through a reduction in cell turnover by a combination of a depletion of stem cells and a decrease of the elimination of the ones becoming senescent (by the reduction on either the abovementioned macroautophagy or of apoptosis, i.e. programmed cell death), which through a characteristic phenotypic change in their secretion profile when becoming senescent, producing the so called Senescent Associated Secretory Profile (SASP), causes a positive feedback loop, turning more cells into a senescent state and increasing the alteration of extracellular matrixes, turning them more rigid, and in turn also affecting more the cell signalling dynamics (see e.g. reviews in Byun et al. 2015; Campisi and Robert 2014). Part of these alterations are caused by the accumulation of primary damage, and

part by the alteration of gene expression (epigenetic changes being classified as one of the “primary mechanism” by Lopez-Otín et al. 2023, although such change may not always be primary in nature; see Braga et al. 2020 for a review).

Probably the most interesting hallmarks from an evolutionary point of view are the antagonistic, as they stand closely in line with Williams (1957) theory. One of those is cell senescence, already discussed partly in the previous paragraph, which is purportedly a mechanism to avoid the over proliferation of cells which have accumulated damage, which would otherwise lead to cancer, with the unfortunate consequence of decrease in tissue regeneration and the mentioned alteration in cell communication with age (as cancer risk is also increasing with age, in link with damage accumulation and decreased surveillance mechanisms’ efficiency; Campisi 2013). Another important one, and probably the most widely studied of these from a comparative perspective in non-model organisms, is nutrient sensing signalling, mainly related to insulin/IGF-1 (Insulin-like Growth Factor 1) and insulin receptor (Lodjak and Verhulst 2020), with all that it entails downstream (IRS, PI3K, PKB, mTOR, FOXO, etc.), together with other related elements, like AMPK and PARP (see e.g. Saltiel and Pessin 2002). These systems are in charge of the integration of the information about the energetic state of the organism, in order to control the switch from catabolism to anabolism, and to also control damage control or reparation mechanisms activation, as well as different cell differentiation and transformation, as in the cases of PARP and FOXO (see e.g. Accili and Arden 2004). These are good examples of pleiotropic genes that are under control of mechanisms as those claimed by Kirkwood (1977) to be the main drivers of ageing modulation. It turns out that the different activity levels of these signalling pathways, both within and between individuals, and even across taxa (see below) can lead to differential ageing rates and longevities (see e.g. Blüher et al. 2003; Parrella and Longo 2010; Dantzer and Swanson 2012; Mathew et al. 2017). Indeed, one of the main interventions found to considerably elongate lifespan is dietary restriction (see e.g. Picca et al. 2017), which would reduce the signalling intensity through these pathways, together with many of the known long-lived mutants in *Drosophila sp.*, *Caenorhabditis elegans* and mice (*Mus musculus*), which have changes in genes related to these mechanisms, like *chico*, the IRS (Insulin Receptor Substrate orthologous from *Drosophila*) or *daf-2* (the IGF/insulin receptor homologous) and *age-1* (also in the insulin/IGF pathway) from *C. elegans* (reviewed on Austad and Hoffman 2018).

This has indeed been taken by the defenders of programmed ageing theories as a proof of their theses: i.e. if ageing can be modulated by gene expression and central physiological pathways depending on certain environmental factors, it must thus be part of a program (which in turn they normally argue that is adaptive, as how would otherwise such a complex system evolve if not by the direct action of natural selection?). These mechanisms should instead be understood as plastic adaptive responses (Moatt et al. 2020; Regan et al. 2020) that help the individuals to direct energy towards growth and/or reparation when enough resources are available and suppress it when not. This is a mechanism similar to what it would be seen more markedly in hibernating animals, which would reduce metabolism when resources are scarce and delay activities as reproduction (Al-attar and Storey 2020). Indeed, hibernation has also shown to correlate with longer lifespan (Wu and Storey 2016).

Insulin/IGF-1 signalling, particularly, are very important not only insofar they regulate the switch between anabolic and catabolic processes, but also because, as we will see later in more detail, insulin is one of the main hormones controlling blood glucose levels, and whose dysregulation leads to metabolic alterations that constitute some of the main ageing-related diseases, in a way which is becoming especially relevant human health: diabetes (see section 3.2.1.). Insulin also

importantly mediates other processes related to ageing, as the degree of fatty acids unsaturation, by inducing the activation of desaturases ( $\Delta 6$ ) (see Brenner 1981), such that the degree of susceptibility to oxidative stress of these cell structural lipids increases (Hulbert 2005).

## 2. Birds as a case study: flying away from ageing

### 2.1. Why studying ageing in birds?

Many different organisms are used for the study of ageing, from short lived ones like *C. elegans*, house mice or killifish, to long-lived ones like naked mole-rats (*Heterocephalus glaber*) (Buffenstein 2005; Holtze et al. 2021). While short-lived species pose obvious benefits for the study of ageing, there are reasons to think that ageing process behaves differently in long-lived species (Turbill and Ruf 2010). Therefore, long-lived models have the advantage of being potentially more translatable to humans, and at the same time, they can teach us something about the resistance or repair mechanisms that favour healthy ageing.

Birds (class Aves) lineage diverged from that of mammals 319 Mya (Kumar et al. 2017) in the Carboniferous period. Still, both groups are highly convergent in many basic aspects of their physiology and behaviour. First, they are endotherms, which means they regulate their body temperature mainly by producing internal heat through metabolism. This makes them able to maintain high levels of activity during most of the day (and sometimes night) and colonize cold areas of the globe, in which they may compete with mammals, and may also be important for their convergently evolved parental care (see Farmer 2000; Koteja 2004). They also share with us a tetracameral heart, which increases the efficiency of circulatory system, as it avoids mixing of oxygenated and non-oxygenated blood, and the presence of complex covering of faneras (feathers versus hair) with mostly thermoregulatory function (although other exapted functions are also very important, as for example communication and flight; see review in Terrill and Shultz 2023). Finally, they also have high cognitive abilities, and in many cases form social bonding with couples or even groups (reviewed in Willemet 2013). Nevertheless, many characteristics are also differentiated. For example, mammals' red blood cells lack nucleus and mitochondria, which makes them metabolically less diverse (Stier et al. 2013). The reason for this loss of the nucleus is not clear, with increased capacity to circulate through blood vessel or reduced oxidative stress being hypothesized (Zhang et al. 2011). Nevertheless, it is not clear why this should not be the case in other vertebrates, especially in birds. At this point, there is something from birds that is very important and differentiate them from most other vertebrates (except bats): active flight. Most birds fly, and the ones who do not are thought to have lost this capacity secondarily, so many aspects of their anatomy and physiology have been coadapted to it. Some examples are their bones, which are hollow and, in many cases, fused, continuous-flow breathing with aerial sacs for increased oxygenation efficiency, their compact morphology, strong hypertrophied pectoral musculature, etc. (Sturkie 2015). But for what matters most in the context of this thesis, they show some physiological peculiarities: the highest mass-adjusted metabolic rate (Lasiewski and Dawson 1967; Speakman 2005), body temperature (King and Farner 1961; Dawson and Hulbert 1970) and average blood glucose values (Polakof et al. 2011) of all vertebrates. All of this is particularly surprising, given other of their peculiarities: their high longevity compared to mammals (Lindstedt and Calder 1976; Speakman 2005; Munshi-South and Wilkinson 2010). This is remarkable given that the aforementioned physiological characteristics are generally believed to negatively impact lifespan and ageing process (see section 1.2.1. for metabolism and 3.2.3 for glucose).

The higher longevity of birds has been known for a long time, as Francis Bacon (1638) already noted that in his *“The historie of life and death”*. More recently, in the XX<sup>th</sup> century, when detailed data from captive populations and banding and recapture studies from the wild allowed us to better know the longevities and age trajectory demographics of many species (see Clutton-Brock and Sheldon 2010; Fransson, et al. 2023), this difference with mammals started to spark the interest in the study of birds’ ageing (Holmes and Austad 1995a; 1995b; Holmes et al. 2001; Holmes and Ottinger 2003; Austad 2011). Since then, a great amount of research has been published about many of the intrinsic characteristics governing lifespan and ageing in birds (reviewed in e.g. Vleck et al. 2007; Travin and Feniouk 2016). The ultimate reason for this higher longevity is thought to be their flying ability, which would reduce their extrinsic mortality and lead to the evolution of a longer lifespan (e.g. Healy et al. 2014), while the mechanistic reasons (some potentially related to flying ability, see Hickey et al. 2012) are diverse and will be described in detail in the next section (2.2.). Within birds, some groups are known to present exceptionally high longevity (e.g. Psittaciformes or Procellariiformes) and several phenotypic traits and ecological conditions have also been linked to such differences in lifespan. Probably, one of the main factors explaining longevity is body size or body mass (as a proxy of size or by its own importance through metabolism) (Lindstedt and Calder 1976; Healy et al. 2014), a main ecological mediator and organizer of life histories (Jeschke and Kokko 2009). Other factors are likely to correlate with longevity through their effects on survival rates, as latitude and clutch size, both of which correlate negatively with survival probability in land-birds (Scholer et al. 2020), although the slope seems to be smaller for the southern hemisphere (note also the lower number of studies in that area of the globe). Other examples of factors that may affect longevity are those observed by Wasser and Sherman (2010), with social and colonial nesting (as a single group) living longer than solitary nesting, herbivorous living longer than carnivorous and omnivorous, and birds nesting in islands living also longer than those doing it in mainland. However, these particular results are to be taken with care, as their analyses were based on family averages (which for categorical variables it is not clear what it means), the diet classification is limited and of uncertain origin and they did not perform any phylogenetic correction on their data. Also, Møller (2006) showed no significant increase in relative maximum longevity with maximum colony size (categorized as 1-5 values in a log<sub>10</sub> scale and argued to be correlated with average size) after controlling for body mass, sampling effort (for the longevity), age at first reproduction and phylogeny.

All of this being said, it is easier to understand why birds have been proposed as another alternative model for ageing research. This of course comes with pros and cons, as discussed by Austad (2011). On the one hand, many species have been bred in captivity for a long time, and their small size and in some cases coloniality makes them suitable for easily maintaining big laboratory populations. On the other hand, many aspects of their physiology or genetics, as well of technics for genetic modification, is not as well-known or developed as in more common model species, like house mice or *Caenorhabditis elegans*. However, there are some species that could still be good candidates as more knowledge including genome sequences, neurology, behaviour, metabolism or endocrinology is available from them, as chicken or zebra finches (Griffith and Buchanan 2010). Another difficulty that can emerge from the study of long-lived animals in general is the longer time needed for detecting age-declining related effects of any experimental treatment, which is nevertheless a price to be necessarily paid if we want to better understand how ageing works in long-lived organisms, particularly important if we want to make the accumulated knowledge more transferable to humans. Finally, to be able to accurately interpret the results obtained from captive conditions, we need to make sure that the

divergences shown from natural environment are not too big. Although that is a concern to be considered for any model species, flying animals (as most birds) require bigger enclosures relative to their body size in order to be allowed to sustain levels of activity that could resemble those in natural habitats. This can be relevant in particular when we may want to assess effects related to metabolic expenditure. It is also possible, as in mammals, to employ cell cultures to compare the variation across different species of properties like resistance to oxidative stress (e.g. Harper et al. 2011; Jimenez et al. 2019), which in whole animals can only be measured taking samples from a limited amount of tissues. This can be done due to the existence in the market of glucose supplemented Dulbecco's modified Eagle medium, more suitable for bird cell cultures. Birds' red blood cells, in comparison with mammal ones, as previously mentioned, contain a nucleus and mitochondria (Stier et al. 2013), which makes it easier to study long-term variation in certain markers as DNA damage, telomere dynamics, epigenetic changes or mitochondrial performance-related metrics by repeated sampling (Stier et al. 2013). This could open us a way to better understand what are the characteristics of birds that make them long-lived, and also how they vary depending on the longevity of different species. In this line, I will briefly explore the known and hypothesized resistance mechanisms existing in birds in comparison to other groups, and their variation within the group in function of different intrinsic and extrinsic aspects, in the next section.

## 2.2. Are birds demigods of anti-ageing mechanisms?

Understanding the functional and structural mechanisms underlying ageing resistance in birds is of uppermost importance if we aim to use them as biogerontology models. Many aspects of their biology have been proposed to explain their relative longevity, several of them intimately related.

Some of these differences in longevity between birds and other vertebrates like mammals can be explained in terms of differential physiology and cell or molecular structure. One of the main mechanisms which has been studied in birds in relation to ageing is oxidative stress. In this line, there are several indicators suggesting that birds are less affected by it than mammals, despite having higher metabolic rates (Lasiewski and Dawson 1967). First of all, their membranes have a higher proportion of monounsaturated fatty acids, which makes them more resistant to lipid peroxidation (reviewed in Hulbert et al. 2007). Also, some studies have shown higher resistance to DNA damage in budgerigars and canaries than in mice (Ogburn et al. 1998), a constitutive activation of NRF2 in Neoaves (to which most extant birds belong), an important regulator of antioxidant enzymes (Castiglione et al. 2020) and a lower production of mitochondrial ROS than similarly sized mammals (pigeons versus rats, see Barja et al. 1994a; Barja et al. 1994b; Barja 1998; Barja and Herrero 1998; Herrero and Barja 1998; Pérez-Campo et al. 1998). The relationship between metabolic rate and longevity, indeed, is supposed to be mediated by ROS production, so mechanisms reducing the superoxide production per O<sub>2</sub> consumed, as higher mitochondrial decoupling rate (Brand 2000), would explain how higher metabolism (O<sub>2</sub> consumption) can actually be related to lower ROS production (Hou et al. 2021; Criscuolo et al. 2005a; Criscuolo et al. 2005b). Indeed, it has been suggested that higher aerobic metabolism and mitochondrial content in birds infers them a higher protection against oxidative stress (Hickey et al. 2012).

Another particularity of birds, their very high blood glucose levels, which seem paradoxical in relation to their longevity, suggest they manifest higher resistance to the pernicious effects of reducing sugars (see section 3.2.3). The factors underlying such resistance are not well known

and it may be mediated by a higher protein turnover (Muramatsu 1990), lower exposition of “glycatable” amino acid residues in proteins like albumin (Anthony-Regnitz et al. 2020) or the possible absence of the Receptor of Advanced Glycation End-products and lower oxidative stress (as a mediator of Advanced Glycation End-products production) (see particularly section 3.2.4. and 4.1.2-3). Also, recent studies show how songbird species with higher glucose levels do not show higher oxidative damage (Vágási et al. 2024), although the opposite seems to be true within species (Vágási et al. 2020). Another factor that may be reducing glycation in birds is a higher level of glycation scavengers, as circulating free aminoacids and polyamines (Szwergold and Miller 2014b), which would also induce higher autophagy (Hofer et al. 2022), an important process in avoiding the accumulation of damaged molecules and therefore promoting proteostasis (López-Otín et al. 2013, 2023). This particular subject will be described in detail in the next sections, as it constitutes the central issue of the thesis.

This is also related to insulin signalling, as birds, in comparison to mammals, seem to show certain differences in insulin sensitivity that may contribute to some of their particularities. Concretely, glucagon seems to be more important in regulating glycaemia than insulin in birds, and some tissues are apparently less responsive to the latter, at least in relation to glucose uptake by cells, as it is the case for fat tissue and partly for skeletal muscle (see section 4.1.1). In this line, (Satoh 2021) proposed that birds’ lineage (represented by theropods), in comparison with mammals, may have evolved “insulin resistance” (understood as a lower response to insulin by some tissues like adipose tissue and skeletal muscle, and mainly through its effects on glucose uptake). However, the whole argumentation shows some deficiencies to be considered, mainly in relation to the hypoxia adaptation hypothesis, and it would be discussed in the later subsection (see section 2.2.1).

Finally, birds show other characteristics that may contribute to their longevity, as a high resistance to cellular damage by multiple agents than similarly sized mammals (Harper et al. 2011) and lower tumour incidence (Ratcliffe 1933; Efron et al. 1977). Curiously, birds’ genome is particularly small for vertebrates, despite genome size being positively related with body mass-adjusted lifespan within birds, even after controlling for mass-adjusted developmental time (Monaghan and Metcalfe 2000).

Within birds, there is considerable variability in life history and in particular in longevity (see also section 2.1.), which is underlined by several physiological factors. One of the most researched traits in this context is probably oxidative physiology. Some examples of these are the comparative studies across several species finding that longer lived species show lower red blood cell mitochondrial superoxide production and higher cardiolipin content independently of body mass (Delhaye et al. 2016) and higher circulating non-enzymatic antioxidant levels and lower plasma MDA (malondialdehyde), adjusting by body mass and developmental period (incubation plus fledging period) (Vágási et al. 2019). Nevertheless, Montgomery, Hulbert and Buttemer (2012) found no differences in mitochondrial ROS production from several tissues between species with very different lifespans (quails and parrots), even when adjusting by tissue mitochondrial content and oxygen consumption, nor differences in several oxidative damage markers and antioxidant compounds, except in the case of glutathione peroxidase, being higher in parrots (Montgomery, Buttemer and Hulbert 2012).



### 2.2.1 Insulin resistance as hypoxia adaptation in birds?

As previously mentioned, Satoh published a hypothesis in 2021 about the macroevolution of birds and their ancestors by insulin resistance, making them more athletic and helping them to get vastly diversified. This would have purportedly been an adaptation to lower levels of atmospheric oxygen during the Permian/Triassic transition and later during the Mesozoic era. This environmental effect was accompanied by gene loss in birds' lineage, that would have led to a higher aerobic metabolism associated with a greater mitochondrial metabolism and biogenesis. This "insulin resistance" would partly explain higher longevity in birds, given the importance of insulin/IGF-1 signalling in ageing (see section 1.2.2) and the fact that this would also lead to a higher oxygen consumption, which in association with a higher decoupling (as suggested by Brand 2000; Speakman et al. 2004), should lead to lower mitochondrial ROS production and therefore lower damage accumulation.

Nevertheless, and although there are possibly some interesting ideas in this proposition, several concerns should be raised about it. First, lowered oxygen levels would have been experienced equally by dinosaur (and therefore bird) and mammal ancestors, so that the differences between both lineages in "insulin sensitivity" would not be solely explained by adaptations to such environment. Here the author tries to explain how mammal and bird ancestors adopted different mechanisms to adapt to such environment. Following his reasoning, insulin signalling, as a mechanism decreasing mitochondrial biogenesis and aerobic metabolism (Katic et al. 2007; Liu et al. 2009), would have been *maintained* in mammals, which would decrease oxygen consumption as an adaptation to hypoxia. Nevertheless, *maintaining* insulin sensitivity cannot be an *adaptation* here. Moreover, he then follows saying that: "Mammals must have adopted this system to minimize reactive oxygen species (ROS) leakage, resulting in suppression of the metabolic rate." (Satoh 2021), which implies the adaptation was actually *to minimize ROS leakage*, metabolic rate changes being a mere consequence, not the actual adaptation. Therefore, the way in which mammals *actually adapted* to hypoxia is not really explained. This discussion is further obscured by the following argumentation on how birds adapted to hypoxia by *increasing* oxygen consumption "so as to maximize the efficiency of oxygen usage" (Satoh 2021). It is unclear how increasing oxygen consumption by itself would lead to an increase in the efficiency of its usage, precisely when the sources that are subsequently cited (Brand 2000; Speakman et al. 2004), (and also as implied in the text itself later), talk about the decrease in "efficiency" (defined as lowered P/O ratio) due to uncoupling in order to reduce ROS production while increasing oxygen consumption. Although purportedly uncoupling proteins present in birds may not be really so, they still have shown to reduce ROS production (Crisuolo et al. 2005b). Nevertheless, here it is then exposed that "ROS leakage may be a potential adverse effect of this system." (Satoh 2021) which not only seems to contradict the expectations coming from the "uncoupling to survive" hypothesis, but would also turn the whole argumentation confusing, as he proposed that mammals decreased oxygen consumption to reduce ROS leakage, and also due to the reported data showing lower mitochondrial ROS production in birds (see above), that he later in the article uses as well as an argumentation of how insulin resistance would lead to higher longevity in birds partially through a lower ROS production by increasing the mitochondrial uncoupling. Furthermore, one could ultimately raise the question of how both increasing and decreasing oxygen consumption could be adaptations to the same condition (i.e. hypoxia), unless other concomitant changes are proposed (like differences in uncoupling levels). Secondly, one of the main arguments used is that gene loss during bird evolution led to loss of insulin sensitivity, as certain of the "lost" genes, as those corresponding to omentin, GLUT4 or UCP1/UCP2, are important in the regulation of this process; nevertheless, at least GLUT4 has

been recently found in avian genome (Huttener et al. 2021), arguing that its apparent absence was possibly due to a high GC content in bird genomes that implied a low sequencing output. This part can probably be excused, as, although GLUT4 presence in avian tissues had been proposed well (Thomas-Delloye et al. 1999; Shi et al. 2014), clearer proofs of it were not evident until the same year this article was published, and it is likely that the author was not aware of such issue, and continued the propagation of this idea, as seen elsewhere in the bibliography (reviewed in Sweazea 2022). Thirdly, the author defends the mutation of *KEAP1* in Neoaves, leading to the constitutive activation of the Nrf2 protein (a transcription factor important in antioxidant enzymes expression), as one of these key adaptations appearing through the evolutions of birds. Although this seems like a reasonable idea, and would at least partially explain how (some) birds adapted to a higher ROS production after increasing oxygen consumption, there seems to be a persistent confusion about basic avian taxonomy somehow obscuring the argumentation. It may seem like the author says Neoaves meaning Neornithes (the group containing all current bird species), as this terminus is mentioned when referring to birds in general, although in a footnote he corrects such possible error making a distinction between Paleognathae and Neoaves (both Neornithes groups), probably confounding this latter group with Neognathae, which again gets corrected by his erroneous inclusion of some Neognathae (chicken) into Paleognathae, introducing however another now evident error, and therefore making the argumentation more confusing and declaring null knowledge about bird taxonomy (which is relevant when posing macroevolutionary hypotheses about big genome and physiological/morphological rearrangement of whole taxonomic groups in face of certain environmental shifts). In fourth place, the reason why hypoxia would lead to “insulin resistance” (or at least favour it after gene loss happened) seems to be partly based on an argumentation inspired by the idea that both insulin and hypoxia activate HIF-1 (Hypoxia Induced Factor-1), both inhibiting mitochondrial biogenesis and activity (Glassford et al. 2007; He et al. 2011); nevertheless, this is exemplified by cases of adipocytes, which are not particularly metabolically active cells, as it is the liver, where birds conserve “insulin sensitivity” (Dupont et al. 2009), and in a context mostly related with obesity, so that this change in mitochondrial number and activity in adipocytes would probably not be of great significance changing overall bird metabolism. Although it is worth considering here the also cited research by Katic et al. (2007), it should be borne in mind that: 1) the authors of this research calculate the metabolic rate dividing the oxygen consumed by the individual mice by Kg of body mass, something a priori inadequate, considering issues as differences in body composition across the tested individuals or groups, as it is indeed the case here between the control and FIRKO (Fat specific Insulin Receptor KO) mice (even if it may be argued that it still represents a difference in whole body metabolism, in this case as an increase in fat tissue oxygen consumption, it would be more adequate to report lean and fat mass metabolic rate separately, as the percentage and total fat mass are anyways calculated in the study) and 2) the effects observed in these mice may differ from what it would be expected in birds, as the general health state of the mice is suggested to be affected by differences in signalling mediated by adipokines like leptin, which is expressed in birds mostly in other parts rather than in adipose tissue, and with different functions as those known in mammals (Friedman-Einat and Seroussi 2019). In the fifth place, the increase in circulating glucose, supposedly as a consequence of insulin resistance, would be “energy substrates [glucose and ketone bodies] needed to maintain a high metabolic rate and to enable birds to do sustained heavy exercise, such as long flight” (Satoh 2021), what would at least not line up with the appearing of this feature as an adaptation in non-avian theropods, and second, is not supported by the fact that birds are prone to use of lipids, in comparison to glucose, mainly during long-term flight (see e.g. Weber 2009), and is not generally believed to be the main

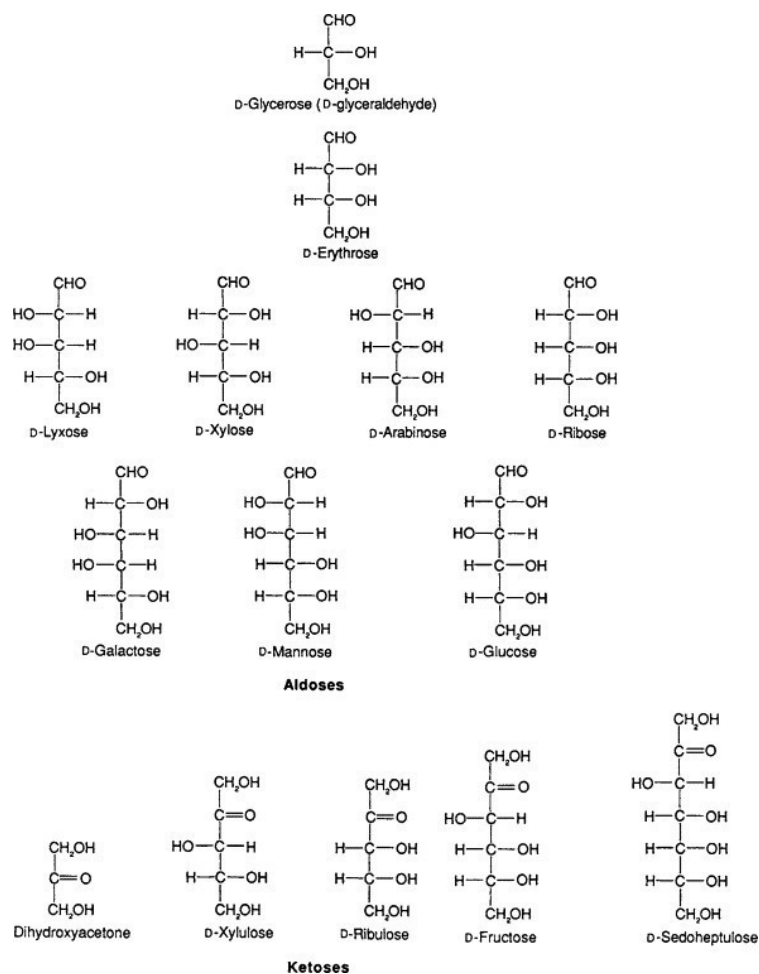
reason why birds have high glycaemia (Braun and Sweazea 2008). Finally, birds conserve main insulin functions related to anabolism, linked to PKB/Akt (Protein Kinase B), and this reported “insulin insensitivity” is related to Insulin receptor and IRS-1 (Insulin Receptor Substrate-1) tyrosine phosphorylation and glucose uptake (Dupont et al. 2009), and mostly with adipocytes (Sweazea 2022), which can be explained by the fact that lipids are synthesized in birds principally in the liver, and then transported by blood to the adipose tissue, where they will be stored (Johan Buyse and Decuyper 2015), so insulin action on adipocytes become much less relevant.

### 3. Glucose as a key “pleiotropic” molecule: metabolism and ageing

#### 3.1. What are “sugars”?

##### 3.1.1 Basic notions and metabolic pathways

Sugars can be defined as solid (at standard conditions), water soluble, white, crystalline and sweet (as tasted by humans) compounds with reducing potential (for “reducing sugars”). This classic definition refers to some of the characteristics that can be used to tell them apart from other compounds, but do not tell us much more about their constitution or their functional importance (except mainly the “reducing” part, as we will see later). Sugars are also carbohydrates, one of the main components of living beings, together with proteins, nucleic acids and lipids. But not all carbohydrates are sugars. Particularly, we call “reducing sugars” a subset of low molecular weight carbohydrates that have the mentioned capacity of having a relatively high reduction potential in the biological environment in which they are found. To understand this, the chemical characteristics of carbohydrates have to be outlined: these are organic compounds formed by a different number of monomers whose carbon chains are substituted with alcohol radicals in every carbon except for a carbonyl in one of them. This carbonyl can be a keto or an aldehyde group, dividing monosaccharides (single monomer carbohydrates) into ketoses and aldoses. Monosaccharides can also have a different number of carbons, from three to seven, being classified thus into aldo/keto-trioses/tetroses/pentoses/hexoses/heptoses (see **Figure 1**), from each of which several stereoisomers with different properties exist. They can also be combined in disaccharides or polysaccharides of different kind, depending on the particular combination of molecules and the kind of bond they form. Despite of all of this variety of forms, some of them are much more common than others in living beings, with variation also depending on the taxonomic group. Interestingly, D isomers are vastly predominant in life forms (although exceptions exist, see Pikuta et al. 2006). Pentoses and hexoses, the more common ones, are normally present in a cyclic form (see Angyal 1984), with their anomeric carbon (the one with the carbonyl group) forming a bond with other of the carbons within the molecules. As this carbonyl group is what gives the molecule its reducing power, only the open linear form is reactive, as the carbonyl group remains free. Nevertheless, the percentage of linear form present in the body of an organism is normally very low, with reported values in aqueous solution at 20° C ranging from 0.002 for glucose to 0.7 % for fructose (Hayward and Angyal 1977). Still, as it will be described later (see section 3.2.2.), some of these linear forms can non-enzymatically react with nitrogen and sulphur groups from other organic molecules, occasioning an alteration on such molecules with possible negative consequences.



**Figure 1.** Examples of some monosaccharides of different kinds (Figure 9.1 from Bhagavan, 2002).

Among the most common reducing sugars, we find glucose, an aldohexose of importance in the metabolism of many organisms. Glucose circulates freely in vertebrates' plasma and it is internalized by cells through membrane transporters by facilitated diffusion, where it is mainly used as a catabolic substrate through glycolysis (see **Figure 2**), producing 2 ATP (Adenosine triphosphate) and 4 NADH (reduced form of Nicotine Adenosine Dinucleotide). As it can be seen in **Figure 2**, the last product of glycolysis is pyruvate, which can in turn be either reduced to lactate in a fermentation process or degraded to acetyl-CoA in the mitochondria, which is in turn combined with oxaloacetate to form citrate, entering this way in the Krebs cycle (see **Figure 3**). This cycle gives as a final product CO<sub>2</sub>, 1 GTP (Guanosine triphosphate, equivalent to ATP) and reducing power in the form of 3 NADH and 1 FADH<sub>2</sub> (see **Figure 3**), which will then be used to feed the respiratory chain, an electron transport chain (ETC hereon) situated in the mitochondrial inner membrane (see **Figure 4**), and that will receive the electrons of these molecules, oxidizing them back to NAD<sup>+</sup> (by complex I) and FAD<sup>2+</sup> (by complex II), and take these electrons through several steps up to an O<sub>2</sub> molecule that will thus be reduced to H<sub>2</sub>O by ETC complex IV (see **Figure 4**). In this process, protons (H<sup>+</sup>) are pumped through the inner membrane to the intermembrane space by complexes I, III and IV such that an electrochemical gradient is formed, generating a membrane potential of about -100 to -140 mV (see Zorova et al. 2018 for a review), that will in turn be used to generate ATP by letting the H<sup>+</sup> go back into the mitochondrial matrix through the ATP synthetase (also referred to as complex V). This process, which is known as oxidative phosphorylation (OXPHOS), and constitutes the main source of energy in the form of ATP within the cell (see Rolfe and Brown 1997), is therefore crucial and

the efficiency of its machinery is of utmost importance for the functioning of the whole organism. However, there are many factors affecting such efficiency, depending on the substrate being used to feed the ETC and the conditions of the system in general (e.g. the temperature). These factors will affect the final outcome, which can be modulated in some cases to support different states adequately (e.g. exercise, ambient temperatures, etc.) (see Rolfe and Brown 1997). This way, for example, the mitochondria can uncouple the conduction of  $H^+$  back into the matrix and the consequent  $O_2$  consumption from the ATP production by employing UCPs (Uncoupling Proteins), in order to generate heat or reduce oxidative stress (Brand 2000; Mozo et al. 2005; Criscuolo et al. 2005).

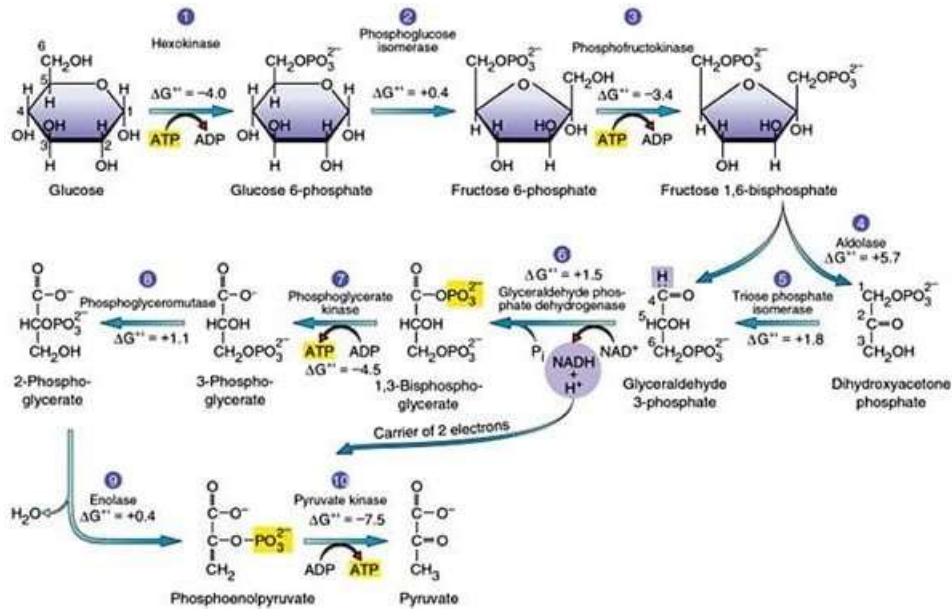


Figure 2. Glycolysis – Embden-Meyerhoff pathway.

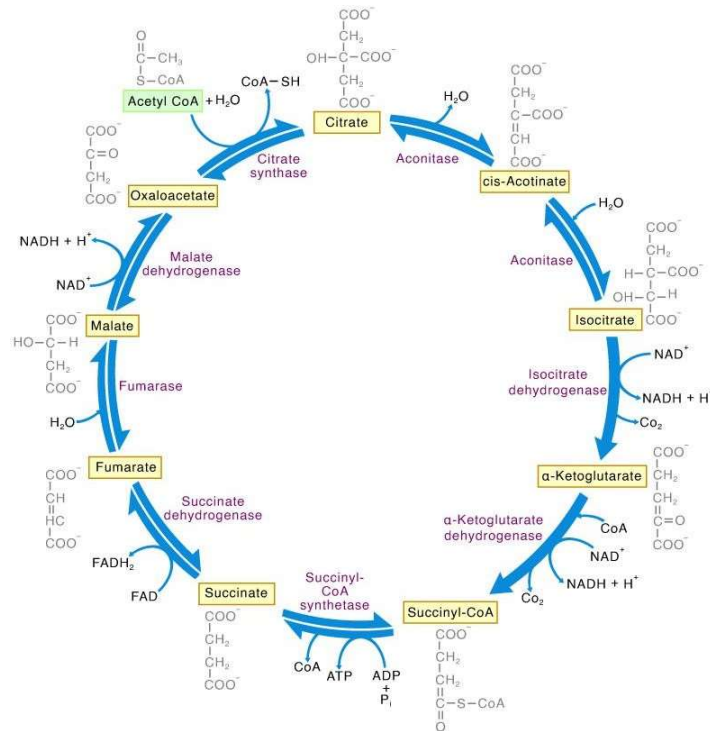


Figure 3. Krebs cycle.

Glucose can also be converted to glycogen, a polysaccharide form, to be used as an energy reserve, mainly in the liver and striated muscles, so it can be used during high and rapid energy demanding activities, or even be transformed into lipids by using acetyl-CoA to build up fatty acids and the trioses generated during glycolysis (glyceraldehyde 3-phosphate and dihydroxyacetone 3-phosphate) to generate glycerol. Lipids release more energy per unit of mass than carbohydrates when totally catabolized (Kleiber 1961) and are hydrophobic (contrary to carbohydrates, that adsorb water), so they are good for accumulating energy for the long term. The balance between these described catabolic and anabolic pathways in the cells is regulated by several hormones, as it will be described further in the next section (3.1.2).

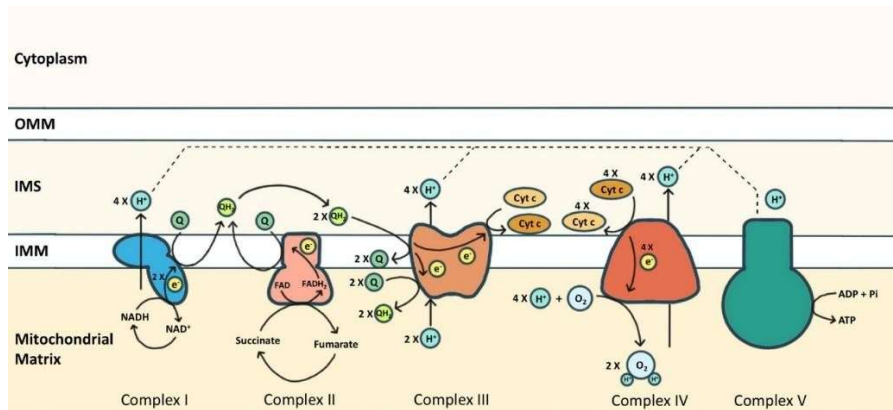


Figure 4. Respiratory ETC.

Glucose can also be directed toward pentose phosphate pathway, a metabolic way in which it gets partially oxidized, releasing  $1\text{CO}_2$ , and producing  $\text{NADPH}+\text{H}^+$  (Nicotine Adenosine Dinucleotide Phosphate), which is very important for synthesis of fatty acids and other macromolecules and other reducing functions, as regenerating glutathione (a very important cell antioxidant), which makes it important for maintaining oxidative status. This pathway can then be used for either generating more substrates for glycolysis or to produce phosphate pentoses for supporting nucleotides synthesis (Figure 5) (see Stincone et al. 2015 for a review).

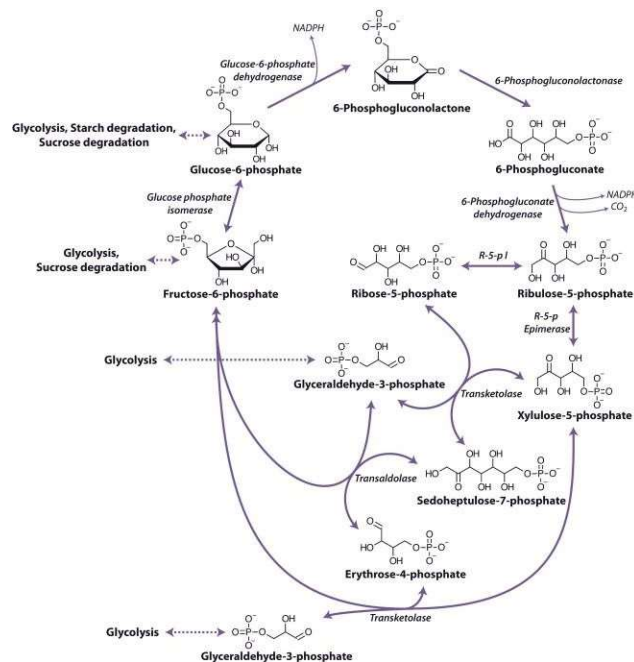
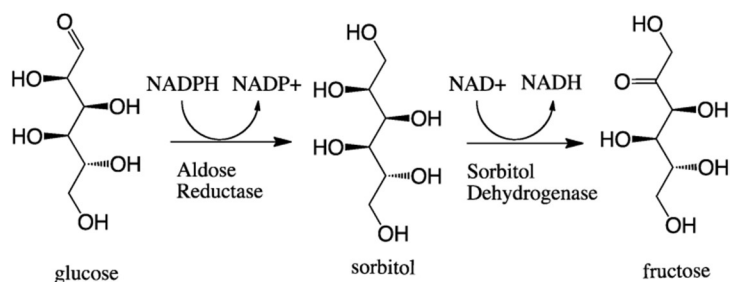


Figure 5. Pentose phosphate pathway.

Finally, there are some other ways in which glucose can be used by the cells, as in enzymatic glycosylation, a process acting as a post-translational modification for many proteins, or to form glucolipids for the membranes (see Springer and Gagneux 2016). It is also important to mention the polyol pathway (**Figure 6**), as it has relevance in the pathogenesis of diabetes, by the accumulation of sorbitol produced by the excess of sugars, leading to osmotic and oxidative stress (see e.g. Chung et al. 2003; Garg and Gupta 2022; Yan 2018). In many species, glucose can also be used as a precursor of the antioxidant ascorbic acid (Linster and Schaftingen 2007), known as vitamin C (as it is essential for humans).



**Figure 6.** Polyol Pathway

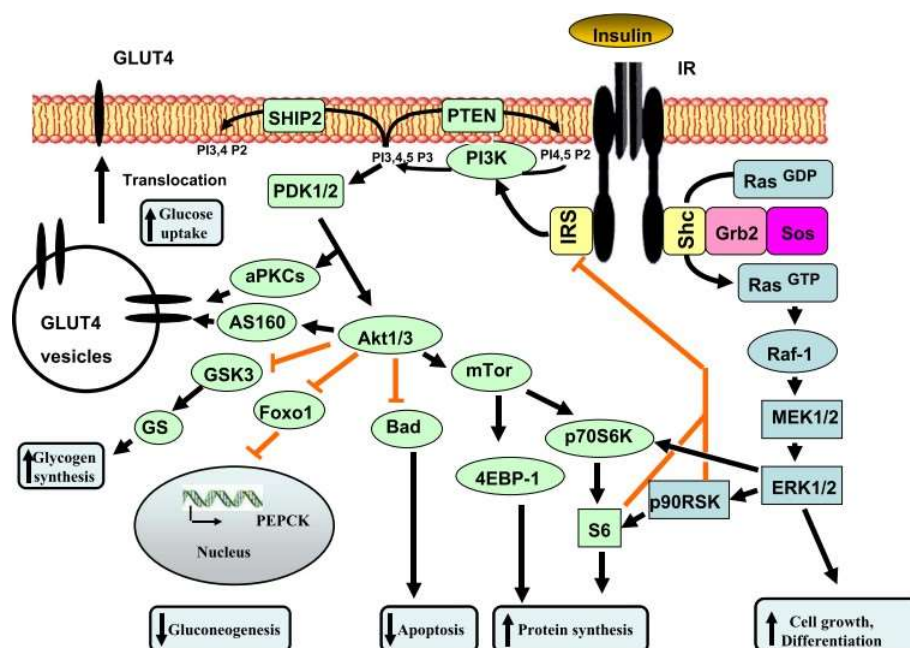
### 3.1.2 Hormonal control

Maintaining sugars circulating levels within a range is a crucial homeostasis process as it is important for cells to have a constant source of energetic substrate (Szablewski 2011). That way, several hormones take part in such process, with the most important in vertebrates being insulin and glucagon, although others like glucocorticoids (see below) are also very important. Despite that, different taxonomic groups will have very different levels and capacities for using different sugars in their tissues. For the case of glucose, and again centring the discussion on vertebrates, this is the most important sugar in the bloodstream, but still varies a lot in level across the different classes. In this sense, amphibians have very low levels, while mammals have higher and birds are clearly at the top, with average levels doubling those of mammals (Polakof et al. 2011). This is why birds are normally referred to as relatively hyperglycaemic, and this is the sense in which it will be used throughout this text.

The reasons why loss of regulation of glucose levels in order to maintain it within a certain limit is problematic are different in the case of hypoglycaemia (too low levels) and hyperglycaemia (too high glucose levels). The reasons for hypoglycaemia are pretty simple: cells would not have sufficient access to it in order to use it for its multiple described usages (see e.g. Frier 2009; Amiel et al. 2021). However, the reasons for hyperglycaemia to be problematic is because this state is related to several pathologies, mostly related to a syndrome known as diabetes (Egan 2018). The mechanism involved in it and its relation with the ageing process will be described in the next section (3.2.).

Going back to the factors regulating blood glucose levels, the main hormones, i.e. insulin and glucagon, are protein hormones produced and released to bloodstream by the pancreatic (or hepatopancreas in groups in which these organs are not differentiated) islets:  $\alpha$ -islets for glucagon and  $\beta$ -islets for insulin. Insulin is, in general, a hypoglycaemic hormone, and one of the main anabolic factors, and glucagon is hyperglycaemic and catabolic. While insulin has higher levels during absorptive phase (i.e. right after eating), glucagon increases its levels during the post-absorptive phase (normally several hours after the ingestion, varying depending on the species). While insulin promotes the internalization of glucose by the location of inducible

transporters into the plasmatic membrane (although see 4.1. for the case of birds) and its usage either by glycolysis (anaerobic catabolism) or for the synthesis of glucagon and lipids (anabolism), glucagon will promote glycogenolysis and lipolysis, to release metabolites into the bloodstream (including glucose). The level of these hormones is regulated mainly by feeding status, being glucose itself, some aminoacids and lipids the main metabolites leading to the release of insulin by pancreatic islets though their catabolism and ATP production, which will work by phosphorylating and thus closing  $K^+$  channels, therefore decreasing membrane conduction of this ion towards the extracellular medium and depolarizing it, which leads to the entrance of  $Ca^{2+}$  and release of vesicles content (i.e. insulin) (see e.g. Pipeleers et al. 1985; Corkey 2014). This way, insulin release depends on the energetic state of the cells, although it also depends on the extracellular medium, as other hormones like glucagon, incretins (stimulatory), somatostatin, or adrenaline (inhibitory) (see e.g. Rorsman and Braun 2013). This way, insulin, and its signalling pathway, becomes one of the central axes of nutrient signalling. This pathway is mainly formed by the IR (Insulin receptor), IRS (Insulin Receptor Substrate), PI3K (Phosphatidylinositol 3 Kinase) and PKB/Akt (Protein Kinase B), acting on TOR (target of Rapamycin) (see **Figure 7**), stimulating the synthesis of proteins, but also on other factors as FOXO, a transcription factor modulating a wide variety of processes from metabolism to cell differentiation (Accili and Arden 2004). However, insulin also plays a role in oxidative status by inducing the activity of  $\Delta 6$  desaturases of fatty acids (Brenner 1981), increasing this way their degree of polyunsaturation and therefore reducing their resistance to oxidation (Hulbert 2005). This way, insulin levels mediate ageing through both regulating nutrient signalling and oxidative status (see section 1.2.2.). Indeed, its role in nutrient signalling, together with IGFs (Insulin-like Growth Factors) released by the liver (mainly IGF-1 during post-embryonic development) by the action of GH (Growth Hormone), which act in a very similar way, is being widely studied in the context of its role on the life-extension consequences of dietary restriction, which underregulates such signalling pathways (Moatt et al. 2020). In this sense, alterations on insulin signalling, as in diabetes, can act accelerating ageing process, not only by its effects on glucose levels, but through the outlined metabolic mechanisms.



**Figure 7.** Insulin signalling pathway as it is known in mammals (from Dupont et al. 2009).



Finally, as mentioned above, some other hormones, so called stress hormones, will act on glucose metabolism and glycaemia (blood glucose levels), when organisms may be facing an unexpected and potentially menacing situation, perceived as a stressor, like a predator or competitor or a change in environmental conditions such as in temperature. These hormones are mainly adrenaline and noradrenaline in the short term (i.e. in the range of seconds), produced by the sympathetic nervous system, and released to the bloodstream by the adrenal medulla, and glucocorticoids like cortisol and corticosterone in the “long-term” (from few minutes on its acute version to several days on its chronic version), produced and released by the fascicular layer of the adrenal cortex (see e.g. Axelrod and Reisine 1984; Kyrou and Tsigos 2009). These hormones induce catabolism of glycogen and lipids, in order to promote the utilisation of metabolites by cells and to release them (glucose and fatty acids) into the circulation so other tissues can benefit from them, in a situation in which a rapid and energetically costly action has to be performed in order to fulfil a task important for survival or reproduction. In the case of glucocorticoids, they also act by inducing gluconeogenesis, which takes place mostly from aminoacids in the liver (also kidneys in birds (Scanes 2015), and in some cases as hummingbirds even the heart: see Gershman et al. 2023), and acts liberating it into the circulation for its usage by other tissues as skeletal muscle and brain. The action of these hormones, together with their induced hyperglycaemia, although very important for short term performance, when produced very frequently or sustained chronically at a high level, can induce a damage on the body that will induce a faster ageing rate as a consequence (see e.g. Hausmann et al. 2012; Hasan and Rahman 2012), mainly through the increase of oxidative stress or, in the chronic version, by actions as reducing bone and muscle mass, leading to higher risk of breakage and a state of frailty (reviewed in Sato et al. 2018).

### 3.1.3 Environmental variation

Blood glucose levels vary normally within a healthy range depending on several factors, mainly food intake, exercise and stressful situations. It also varies daily with a circadian rhythm: in diurnal humans, for example glucose levels are higher in the morning, but also insulin, while hepatic glucose production is lower, which leads to a lower postprandial hyperglycaemia. All of this is opposed during the inactive phase. Indeed, alterations of these rhythms can lead to disfunction like an increased type 2 diabetes mellitus (T2DM) risk in nightshift workers (Wang et al. 2011) and worsened glucose regulation when feeding schedule is irregular (Bazotte, et al. 1989). Other factors clearly alter glucose homeostasis through insulin resistance or other mechanisms, like diet, with high fat diet increasing insulin resistance (e.g. Blázquez and Lopez Quijada 1968; Kim et al. 2000; Clegg et al. 2011) while fibres improve glucose tolerance and glycaemia control (Goff et al. 2018). Some of these mechanisms show a neural control, either from the autonomous or the central nervous system (Arble and Sandoval 2013). Most of the unpredictable environmental changes are nevertheless regulated through stress mediating hormones as catecholamines like adrenaline and noradrenaline and glucocorticoids (see section 3.1.2).

In other animals, several environmental factors have been observed to affect glycaemia. Higher temperature and longer day length were related with lower glucose levels in zebra finches (*Taeniopygia guttata*), while harsher environment (higher brood size and more costly foraging) are related to higher glucose (Montoya et al. 2018). This is paralleled in striped mice (*Rhabdomys pumilio*), where higher food abundance and solitary living is related to higher blood glucose levels, while higher daily minimum temperature is related to lower glucose levels (Schradin et al. 2015). Circadian (Frelin 1974; Siegel et al. 1976; Jenni-Eiermann and Jenni 1997; Ramage-

Healey and Romero 2000; Lobban et al. 2010; Downs et al. 2010) and seasonal variation (Bairlein 1983) has also been observed in some species of birds, with higher glucose during migration in some cases (e.g. Bairlein 1983; Remage-Healey and Romero 2000). Differences between years and habitats in glucose values also suggest environmental effects playing a role on it (e.g. Kalinski et al. 2014; Skwarska and Wawrzyniak 2015 in tits).

## **3.2. Facing a sweet death: dysregulation of sugar metabolism in disease and ageing**

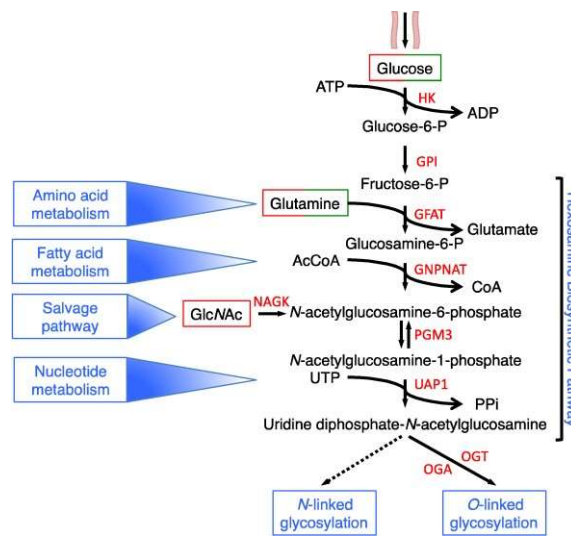
### **3.2.1 Blood glucose dysregulation and diabetes**

Dysregulation of glucose metabolism can appear in three “flavours”: hypoglycaemia, hyperglycaemia and poor glycaemic control (i.e. inadequate fluctuations). Also, these alterations can be referred at different phases of metabolism, mainly in relation to food intake, as postprandial (shortly after ingestion), post-absorptive (several hours after ingestion, depending on the species) and fasting glucose levels (long after ingestion). The first case, hypoglycaemia (i.e. levels lower than certain limit determined considering the species normal values), is clearly noxious, given the importance of glucose in metabolism (see section 3.1.2.). Hyperglycaemia (i.e. glucose levels above certain limits determined for a species), which can be transient or chronic (i.e. sustained), can lead to several health issues, mainly those related to the pathological state known as diabetes (see below). Finally, inadequate fluctuations in blood glucose levels, i.e. consistently present variations in glucose levels out of those that would be the norm in healthy individuals (e.g. transient postprandial hyperglycaemic peaks significantly higher or longer than a certain limit), could also be noxious (see discussion in section 3.2.3). Also, poor glucose tolerance, measured for example as a higher AUC (Area Under the Curve) of the blood glucose concentrations over a certain standardized period of time in a GTT (Glucose Tolerance Test), in which an individual receives a known dosage of glucose (orally or injected) can be a predictor of pathology (see e.g. Schnell and Standl 2006; Sakaguchi et al. 2016) beyond fasting plasma glucose or glycosylation levels (see below).

In this context, variations in glucose levels related mainly with higher glycaemia and poorer glycaemic regulation are part of the pathogenesis of diabetic syndrome (i.e. diabetes mellitus). Diabetes is a complex set of pathologies based on metabolic disorders leading to several pathologies which all have in common the prevalence of sustained hyperglycaemia and normally poor glucose levels regulation. It is normally classified in type 1 and type 2 diabetes, depending on if it is caused by deficiency on the insulin itself or its production (type 1) or by insulin signalling resistance (type 2) but there is a whole lot of other types, like gestational diabetes or mature-onset diabetes of the young (MODY), with several genetic or environmental causes. It has been studied mostly in humans, where it is diagnosed, as accepted by the WHO (World Health Organization) by fasting (at least 8 hours after ingestion) plasma glucose level  $\geq 7$  mM or  $\geq 11.1$  mM 2 hours after an oral GTT of 75 g of glucose dissolved in water, or instead  $\geq 11.1$  mM at any moment if it comes with symptoms like polydipsia (excess drinking), polyuria (excess urination) and mass loss. It can also be diagnosed by glycated haemoglobin levels  $\geq 6.5$  %, which represent the state during the last 2-3 months although this last measurement is not valid under certain conditions (see Egan and Dinneen 2022). Following recent reports, it affects (by 2021) 10.5% of the human population (536.6 million individuals), and it is expected to rise to 12.2% (783.2 million) in 2045 (Sun et al. 2022). Out of humans, different cases of this pathological state has been reported, varying on its characteristics, for example on dogs, cats, cattle, horses, several primates and some bird species, mostly raptors and parrots (Niaz et al. 2018; see section 4.1.2).

This syndrome is related to several pathologies, affecting multiple organ systems, causing mainly cardiovascular diseases (principally atherosclerosis, but also leading to increased heart infarction risk), and nephropathies (chronic kidney disease), neuropathies (as peripheral neuropathies or dementia), retinopathies, cataracts, etc (see e.g. Nathan 2014; Kiziltoprak et al. 2019; Harding et al. 2019).

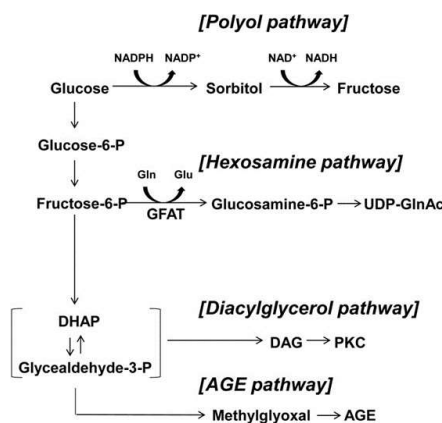
Several mechanistic factors are believed to underlie the pathophysiology of this disorder: 1) hypertension linked to the higher circulating levels of glucose and polyols (derived from higher polyol pathway flux, see section 3.1.1), 2) increased oxidative stress (from several sources), 3) augmented non-enzymatic glycation and the consequent formation and accumulation of AGE, 4) elevated hexosamine pathway flux (**Figure 8**) and 5) enhanced PKC (Protein Kinase C) activation (Brownlee 2001; Brownlee 2005).



**Figure 8.** Hexosamine pathway.

Some of these different mechanisms are interconnected (see **Figure 9**), with polyol pathway producing fructose, which has higher glycation activity than glucose (see e.g. Schalkwijk et al. 2004), oxidative stress enhancing glycation and AGE formation or AGE inducing oxidative stress through inflammation.

Among all the abovementioned processes, during the next sections (3.2.2. and 3.2.3), I will dig further on glycation and AGE, as they are the most importantly addressed within the context of this thesis.

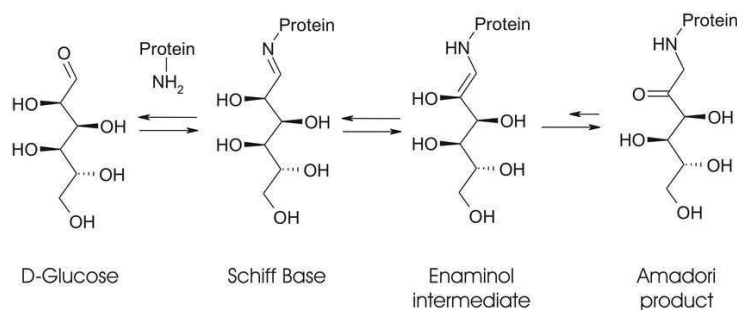


**Figure 9.** Pathways promoted by hyperglycaemia.

### 3.2.2. Glycation and AGE formation and diversity

One of the main and most studied of these mechanisms is glycation and AGE formation. Non-enzymatic glycation consists of a reaction of reducing sugars with nitrogen or sulphur-based residues, contained in aminoacids (free or within proteins), nucleic acids, lipids like phosphatidylethanolamine, etc. It is important to mention that not all reducing sugars have the same reactivity, with aldoses (like glucose) being more reactive than ketoses (like fructose or ribose), and the degree of reactivity depending on the percentage of the molecule present in the open versus the circular form. This may have affected the evolution of the abundance of different sugars and their usage as a metabolic substrate (Bunn and Higgins 1981).

This reaction, firstly described by Maillard (1912) after observing the “browning” of heated foods, happens by a Schiff base formation between the carbonyl group of a sugar (see section 3.1.1.) and the amino, guanidine or thiol group of another molecule, which then undergoes an Amadori rearrangement (**Figure 10**) and becomes irreversibly bound to such molecule. These reactions can also undergo further steps, producing other compounds like dicarbonyls (**Figure 10**), even more reactive than the initial ones, which in turn may react back with the same nitrogenated or sulphur compounds to form AGE. These AGE come in many different forms, depending on the kind of dicarbonylic compound and what it reacts with, which generally consists of either free aminoacids (lysine, arginine or cysteine) or those bound to peptides/proteins, forming low or high molecular weight AGE, respectively (see **Figure 11**). Although, as mentioned, such process was initially studied on foodstuffs, it was later recognised to happen also *in vivo*, as body temperatures can certainly promote it at a level particularly increased when sugars have higher concentrations in the inner medium (Twarda-clapa et al. 2022).



**Figure 10.** Amadori reaction (from Laga 2008, adapted from Ulrich and Cerami 2001).

Some of these mentioned reactive compounds are dicarbonyl like glyoxal, methylglyoxal, diacetyl, 3-deoxyglucosone, 1-deoxyglucosone, but sometimes the reaction can happen also with monocarbonylic compounds other than sugars, like glycolaldehyde or glyceraldehyde. Also, the source of these is not necessarily the rearrangement of Amadori product, as other reactions can produce them, as is the case of the autooxidation of sugars, promoted by oxidative stress (Wolff et al. 1991). In the case of methylglyoxal, it can also be produced from trioses phosphate from glycolysis, acetone from lipids or aminoacetone from aminoacids' catabolism, respectively (Kalapos 2008). Glycolysis is a particularly relevant source of methylglyoxal (Allaman et al. 2015), as when glucose concentration within the cell becomes sufficiently high, and/or OXPHOS cannot handle efficiently all the NADH produced by its catabolism, so NAD<sup>+</sup> is not regenerated, trioses phosphate may accumulate and be converted into methylglyoxal (**Figure 12**). Therefore, cell redox state can this way also affect indirectly (methylglyoxal derived) AGE formation (Hipkiss 2010).

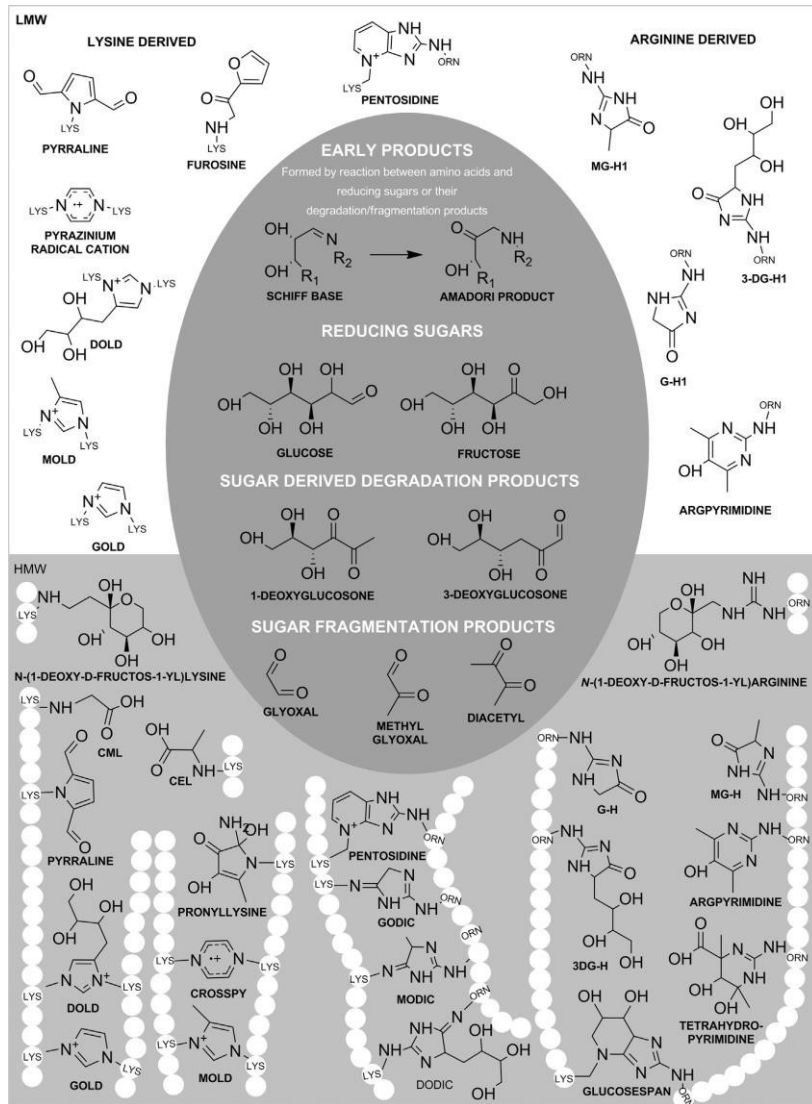


Figure 11. AGE classification (from Poulsen et al. 2013).

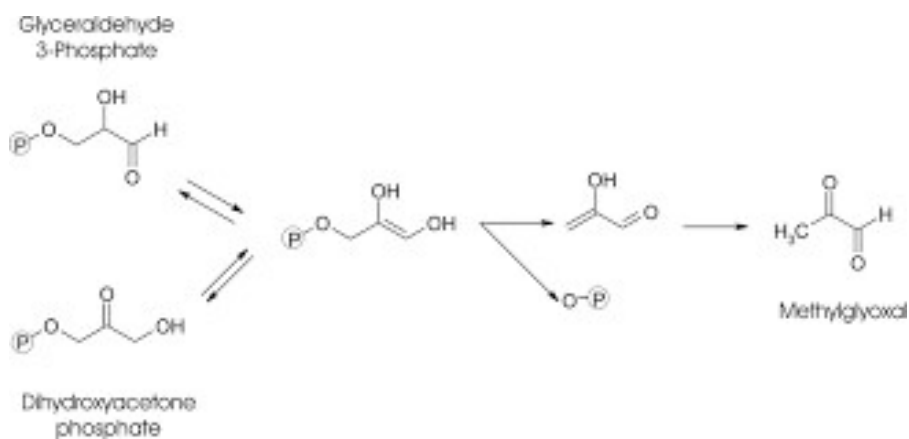


Figure 12. Methylglyoxal production by glycolysis (from Laga 2008, adapted from Thornalley 1990).

### 3.2.3 Glucose, glycation and AGE in pathogenesis, including ageing

As mentioned in the previous sections, glycaemia dysregulation can lead to several health issues. In the case of hypoglycaemia, defined arbitrarily in humans as (1) glucose values under 50 mg/dl with (2) symptoms as neuroglycopenic damage, i.e. nervous system failures due to low glycaemia, that (3) solve after glucose levels rise back to normal, a set of traits known as Whipple's triad (see Brown 2021), or less than 40 mg/dl without them (see e.g. Szablewski 2011), it can be dangerous mainly because of the dependence of the brain on glucose as a catabolic substrate, causing several unspecific symptoms like dizziness, fatigue, loss of consciousness, etc. Nevertheless, this definition has some limitations (Ademolu 2020), and its symptoms can also be confounded with those of a panic attack, as it goes accompanied by a sympathetic response (Szablewski 2011), with a liberation of catecholamines to stimulate the liberation of glucose reserves (Lins et al. 1986). Although it rarely happens in non-diabetics, it remains one of the main worrying of diabetic patients, and it can not only produce death by itself, but greatly increase future death risk, including through cardiovascular disease (Amiel et al. 2021).

Hyperglycaemia, on the other hand, is normally associated to deficient insulin secretion or insulin action (insulin resistance), and constitutes one of the main definitory characteristics of diabetes (see section 3.2.1.), and is normally related to hypertension, increased LDL (Low Density Lipoprotein), or at least a shift in their kind, and decreased HDL (High Density Lipoprotein) (see e.g. Simonson 1988; Laakso 1996; Latha et al. 2023). There are several kinds of hyperglycaemia: fasting hyperglycaemia (defined in humans as a blood sugar higher than 90-130 mg/dL/5-7.2 mmol/L after fasting for at least 8 hours), and postprandial hyperglycaemia (in humans, blood sugar usually greater than 180 mg/dL/10 mmol/L) (see Szablewski 2011). This can be related with several symptoms like vision problems, frequent skin or vaginal infections, impaired wound healing, polydipsia (frequent drinking), polyuria (frequent urination), headaches or weight loss (see Szablewski 2011). Nevertheless, these levels are not normally pathological if they return to normal after 12-24 hours and if they are caused by punctual event like a very large meal.

Nevertheless, high glycaemia variability may be of higher concern than mere high mean glucose levels, although its relation to pathological outcomes is not yet as clear (Siegelar et al. 2010). Glucose variability can be measured in many different ways, the simplest one as the Standard Deviation (SD) or Coefficient of Variance (CV) of a set of measures. The problems with this measure are, however, first that SD is, by definition, not adequate for non-normally distributed variables (as it is the case with blood glucose concentration), which may nevertheless be solved by log-transforming the values (although that may bring other problems as underestimating the effect of extreme values) and second that individuals with the same mean and SD in glucose values can have very different variation profiles, as e.g. a cyclic variation within certain limits or sustained very low levels followed by sustained very high levels within the same limits. There are other ways of measuring glucose variation, like MAGE (mean amplitude of glycaemic excursions), considering excursions variations above 1 SD, CONGA (continuous overlapping net glycaemic action), which consist into the SD of the differences between an observation and another observation  $n$  hours before, or MODD (absolute mean of daily differences), designed to take into account interday variations, and it is calculated as the mean absolute value of the differences in glucose levels between consecutive days at the same time of the day (this has the problem of possible differences in meal hours or different activities performed at different days). Another considered option to measure glucose excursions is concentrations of 1,5-anhydroglucitol (1,5-AG), a polyol whose levels decrease with hyperglycaemia, as its recapture

in urine is inhibited, but it stops being correlated with glucose values when hyperglycaemia is sustained (see Siegelaaar et al. 2010 for a review on these indexes). Although some of those do not show the mentioned problems of SD, this parameter is still potentially useful as it has been shown to positively correlate with mortality in non-diabetic critically ill patients, i.e. those in Intensive Care Unit (ICU). The way this is supposed to act is by increasing oxidative stress, although the results are not consistent, with experimental studies in vitro and in rats differing from some studies in humans (reviewed in Siegelaaar et al. 2010). Also, out of such severely ill individuals, long-term variability seems more important than short-term one, although more research is needed.

One of the main outcomes of hyperglycaemia is protein glycation. Among these, the proteins that have more extensively been studied are haemoglobin and albumin. Glycated haemoglobin (being HbA1c the most commonly measured version in humans) is a more classical proxy of diabetes, and it has been widely studied since it was discovered in the 60s and 70s (see Rahbar 2005), its usage being rapidly implemented for clinical assessment (see e.g. Koenig and Cerami 1975; Koenig et al. 1976; Jovanovic and Peterson 1981). There is an extensive bibliography on its usage as a marker of several diabetes-related pathologies (see e.g. Rezaee et al. 2016; Liu et al. 2021; Casadei et al. 2021; Bao and Gu 2021; but also Dunn et al. 2023 for an improvement of the index) and certain levels of it are employed as thresholds for diabetes diagnosis (see section 3.2.1). This is justified because glycated haemoglobin levels reflect average glucose levels during the previous weeks (Cefalu et al. 1994), and up to 120 days (4 months) (see e.g. Bunn 1981; Makris and Spanou 2011), the mean lifespan of human erythrocytes. More concretely, its variation seems to be explained at 50% with the current month, 25% with the previous one and the remaining 25% by the rest (Tahara and Shima 1995). Nevertheless, HbA1c levels have also been found to vary with ethnicity (e.g. Selvin et al. 2011) and potentially other factors as Hb variants or anaemia, although Cavagnoli et al. (2015) found no effects on a meta-analysis of sickle cell anaemia (HbS) nor uraemia, but the effects of iron deficiency/iron deficiency anaemia were inconclusive. It has also been shown to increase with age (in humans, Kilpatrick et al. 1996) and positively correlate with blood glucose (e.g. Makris and Spanou 2011; Lledó-García et al. 2013). Indeed, given these kinds of concerns about variability, Hempe et al. (2002) suggested the use of Haemoglobin Glycation Index (HGI), based on the number of SD HbA1c levels deviated from those expected for the given glucose levels. Wu et al. (2021) showed this index to be positively related to composite cardiovascular disease (CVD) and all-cause mortality in a meta-analysis of prospective studies on type 2 diabetic patients, but the results being non-significant when also accounting for HbA1c indicate that the index itself may not provide too much of an additional insight. Indeed, glycated haemoglobin has been related to, among others, cardiovascular disease (Selvin et al. 2004) and retinopathies (The Diabetes Control and Complications Trial Research Group 1995).

In this line, glycated albumin (the most abundant plasma protein) has also increasingly been considered as a diagnostic tool (see e.g. Kim and Lee 2012; Furusyo and Hayashi 2013). Given that plasma proteins are much more exposed to blood glucose, and reparation enzymes are not present freely in plasma as within the cells (see section 3.2.4), the levels of glycated albumin are much higher than those of haemoglobin (Guthrow et al. 1979). Also, they indicate glucose values over shorter time spans, potentially of around 2-4 weeks in humans (see Guthrow et al. 1979; Yue et al. 1980; Dolhofer and Wieland 1980; Armbruster 1987), given that the half-life of human albumin is 17 days (Levitt and Levitt 2016), and it is much lower in other cases, as around 2 days in rats, regardless of its glycation state (Day et al. 1979), and have been shown to reflect glucose excursions better than HbA1c (Yoshiuchi et al. 2008; Suwa et al. 2010), which makes it a more

reliable marker to study more short-term glucose homeostasis deregulation. Glycation affects albumin function (Rondeau and Bourdon 2011) and it can increase oxidative stress (Cohen et al. 2003) and AGE formation (Kim and Lee 2012), which could be part of what explains why its glycation levels are also positively correlated with microvascular dysfunction (Selvin, Francis, et al. 2011), higher glycation levels seem also to be related with atherosclerosis (Moon et al. 2012; Song et al. 2012; Furusyo et al. 2013) and certain macrovascular pathologies (Pu et al. 2007) and can be used to predict diabetes (Furusyo et al. 2011) and mortality (Rooney et al. 2022). Interestingly, although albumin and haemoglobin glycation levels are positively correlated, the higher the plasma albumin levels, the lower the haemoglobin glycation rate, which may indicate that albumin may act as a protector against haemoglobin glycation (Kulkarni 2018). Furthermore, albumin glycation rate increases with plasma glucose, albumin concentration and age (Suzuki et al. 2012). Finally, it is also worth mentioning that, opposite to what is shown with glucose and glycated haemoglobin, body mass index (BMI) and glycated albumin levels have been found to be negatively correlated in humans (Selvin et al. 2018), although the reasons for it are not yet well understood.

A last option to measure glycation levels is fructosamine, which consists into a general marker of total plasma protein glycation, and therefore is more useful for reliably monitoring plasma glucose levels over shorter periods of time (2-4 weeks) than haemoglobin (3-4 months), in a similar way as glycated albumin. It has been shown to reflect glucose and haemoglobin glycation levels and to be an independent predictor of risk of development of pathologies as diabetes, retinopathies or chronic kidney disease, and also for cardiovascular, heart coronary disease and all-cause mortality, but not independent from haemoglobin glycation levels in such case (reviewed in Ribeiro et al. 2016). It represents thus a potential complement for haemoglobin glycation measurements and an alternative for when certain factors may alter haemoglobin glycation values independently of glucose levels. It has also been measured to monitor diabetes and estimate condition in the presence of pollutants in some birds (Gancz et al. 2007; Kothe and Mischke 2009; Ma et al. 2020).

Finally, hyperglycaemia can lead, partly through glycation, to the formation of AGE (see section 3.2.2). These compounds, which comprise a wide variety of molecules, mainly divisible between low and high molecular weight ones, can lead to several pathologies when they accumulate into tissues, as in atherosclerosis (Brownlee 2005) or chronic kidney disease, or impair protein function by the formation of cross links like in cataracts when they are formed in the lens. They are mostly eliminated by the kidneys, where they are filtrated and subsequently recaptured for apparent degradation of peptides into free AGE, so kidney failure highly increases their levels (see e.g. Thornalley 2005). AGE accumulation have been associated to ageing and some of them, as CML (Carboxymethyl lysine), have been linked to mortality (Semba et al. 2009). Other AGE, as pentosidine, have been extensively studied in relation to their association to extracellular matrices, joining to long-lived proteins as collagen and contributing to the pathologies of bones and articulations with age (Yamagishi 2011). They are also thought to be one of the main sources of diabetic complications like neuropathy, nephropathy or macroangiopathy (Singh et al. 2014; Chaudhuri et al. 2018).

One of their main sources of pathological insult is inflammation, as they activate the so-called RAGE (Receptor of Advanced Glycation End-products), a family of receptors that are activated by so called DAMP (Danger Associated Molecular Patterns), and join to multiple ligands like S100/calgranulins, high mobility group box 1 protein (HMGB1) or  $\beta$ -amieloids (Ramasamy et al. 2011; Daffu et al. 2013). These receptors induce the activation of the NF- $\kappa$ B transcription factor



and in some cells induce the activation of NOX (NADPH oxidases), increasing proinflammatory cytokines release and oxidative stress, which contributes to the development of vascular diseases (D'Agati and Schmidt 2010; Ramasamy et al. 2011). Nevertheless, other versions of these receptors exist, as the esRAGE (endogenous secretory RAGE) and the sAGE (soluble RAGE), soluble forms, that are released (the second by RAGE cleavage) to the circulation, acting as scavengers of AGE and reducing their pathogenesis (Bowman and Schmidt 2013)(see section 3.2.4).

In non-model species, most of the research on the relationship between glycation with age, fitness or life-history has been performed in birds (see Duval et al. 2024 for a review and section 4.1.3.). For AGEs, pentosidine (a particular kind of AGE) has been found to accumulate in skin collagen of several mammal species with age at a rate inversely related to the log of Maximum Lifespan (MLS) (Sell et al. 1996). This increase of pentosidine in skin with age has also been reported in turtles (Iverson et al. 2017), *Fukomys anelli* mole-rats (also glucosepane and total AGE, Dammann et al. 2012) and in some birds, but not in others (see section 4.1.3). A comparative study on AGE on several species of birds and mammals also exists, suggesting higher levels in birds but no relationship with body mass or age (Baker et al. Gabriela 2022; see section 4.1.3 for further discussion on these results).

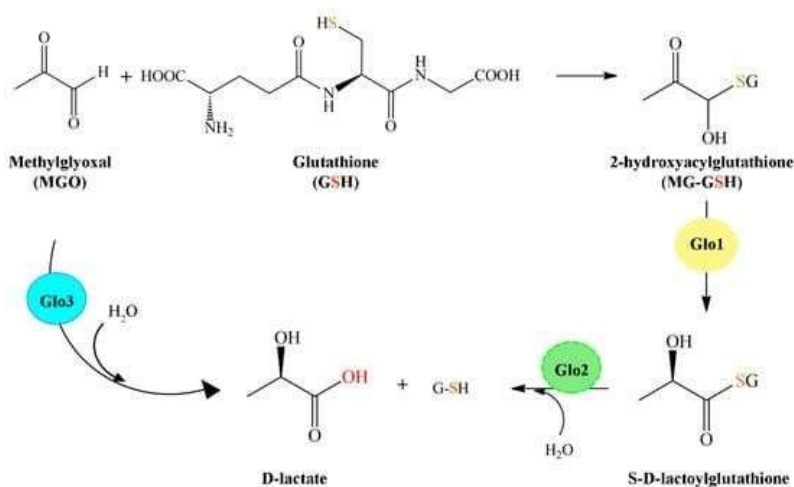
### 3.2.4 Mechanisms acting against glucose related damage

Some mechanisms have been reported or hypothesized to act in order to protect organisms from the noxious effects of sugars. The first that could be considered is to avoid or reduce the use of reducing sugars at all. In plants, for example, the main circulating carbohydrate through the phloem is saccharose (disaccharide of glucose and fructose) (Yoon et al. 2021), which does not have reducing power, given that the anomeric carbon of glucose is forming a bond with fructose, and both are kept in their circular form. However, animals do not use saccharose for circulation, and the use of reducing sugars is extended. An efficient regulation of circulating levels by the insulin/glucagon system is required to avoid high and long hyperglycaemia peaks, as discussed above (sections 3.1.2 and 3.2.1.). In some species with high sugar intake, this is of particular relevance. This is the case of nectar feeding bats, for example, in which high levels of activity (Kelm et al. 2011) and a potentially high metabolic flexibility (*sensu* Kelley and Mandarino 2000; Saltin and Goldnick 1983 i.e. fast switch in fuel usage in response to an environmental change, as ingestion; see (Suarez, et al. 2011; Suarez and Welch 2017; Welch et al. 2022) allow them to rapidly downregulate blood sugar levels. Nevertheless, in hummingbirds, ecologically very similar, the same would apply but still post-absorptive blood sugar would be kept at high levels (Beuchat and Chong 1998). Thus, they must have different ways of fighting against the deleterious effects of reducing sugars. Regarding glycation, more concretely, there are several mechanisms to protect against it, either by avoiding it or by restoring the damage. To avoid it, a reduced number of exposed “glycatable” aminoacids, like lysine or arginine, in certain proteins is to be expected if blood glucose levels are higher in a particular species. This may be the case for birds, as proposed by (Anthony-Regnitz et al. 2020) after looking at chicken serum albumin. Also in birds, and in some mammal species, it has been found that mature red blood cells’ metabolism hardly depends on glucose, but rather catabolizes glutamine and nucleosides as its main energy source (Johnstone et al. 1998), potentially contributing to the low haemoglobin glycation levels found in some species (Brun, Hernandez-alba, et al. 2022). High levels of free aminoacids or polyamines have been suggested to act as glycation scavengers (Nandhini et al. 2004; Szwergold and Miller 2014b), to avoid proteins to be glycated and lose function or form high molecular weight AGE that would be much more prone to accumulate and

produce pathology. Polyamines also promote autophagy (see Hofer et al. 2022), which would reduce accumulation of damaged molecules within cells.

For restoring protein original state, some deglycases have been found to be acting within cells, as the 3-fructosamine kinase (3-FNK) and FN3K-related protein (FN3K-RP), hydrolysing ATP to remove a glucose molecule from a lysine residue (Delplanque et al. 2004). Interestingly, 3-FNK activity levels have been found to be higher in erythrocytes from species like humans, mice and rats, where glucose levels are high, than in pigs or chicken, where glucose levels are almost null (Delplanque et al. 2004). For circulating proteins like albumin, nevertheless, protein turnover is probably the most efficient way of eliminating glycated ones, as it serves for those damaged by oxidative stress (Wada et al. 2016; Wada et al. 2018).

Finally, protection against AGE-related damage can be accomplished through multiple ways. Apart from the already mentioned mechanism to avoid the accumulation of early stage glycation-related damage, that could lead to the formation of AGE, and any mechanism protecting against oxidative stress, that would also in turn decrease AGE formation, particular pathways to detoxify reactive carbonyls can be used. Among these we can find aldose and ketoses reductases (ALDH and AKR), betaine aldehyde dehydrogenase, and 2-oxoaldehyde dehydrogenase, a series of enzymes that act reducing excess carbonyls that could lead to AGE formation (see Turk 2006; Turk 2010). For the particular case of methylglyoxal, the glyoxalase system, composed by several enzymes that catabolize the reduction of methylglyoxal to form lactic acid at the expense of glutathione oxidation (Rabbani et al. 2014; Rabbani and Thornalley 2014), constitutes one of the main detoxification systems avoiding the accumulation of methylglyoxal (**Figure 12**).



**Figure 12.** Glyoxalase pathway.

Another important component of the AGE-related damage is their relation with inflammation (see section 3.2.3). This is mediated by RAGE (Hofmann et al. 1999; Schmidt et al. 2001). However, there are different kinds of AGE receptors with esRAGE and sRAGE being soluble versions, from which at least esRAGE is supposed to mediate beneficial effects, acting as AGE scavengers (Vazzana et al. 2009; Raposeiras-Roubín et al. 2010).

## 4. Finally addressing the question: how do birds face relative hyperglycaemia?

As already mentioned, birds have the highest circulating glucose levels of all vertebrates, doubling that found in mammals (Polakof et al. 2011), while living much further for a given body mass (Lindstedt and Calder 1976). For all of the abovementioned reasons about the relationship between glucose and ageing, this makes them a really interesting subject of study. Also, some aspects of their glucose regulation differ from what has previously been exposed in particular ways. Nevertheless, not many studies have been performed in order to disentangle how birds can sustain such high glucose levels and still be so long-lived. The present section will thus outline the main knowledge up to date on glucose, glycation and AGE in birds, in order to prepare the reader for the following corpus of the thesis, in which I will lay out the results from the different studies I performed in order to try to add to this knowledge by exploring the issue from different perspectives.

### 4.1. Glucose, glycation and AGEs in birds

#### 4.1.1. Glucose regulation in birds

Some aspects of glucose regulation vary in birds in relation to what has been explained before (see section 3.1.2.), although it must be said that most that is known comes from chicken, and Neoaves are by far underrepresented in the extant studies. First of all, glucose is much higher in birds' blood, with most of it being in the plasma, as their erythrocytes lose most of their ability to internalize it as they mature (Johnstone et al. 1998). This relative hyperglycaemia is suggested to be an adaptation to flight, such that readily accessible circulating glucose serves for rapid take-off, short term flight and manoeuvres (Braun and Sweazea 2008). Secondly, insulin levels are much lower than in mammals (Joëlle Dupont et al. 2004), while glucagon levels are higher, which potentially contribute to maintain such a relatively hyperglycaemic state (Sweazea 2022). Indeed, anti-insulin antibody injection to chicken have demonstrated to be able to increase glucose levels, demonstrating a role of this hormone in glycaemia regulation, although seemingly more through the ratio of both insulin and glucagon than by direct insulin action itself (reviewed in Sweazea 2022).

Despite acting on the same receptor (IR), insulin seems not to drive the same cellular response, depending on the tissue, developmental state and species. Adipocytes have shown to have a very low level of response to insulin signalling, with very low glucose uptake rates, which makes sense when we think that lipids are mostly synthesized in the liver (Leveille et al. 1968). They also seem not to be implied in satiation signalling, as leptin, an anorexigenic hormone they produce in mammals, seems to be produced in birds mostly in other organs and with other functions (Friedman-Einat and Seroussi 2019).

Insulin signalling in muscles seems also to vary to a certain point in relation to what is known from mammalian models, and the effect of insulin on glucose uptake by muscle cells is discussed, with some studies that showed it to be the case, even showing an increase of certain GLUT (Glucose Transporters) proteins by insulin, while others do not succeed in doing so. Indeed, GLUT1-6, 8-10 and 12 have been reported in birds, at least at the genome level (Sweazea 2022), and particularly GLUT4, that is crucial in insulin and contraction-dependent glucose internalization by the striate muscle in mammals (reviewed in Shepherd and Kahn 1999), has only recently been allegedly found in chicken (Shi et al. 2014), although its presence in ducks

was suggested many years ago (Thomas-Delloye et al. 1999). Its expression seems limited to striated muscle and very scarcely to liver, varying across development, with a very complex transcription regulation, including several alternative splicing versions which may have led to the difficulties on its finding (Luo et al. 2023). It is possible that some of these mentioned GLUTs show a decreased expression during adulthood in comparison to embryos and chicks, which would explain some of the contradictory results (Luo et al. 2023). It has also been shown that insulin acts increasing glomerular filtration rate (GFR) in mourning doves (*Zenaida macroura*) (Sweazea et al. 2017), which could explain the hypoglycaemic response of this hormone even if it does not induce tissue glucose uptake (Sweazea et al. 2006).

Nevertheless, insulin action on anabolism seems to be working on birds as well, both through PKB and MAPK (Mitogen Activated Protein Kinases) pathways. Still, some differences in the signalling pathways have been reported, and IRS-1 and PI3K, for example, seem not to be responsive to insulin, although insulin signalling activates MAPK (Mitogen Activated Protein Kinases) and PKB (Protein Kinase B) (reviewed in Sweazea 2022). Moreover, although IGFs are hypoglycaemic-inducing in chicken (J. Buyse and Decuypere 1999), IGF-1 do not induce glucose uptake by house sparrow (*Passer domesticus*) skeletal muscle *in vitro* (Sweazea and Braun 2005), suggesting again that muscle glucose uptake is not very important in the insulin hypoglycaemic response of birds. Much more work is still needed to better integrate this knowledge, as to understand how the action of insulin/insulin signalling differs from mammals and even other vertebrates.

#### 4.1.2. Glucose variation in birds and its relation with fitness and ageing

Several bird groups have been explored in relation to whole blood or plasma glucose values, with more than 20 orders (including data on this thesis, see Chapter 2; the exact number depends on the classification being accepted) with data available. Although this data is biased towards Galloanserae, many parrots (Psittaciformes) and songbirds (Passeriformes) have also been measured, and species from many other groups with different diets and ecological niches, like carnivore raptors and aquatic birds, terrestrial, flying and non-flying species have been reported (see Table 1 from Sweazea 2022 for an example). Differences across species depend on several factor: it has been repeatedly reported to decrease with body mass (Umminger, 1975; Braun and Sweazea, 2008; Kjeld, 2008; Tomášek et al. 2019, 2022; although Beuchat and Chong 1998 and Scanes 2016 found non-significant trends) and increase with clutch mass (Tomášek et al. 2019; Tomášek et al. 2022), interpreted as a coevolution of glucose levels with life history in the context of the pace of life (POL) hypothesis (Wikelski and Ricklefs 2001; Ricklefs and Wikelski, 2002; Wikelski et al., 2003; see Chapter 2). In this line, increase on basal glucose levels with latitude and altitude also points towards this idea (Tomášek et al. 2022), as normally faster life pace would evolve with latitude, with lower survival in higher latitude (Scholer et al. 2020). In the case of altitude, this increase in baseline glucose is not related with an increase in fecundity, so that montane birds would show a “faster physiology” with a slower life pace, as hypothesized by Hille and Cooper (2014). In terms of stress induced hyperglycaemia measured as levels 30 mins after capture and the amount of change in comparison to baseline, both significantly increased with altitude in temperate but not tropical birds in Tomášek et al. (2022), with would relate also with observed increase in survival with altitude in temperate birds (Boyle et al. 2015). On addition, Tomášek et al. (2019) and Tomášek et al. (2022) show a negative correlation between glucose levels (measured during reproductive period) and migration distance in Passeriformes, which seems opposed to what was observed by Pistone et al. (2017) for Passeriformes from Texas, where the migrant species showed higher glucose levels, which could

be understood in line with POL hypothesis given the lower survival of migratory birds reported by Scholer et al. (2020). Still, the relationship between migration and plasma glucose levels is probably not straightforward, as long-term flight, as during migration, is mostly fuelled by lipids (see review in Weber 2009), with no differences in glucose levels before and after migration being detected on Bar-tailed godwits, (*Limosa lapponica taymyrensis*) (Landys et al. 2005), but higher glucose found during the migration period in captive garden warblers (Bairlein 1983). In relation to diet, Tomášek et al. (2022) find increased glucose levels on species with higher levels of nectar and fruits intake and higher stress related hyperglycaemic response in herbivore/granivore species, while Tomášek et al. (2019) does not find any effects of diet (see Chapter 2). Sex differences has also been explored, with higher levels in females during the reproductive season and in males out of it found by Tomášek et al. (2022).

Within individual variation in circulating glucose levels has also been reported for birds, with several studies performed on different species showing variation on glucose either through day hours (Frelin 1974; García-Rodríguez et al. 1987; Klandorf et al. 1999; Remage-Healey and Romero 2000; Pérez-Rodríguez et al. 2008; Downs et al. 2010; Lobban et al. 2010) or seasonally (Jenni-Eiermann and Jenni 1996; Remage-Healey and Romero 2000).

Short-term variations in relation to exercise or stress are also studied in some species (Schwalme and Mackay 1985; Ryan et al. 2023). Nevertheless, changes in food amount or composition has shown to rarely have sustained effects on glucose levels in birds (Fokidis et al. 2011; Basile et al. 2022). Finally, variation with age or stage of the life cycle has also been reported, with a linear increase in plasma glucose with age taking place during nestlings' growth in some species, even after asymptotic mass is reached (Lill 2011) or stopping half way through development in European Starlings (Juráni et al. 2004). Chicks values are reported as lower than adults in some studies (Work 1996; Dobado-Berrios et al. 1998; Ferrer and Dobado-Berrios 1998; Villegas et al. 2002), but not in others (De le Court et al. 1995; Bowerman et al. 2000; Casado et al. 2002), probably due to the clustering of different ages together within the same category.

Out of the healthy range, pathological glucose levels have also been reported, with several cases of diabetes identified in the literature (reviewed in Van de Weyer and Tahas 2024, cases reported in e.g. Altman & Kirmayer, 1976; Douglass 1981; Ryan et al. 1982; Appleby 1984; Candeletta et al. 1993; Kahler, 1994; Pollock, et al. 2001; Pilnydvm et al. 2005; Pendleton et al. 2007; Gancz et al. 2007; Desmarchelier et al. 2008; Kothe and Mischke 2009).

However, very few studies have related plasma glucose levels with fitness outcomes, or in particular with the ageing process. It is remarkable, for example, the work of Montoya et al. with zebra finches, showing how adult glucose levels are increased with early life harsh conditions and are negatively linked with survival (Montoya et al. 2018), and how glucose tolerance measured through the response to intraperitoneal GTT, a repeatable trait to assess glucose dynamics (Montoya et al. 2020), is positively correlated with survival in old individuals (Montoya et al. 2022). Interestingly, higher AUC (lower glucose tolerance) were observed at warmer temperatures and at “easy foraging” conditions (versus “hard conditions”, in which birds had to hover to access the food within the feeders) (Montoya et al. 2022). This would suggest a lower AUC when basal glucose is already elevated (colder temperature and harsh foraging conditions), but Montoya et al. (2022) showed no effect of basal glucose on AUC and Tomášek et al. (2022) no effect of basal glucose on glucose stress response (difference between basal glucose and glucose levels 30 mins after capture). How this lowered glucose tolerance may mediate survival is not well known, although it could be related to the fact that higher basal glycaemia induces higher oxidative stress in house sparrows (*Passer domesticus*),

while acute stress-induced oxidative stress seems to blunt glucose response, in a feedback loop (Vágási et al. 2020). However, at the interspecific level, no relationship between either basal or stress glucose values with oxidative status markers was found (Vágási et al. 2024).

In the wild, few studies have related glucose with fitness. For example, in tits (*Cyanistes caeruleus* and *Parus major*) average nestling glucose levels within a brood have been shown to negatively relate with body mass and fledging success, but only in the environment in which they showed higher glucose levels for *Parus major*, potentially reflecting that this negative effect only shows up at the top percentiles (Kaliński et al. 2014; Kaliński et al. 2015). In some passerines, glucose has also been found to negatively correlate with haemoglobin (Gładalski et al. 2015; Minias 2014), probably associated with hyperglycaemia related anaemia (Winkler et al. 1999), while haemoglobin is shown to be an indicator of breeding success (Gładalski et al. 2015; Kaliński et al. 2018), which could explain why fledging success is reduced at highest glucose levels. Nevertheless, all of these measures in chicks may not be totally extrapolable to adult values, for which plasma glucose has been explored as a body condition indicator, being positively related with either body mass directly (Jenni-Eiermann and Jenni 1994; Bendová 2020) or its residual in respect to structural size (Azeredo et al. 2016, although the opposite was found out of the breeding season; McGraw et al. 2020). However, different results were found by Lill (2011) for each of several species tested (see Chapter 3 for a further discussion), and other studies did not find such correlation (Vágási et al. 2020).

#### 4.1.3. Glycation and AGE in birds

Very few studies have measured glycation in birds. Most of them have assessed haemoglobin glycation (Rendell et al. 1985; Miksik and Hodny 1992; Rosa 1993; Andersson and Gustafsson 1995; Beuchat and Chong 1998; Ardia 2006; Récapet et al. 2016; Borger 2024), a few albumin glycation (Zuck et al. 2017; Ingram et al. 2017; Anthony-Regnitz et al. 2020; Brun et al. 2022), and some others have relied on an indicator or general protein glycation levels, like fructosamine values (Rosa et al. 1993; Holmes et al. 2001; Gancz et al. 2007; Kothe and Mischke 2009; Ling et al. 2020).

One of the problems with many of these studies, in particular those measuring haemoglobin glycation, is that the methods used are often inaccurate, based on boronate affinity columns to separate glycated versus non-glycated proteins and then using a Bradford protein quantification test on each extracted fraction (Rendell et al. 1985), by immunodetection of reduced glycated lysine residues (Holmes et al. 2001) or using kits developed for humans HbA1c. This is to be taken in consideration, as the mere presence of different haemoglobin variants within humans can already bias the results with different techniques (Suo et al. 2019), not to say when testing other species. This methodological diversity and non-specificity make difficult to rely on the correspondence between the values reported and the actual values. Nevertheless, some studies have started using different variations of mass spectrometry techniques, which is considered as the gold standard (Jeppsson et al. 2002), to measure albumin glycation levels (Zuck et al. 2017; Ingram et al. 2017; Brun et al. 2022).

Still, in the cases in which measures have been done, fewer go further than a mere description of the levels of glycation and a rough comparison among species. In this line, Holmes et al. (2001) found lower general glycation levels in budgerigars' plasma than in humans' plasma, while Rendell et al. 1985 found haemoglobin glycation rates to be lower in all bird species he surveyed than in mammals (except in pigs, which have shown low levels of glucose permeability in their erythrocytes, similarly than birds; Zeidler and Kim 1982), despite showing

(birds) relatively high albumin glycation levels, in line with other results, like Brun et al. (2022) with zebra finches versus humans, and Anthony-Regnitz et al. (2020) with chicken versus BSA (Bovine Serum Albumin). Interestingly, Zuck et al. (2017) rather found higher albumin glycation levels in chicken than in humans, but those did not increase after increasing the concentration of glucose, as human values did, becoming then higher than in chicken.

Nevertheless, some have tried to relate these values with some other parameter. Here we can find the examples of Récapet et al. (2016), which detected a variation in haemoglobin glycation with age (decreasing up to five years and increasing afterwards, in a cross-sectional design) and a negative relationship with survival probability in the collared flycatcher (*Ficedula albicollis*); Andersson and Gustafsson (1995), that found a positive relationship with fledgling production and a negative one with arrival date at the breeding grounds in the same species; Ardia (2006), that found a positive relationship with chick growth in American kestrels (*Falco sparverius*) and Borger (2024) that found a positive relationship of females haemoglobin glycation (after breeding) and number of offspring produced in captive zebra finches.

In the case of AGE, some attention has been put into the rate of accumulation of some of them, like pentosidine, in tissues, and its consequentially potential usefulness to determine age. Examples of these studies are Gu (2013), measuring it in plasma, and the following in skin (Iqbal et al. 1999; Klandorf et al. 1999; Klandorf et al. 2001; Chaney et al. 2003; Fallon et al. 2006a; Fallon et al. 2006b; Coe and Virginia 2008; Coe et al. 2010; Le Souëf 2012; Campion 2015; Rattiste et al. 2015; Dorr et al. 2017; Iverson et al. 2017; Labbé et al. 2019; Aleksieva et al. 2020). From these, (Iqbal et al. 1999; Klandorf et al. 1999; Fallon et al. 2006a; Fallon et al. 2006b; Coe and Virginia 2008; Coe et al. 2010; Dorr et al. 2017) found an increase with age, while others did not (Le Souëf 2012; Campion 2015; Rattiste et al. 2015; Labbé et al. 2019). Aleksieva et al. (2020) detected a significant correlation only in 1 out of 4 measured species, discussing, as others did before, that pentosidine accumulation may thus not be an adequate parameter to determine age in long-lived species like some seabirds. Dorr et al. (2017) also detected differences between breeders and non-breeders and between sexes. Gu et al. (2013) also explored the relationship between plasma glucose and pentosidine, indicating that, if two outliers with very high glucose levels were removed, pentosidine increased with glucose. Nevertheless, those results are based on 8 individuals (6 without the outliers), so that the evidence shown is very scarce. Finally, dietary restriction was found to reduce pentosidine accumulation with age in skin of chicken, which, moreover, have lower levels than those reported in mammals (Iqbal et al. 1999), and ingestion of certain compounds like alopurinol (inhibitor of uric acid synthesis) and hemin can increase it, probably by increasing oxidative stress (Klandorf et al. 2001).

Finally, Baker et al. (2022) made a recent attempt to perform a comparative analysis of AGE levels across birds and mammals, assessing it over a total of 23 species (7 birds and 16 mammals). They found that birds have higher levels of plasma AGE-modified proteins than mammals, and that these increase with age in mammals, but with the effect however disappearing with phylogeny correction, or when age divided by MLS is considered instead. Nevertheless, as it happens with other studies, they use a commercial kit for AGE determination, which makes it very difficult to properly interpret their results, as these kits are based on an ELISA (Enzyme-Linked ImmunoSorbent Assay) which uses “anti-AGE” antibodies with AGE-modified BSA (Bovine Serum Albumin) as a reference standard, which is quite ambiguous, and also, as AGE come in very different shapes and sizes, the whole diversity of them is likely not to be captured by such a method. This is important as some AGE probably do not reflect the same

thing as others, and may pose different health risk, mainly depending on if they are formed in free small molecules or macromolecules as whole proteins, and also on the tissue on which they may accumulate (see e.g. Chaudhuri et al. 2018; Twarda-Clapa et al. 2022).

The effects of AGEs on birds' health is still nevertheless not well known, and it has been suggested that the reported absence of RAGE in avian vasculature (Eythrib 2013) could be linked to their resistance to some of the negative effects of those (Szwergold and Miller, 2014a). Nevertheless, recent evidence suggests that AGEs do induce proinflammatory responses in chicken, with galectin 3 being suggested as a candidate for RAGE in immune cells (Wein et al. 2020).

## 4.2. Different methods of research for the same end: comparative, correlational and experimental

In order to generate scientific knowledge about a particular topic, different methods can be used. Some of the more important are the experimental, the observational (or correlational) and the comparative methods. Each of them has their pros and cons, and provide different insights into the thing being studied. Experiments, in the first place, can allow us to obtain more precise information with which to perform causal inferences: a modification is made on a system and the outcomes are measured, in comparison to a non-manipulated same kind system. However, sometimes the needed level of control over the target system and its environment cannot be exerted, due to technical, logistical or ethical issues. This is frequently the case for the study of animals, particularly wild ones. This is when observational studies come in handy: despite the difficulties they pose when trying to extract causal inferences from the data obtained, they are relatively easier to perform, sometimes even retroactively (i.e. on data previously collected with, potentially, even other or at least somehow undefined intention) and give us abundant information on patterns that can later be confirmed by more precisely organized experiments. They may also have the advantage of being easier and cheaper, especially when employing already collected data. This is particularly the case when long-term studies are done over particular populations (Sheldon et al. 2022). Finally, comparative studies, which are a particular kind of correlational study, consist into the interpretation of associations present on different variables among them on the same target individuals (individual organisms, species, etc.). In evolutionary biology, this last case is particularly useful, as macroevolution cannot be replicated, and in order to understand the reason why certain traits evolved in the way they did, it is interesting to look at what environment, both external and internal, i.e. other traits within the same (statistical) individual, they are related to. This way, and looking at the distribution of trait values across species, we can try to make inferences about the possible evolutionary causes of the observed trait values, either by natural selection or phylogenetic constraints. This is an advantage against experiments or other correlational studies, that are normally better to improve our understanding of current processes and (proximal) causes, which suit much more the generation of mechanistic knowledge (see section 1.).

### 4.2.1. Main thesis questions and structure

With these thoughts in mind, this thesis tries to improve our knowledge on both the proximate and ultimate causes of glycaemia and glycation levels in birds, and its relationship with ageing and fitness. For this, three main tasks have been performed: 1) a comparative study on birds' plasma glucose and glycation levels (as albumin glycation) in relation to diet and life-history



traits (**Chapter 2**); 2) a correlational study on plasma glucose and albumin glycation in a wild population of Alpine swifts (*Tachymarptis melba*, Linnaeus 1758) (**Chapter 3**) and 3) an experimental approach trying to manipulate glucose and glycation levels in captive zebra finches (*Taeniopygia guttata*, Vieillot 1817) (**Chapters 4-6**). A final discussion of these results altogether will be performed in a last section (**Chapter 7**). The comparative study allows us to explore how variable plasma glucose and glycation levels are across taxa, and how it has coevolved with life-history, trying to determine if longevity, developmental time or investment into reproduction (measured as clutch mass) influenced the variation of these physiological traits, as it would predict the Pace of Life (POL) hypothesis. We also explore how diet may influence these values, potentially generating well adapted phenotypes to an environmental factor that determine their level of exposure to sugars. In the second task, intraspecific variation is explored, taking as an example a highly aerial bird, the Alpine swift, from a population that has been followed by several decades, such that good demographical data is available (including individual age). This model allows us to establish relationships between glycaemia and glycation and fitness in a wild population, as well as hint at potential variation with age, in a relatively long-lived bird with a particularly special lifestyle, consisting into a continuous flight, that is only interrupted for certain tasks related to reproduction, and therefore high energetic demands. Finally, the third task gives us the opportunity to explore more in detail the intraindividual variability of blood/plasma glucose and albumin glycation levels in relation to age and other parameters known to be related with the ageing process. This allows us to obtain a more detailed and mechanistic picture relating these traits with fitness within experimental conditions in a model species. All of this together should help significantly enhancing our up to date limited knowledge on the intra and interspecific variation of circulating glucose in relatively hyperglycaemic yet long-lived birds and how it affects their fitness and the ageing process.

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# Chapter 1

Comparative approach: glycaemia and glycation across the birds' tree





## Résumé

L'hypothèse du syndrome du rythme de vie (POLs) suggère que les traits physiologiques et comportementaux des organismes devraient co-évoluer avec les traits d'histoire de vie. Dans ce cadre, la manière dont la glycémie (c'est-à-dire les niveaux de glucose dans le sang) et sa réaction avec les protéines et d'autres composés (c'est-à-dire la glycation) covariant avec les traits d'histoire de vie reste relativement peu étudiée, malgré les conséquences bien documentées du glucose et de la glycation sur le vieillissement, et donc potentiellement sur l'évolution des histoires de vie. Les oiseaux sont particulièrement pertinents dans ce contexte, étant donné qu'ils ont les niveaux de glucose sanguin les plus élevés parmi les vertébrés et encore une longévité ajustée à la masse plus élevée par rapport aux organismes ayant une physiologie similaire, comme les mammifères. Nous avons donc réalisé une analyse comparative du glucose et du taux de glycation de l'albumine chez 88 espèces d'oiseaux appartenant à 22 ordres, en fonction des traits d'histoire de vie (masse corporelle, masse de la couvée, durée de vie maximale et temps de développement) et de la diète. Les niveaux de glucose montraient une corrélation positive avec les taux de glycation de l'albumine de façon non linéaire, ce qui suggère une résistance à la glycation chez les espèces ayant des niveaux de glucose plus élevés. Les niveaux de glucose plasmatique diminuent avec l'augmentation de la masse corporelle mais, contrairement à ce que prévoit l'hypothèse POLs, les niveaux de glucose augmentent avec la durée de vie maximale avant d'atteindre un plateau. Finalement, les carnivores terrestres ont montré une glycation de l'albumine plus élevée que les omnivores, bien qu'ils n'aient pas montré une glycémie plus élevée, ce qui, selon nous discutons, pourrait être lié à d'autres facteurs tels que des niveaux différentiels d'antioxydants ou la composition du régime alimentaire en termes de fibres ou d'acides gras polyinsaturés. Ces résultats augmentent nos connaissances sur la diversité des patterns de glycémie et de glycation chez les oiseaux, et suggère l'existence de mécanismes de résistance à la glycation chez les oiseaux, dont la glycémie est comparativement élevée.



1 **Variation in albumin glycation rates in birds suggests**  
2 **resistance to relative hyperglycaemia rather than**  
3 **conformity to the pace of life syndrome hypothesis**

4 *Manuscript accepted on eLife*

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22 Key words: birds, comparative method, glucose, glycation, life history, longevity

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## 25 **ABSTRACT**

26 The pace of life syndrome hypothesis (POLS) suggests that organisms' life history, physiological  
27 and behavioural traits should co-evolve. In this framework, how glycaemia (i.e., blood glucose  
28 levels) and its reaction with proteins and other compounds (i.e. glycation) covary with life history  
29 traits remain relatively under-investigated, despite the well documented consequences of  
30 glucose and glycation on ageing, and therefore potentially on life history evolution. Birds are  
31 particularly relevant in this context given that they have the highest blood glucose levels within  
32 vertebrates and still higher mass-adjusted longevity when compared to organisms with similar  
33 physiology as mammals. We thus performed a comparative analysis on glucose and albumin  
34 glycation rates of 88 bird species from 22 orders, in relation to life history traits (body mass,  
35 clutch mass, maximum lifespan and developmental time) and diet. Glucose levels correlated  
36 positively with albumin glycation rates in a non-linear fashion, suggesting resistance to glycation  
37 in species with higher glucose levels. Plasma glucose levels decreased with increasing body mass  
38 but, contrary to what is predicted to the POLS hypothesis, glucose levels increased with  
39 maximum lifespan before reaching a plateau. Finally, terrestrial carnivores showed higher  
40 albumin glycation compared to omnivores despite not showing higher glucose, which we discuss  
41 may be related to additional factors as differential antioxidant levels or dietary composition in  
42 terms of fibres or polyunsaturated fatty acids. These results increase our knowledge about the  
43 diversity of glycaemia and glycation patterns across birds, pointing towards the existence of  
44 glycation resistance mechanisms within comparatively high glycaemic birds.

## 45 **INTRODUCTION**

46 The pace of life (POL) hypothesis [1-3] postulates that organisms behavioural/physiological  
47 characteristics and life histories have co-evolved in answer to specific ecological conditions  
48 forming pace of life syndromes (POLS) along a fast-slow continuum (but see [4] for the more  
49 recent consideration of other axes). According to the classical approach from life history theory,  
50 slower organisms would have, e.g. large body mass, late maturation, slow growth rate, small  
51 number of large offspring and high longevity, whilst faster ones representing the opposite trait  
52 values (see [5-8]) within this continuum. Pace of life hypothesis added physiology to this  
53 continuum, with studies testing its predictions focusing often on metabolic rate (i.e. higher or  
54 lower metabolic rate corresponding to faster or slower pace of life, respectively; see e.g. [9-12]).  
55 However, key energy substrates related to metabolic performance, such as glucose

56 concentrations in tissues, have been largely overlooked (but see [13-16]). Notably, glucose, a  
57 central energy source for many organisms, plays a pivotal role in metabolism and there are some  
58 indications that its circulating blood or plasma levels correlate positively with metabolic rate per  
59 gram of mass at the interspecific level [13,17], and with whole body metabolism at the  
60 intraspecific level ([18] for the case of non-diabetic humans).

61 Research focusing on plasma glucose becomes highly relevant as high glycaemia can entail costs  
62 that accelerate ageing. Along with other reducing sugars, glucose can react non-enzymatically  
63 with free amino groups of proteins, lipids or nucleic acids, a reaction known as glycation [19-20].  
64 This reaction, after several molecular reorganizations, can lead to the formation of a plethora of  
65 molecules called Advanced Glycation End-products (AGEs) [21]. AGEs can form aggregates in  
66 tissues that are well known to contribute to several age-related diseases related, such as  
67 diabetes (e.g., reviews by [22-25]). AGEs are also known to promote a proinflammatory state  
68 through their action on specific receptors called RAGEs [26-27].

69 Remarkably, birds show circulating glucose levels much higher than other vertebrate groups, on  
70 average almost twice as high as mammals [28]. These relatively high glucose levels might  
71 support the higher metabolic rates [29, 11] and body temperatures observed in birds compared  
72 to mammals [30-31]. In addition, elevated glycaemia is thought to be an adaptation to flight,  
73 providing birds with rapid access to an easily oxidizable fuel during intense bursts of aerobic  
74 exercise (see [32-33]), such as during take-off and short-term flapping flights [34-35]). However,  
75 the presence of high glycaemia in birds is also paradoxical, given their remarkable longevity  
76 compared to their mammalian counterparts — living up to three times longer than mammals of  
77 equivalent body mass [36, 11].

78 Several non-excluding hypotheses have been proposed to explain how birds apparently resist  
79 the pernicious effects of a high glycaemic state. One possibility is that birds might resist protein  
80 glycation through a lower exposure of lysine residues at the surface of their proteins [37].  
81 Another hypothesis suggests that increased protein turnover may play a role [38-39].  
82 Additionally, birds might benefit from more effective antioxidant defences [40-42] (although  
83 within Passeriformes, [16] shows no coevolution between glycaemia and antioxidant defences)  
84 or even the presence of “glycation scavengers” such as polyamines which circulate at high  
85 concentration [35]. Moreover, it is generally considered that RAGEs are not present in avian  
86 tissues, suggesting possible adaptive mechanisms that would allow birds to avoid the  
87 inflammatory consequences associated with RAGE activation [43-44]. However, a putative



88 candidate for RAGE and a case of AGE-induced inflammation were identified by Wein et al. [45],  
89 warranting further investigation on the occurrence of RAGEs in birds.

90 To date, although protein glycation levels have been measured in birds in several species, these  
91 results were mostly descriptive and concerned a small number of species [46-54]. Additionally,  
92 many of these studies employ commercial kits that were not specifically designed for avian  
93 species, making it challenging to interpret the results [53]. These limitations restrict our ability  
94 to draw general conclusions from these studies. Here, we performed a comparative analysis on  
95 88 species belonging to 22 orders (see ESM6) and assessed whether and how bird glycaemia and  
96 glycation rates are linked to ecological and life-history traits (including body mass). While doing  
97 this, we also checked whether glycation levels are influenced by circulating plasma glucose  
98 concentrations. Finally, as diet can influence glycaemia (see e.g. [15, 55-56] but [14] found no  
99 significant effects) and glycation, we included it in our analyses. We hypothesized that  
100 carnivorous birds would exhibit higher glycaemia and glycation levels than omnivorous or  
101 herbivorous species, as diets with low-carbohydrates, high-proteins and high-fat are associated  
102 with comparatively high glycaemia and reduced insulin sensitivity in some vertebrates (e.g.; [57-  
103 59]). Accordingly, a recent comparative study in birds showed higher blood glucose levels in  
104 carnivorous species [56]. This may be attributed to high levels of constitutive gluconeogenesis,  
105 which has been confirmed in certain raptors [60-61]. Conversely, bird species with high sugar  
106 intake, such as frugivores or nectarivores, are expected to exhibit high glycaemia, as observed  
107 in hummingbirds [49] and Passeriformes with such diet [15] (although the opposite was found  
108 in [56]). In line with POLS hypothesis, we also hypothesized that species with a slower POL should  
109 exhibit a lower glycaemia and higher glycation resistance (glycation levels for a given glycaemia)  
110 compared to species with a faster POL. Hence, we included the following four parameters in our  
111 analyses of glycaemia and glycation levels: body mass, maximum lifespan, clutch mass and  
112 developmental time. Body mass is one of the main factors underlying life history, with higher  
113 body mass associated with “slower” strategies and vice versa [62]. Previous studies have  
114 reported a negative relationship between body mass and glycaemia [63, 34, 13-14, 56] (but see  
115 [49, 64] with non-significant trends in birds, and [15] only for temperate species within  
116 Passeriformes). Therefore, we predicted that bird species that live longer, develop more slowly  
117 and invest less per reproductive event (see ESM6 for further justification of the chosen variables)  
118 should show lower plasma glucose levels and albumin glycation rates (after controlling for body  
119 mass, phylogeny, diet and glucose in the case of glycation; see methods).

## 120 MATERIALS AND METHODS

### 121 Species and sample collection

122 A total of 484 individuals from 88 species measured were included for this study (see **Table**  
123 **ESM6. 1**). A detailed list with the provenance of the samples is provided in ESM3, with a textual  
124 description in ESM6.

125 Blood samples were collected from the brachial or tarsal vein, or from the feet in the case of  
126 swifts, using heparin-lithium capillaries or Microvette® (Sarstedt). Samples were centrifuged at  
127 4°C, 3500 g for 10 min and plasma was aliquoted when a large volume was available.  
128 Subsequently, they were transported on dry ice to the IPHC (Institut Pluridisciplinaire Hubert  
129 Curien) in Strasbourg and stored at -80° C until analysis. Glycaemia was measured in the  
130 laboratory on the remaining plasma after taking an aliquot for glycation assessment, using a  
131 Contour Plus® glucometer (Ascensia diabetes solutions, Basel, Switzerland) and expressed in  
132 mg/dL. These of point-of-care devices have previously been assessed for its usage in birds [65-  
133 66], with several examples of its usage in recent literature related to the topic presented here  
134 (e.g. [14, 67-69]. We also performed an assay with this particular brand on a subset of 46  
135 samples from this study, coming from 9 species distributed across the whole range of glucose  
136 values (3 “low”, 3 “medium” and 3 “high”, from both captive and wild populations), confirming  
137 a positive linear correlation ( $R^2_{\text{marginal}}=0.66$ ;  $R^2_{\text{conditional}}=0.84$ , for a model including the species as  
138 random factor) of this device with Randox GLUC-PAP colourimetry kits (P-value<0.001,  
139 unpublished data). Due to methodological constraints, we could not measure glycaemia values  
140 of 95 individuals of those that we sampled and in which glycation levels were assessed  
141 (belonging to 40 species coming from different sources). For these species, if not a single  
142 individual had a glucose measurement (which was the case in 13 species), we obtained mean  
143 plasma glucose values reported for the species from the ZIMs database from Species360  
144 (Species360 Zoological Information Management System (ZIMS) (2023), zims.Species360.org).  
145 This database provides plasma glucose data measured by colorimetric glucose kits on zoo  
146 specimens not necessary corresponding to those in which we measured glycation values. The  
147 sample sizes for both glucose and glycation measurements, either from our measured  
148 individuals or from the ones from ZIMs, are reported on the ESM5.

149

## 150 **Glycation levels**

151 Glycation levels for each individual were determined using liquid chromatography coupled to  
152 mass spectrometry (LC-MS), which is considered the gold standard for the assessment of protein  
153 glycation levels (see e.g. [70]) and has previously been used for birds [52-53, 71]. The samples  
154 were analysed across several instances within a period of less than 2 years (2021-2023). Only  
155 one sample from each individual was measured, given logistic limitations on the total number  
156 of samples that could be processed. Briefly, 3  $\mu$ l of plasma were diluted with 22  $\mu$ l of distilled  
157 water containing 0.1% of formic acid, followed by injection of 5  $\mu$ l into the system. The glycation  
158 values used in the analyses represent the total percentage of glycated albumin, obtained by  
159 adding the percentages of singly and doubly glycated albumin. These percentages were  
160 calculated by dividing the areas of the peaks corresponding to each glycated molecule form  
161 (albumin plus one or two glucose) by the total albumin peak area (sum of the areas of glycated  
162 plus non-glycated molecules) observed in the spectrograms obtained from the mass  
163 spectrometry outcomes. These spectrograms represent the different intensities of signal for the  
164 components differentiated in the sample by their TOF (Time Of Flight), which depends on the  
165 mass to charge ratio ( $m/z$ ) of the ionized molecules. More detailed information regarding  
166 functioning of the method and data processing can be found in [53]. In cases where albumin  
167 glycation values dropped below the limit of detection, resulting in missing data, these individuals  
168 (10 individuals from 4 species) were excluded from the statistical analyses, as outlined below.

## 169 **Data on ecology and life-history**

170 Diets of individual species were extracted from the AVONET dataset ([72], coming from [73], and  
171 those adapted from [74]) and life history traits from the Amniote Database [75]. After realizing  
172 that diet classifications from AVONET were not always suitable for our purposes, minor changes  
173 were made after consulting the original Wilman et al. [74] database (see ESM6).  
174 For missing data after this stage, we extracted values, in order of preference, from the AnAge  
175 database [76] or the Birds of the World encyclopaedia [77], by calculating mean values if an  
176 interval was given, and then averaging if male and female intervals were provided separately.  
177 For European species, longevity records have always been checked against the most recent  
178 Euring database [78]. Maximum lifespans from *Chionis minor* and *Eudypetes chrysolophus* (not  
179 available from the previous sources) were extracted from the Centre d'Etudes Biologiques de  
180 Chizé, Centre National de la Recherche Scientifique (CNRS-CEBC) database. Several species from

181 the zoo had maximum lifespan values available on the ZIMs from Species 360 (Species360  
182 Zoological Information Management System (ZIMS) (2023), zims.Species360.org), so these were  
183 also compared with the data we had from other sources.

184 In the case of variables for which two different sources provided different records, an average  
185 value was calculated. For the maximum lifespan value, available sources were cross-checked  
186 and the highest value was always used. In the absence of satisfactory support from another  
187 source, values for maximum lifespan indicated as being anecdotal and of poor quality have been  
188 excluded from the analyses. A table with the adequate citations for each value is provided as  
189 part of the online available data (see Data accessibility section). When the source is AVONET  
190 [72], this database is cited, and when it is Amniote Database [75], we cite the sources provided  
191 by them, so the references can be checked in [75]. A list with all the additional references not  
192 coming from any of these databases or not provided by their authors (and that are not already  
193 in the main text) is given as ESM4.

## 194 **Statistical analyses**

195 All analyses were performed in R v.4.3.2 [79]. Alpha ( $\alpha \leq 0.05$ ) was reported as significant, and  
196  $0.05 < \alpha \leq 0.1$  as trends. General Linear Mixed Models with a Bayesian approach (*MCMCglmm*  
197 function in R; [80]) were performed. All models were run with  $6 \times 10^6$  iterations, with a thinning  
198 interval of 100 and a burn-in of 1000. The models were simplified by eliminating quadratic terms  
199 where they were not significant and selecting models with lower AIC (Akaike Information  
200 Criterion) and BIC (Bayesian Information Criterion). Gaussian distribution of the response  
201 variables was assumed (after  $\log_{10}$  conversion for glucose; see ESM6 for further discussions). The  
202 priors were established assuming variance proportions of 0.2 for the G matrix and 0.8 for the R  
203 matrix, with  $\nu=2$ . For the phylogenetically controlled analyses, consensus trees (each with the  
204 species included in the model) were obtained by using the *consensus.edges()* function (from the  
205 *phytools* package from R [81] from a total of 10,000 trees downloaded from Birdtree.org (option  
206 “Hackett All species” [82])). For such purpose, a list of species with the names adapted to the  
207 synonyms available in such website was used (see ESM5), including the change of *Leucocarbo*  
208 *verrucosus* for *Phalacrocorax campbelli*, as the former was not available and it was the only  
209 species from the order Suliformes in our dataset, so that neither the position in the tree nor the  
210 branch length would be affected by this change. The consensus trees were included as *pedigree*  
211 in the models. We performed models with either glucose or glycation as dependent variables  
212 and with the diet, body mass and life history traits as predicting variables (and glucose in the

213 models with glycation as a response to assess glycation resistance; see introduction). Variance  
214 Inflation Factors (VIFs) were calculated for all the models with more than one predictor variable  
215 to assess the collinearity of them, as it may be expected for life history traits (see results on  
216 ESM1). Models testing the effects of age and sex on glucose and glycation levels and the number  
217 of exposed lysine residues on glycation were also carried. Finally, we performed models on  
218 glucose and glycation values controlling for the taxonomic orders included in the dataset (see  
219 ESM5). The effects of phylogeny on all models was determined by calculating the ratio of the  
220 variance estimated for the “animal” variable, representing the *pedigree* (see ESM6) by the total  
221 variance (all random factors plus *units*). A thorough description of all of the models, including  
222 transformations of the variables and other details is given on the ESM6.

## 223 RESULTS

### 224 Plasma glucose and albumin glycation variability across the birds’ tree

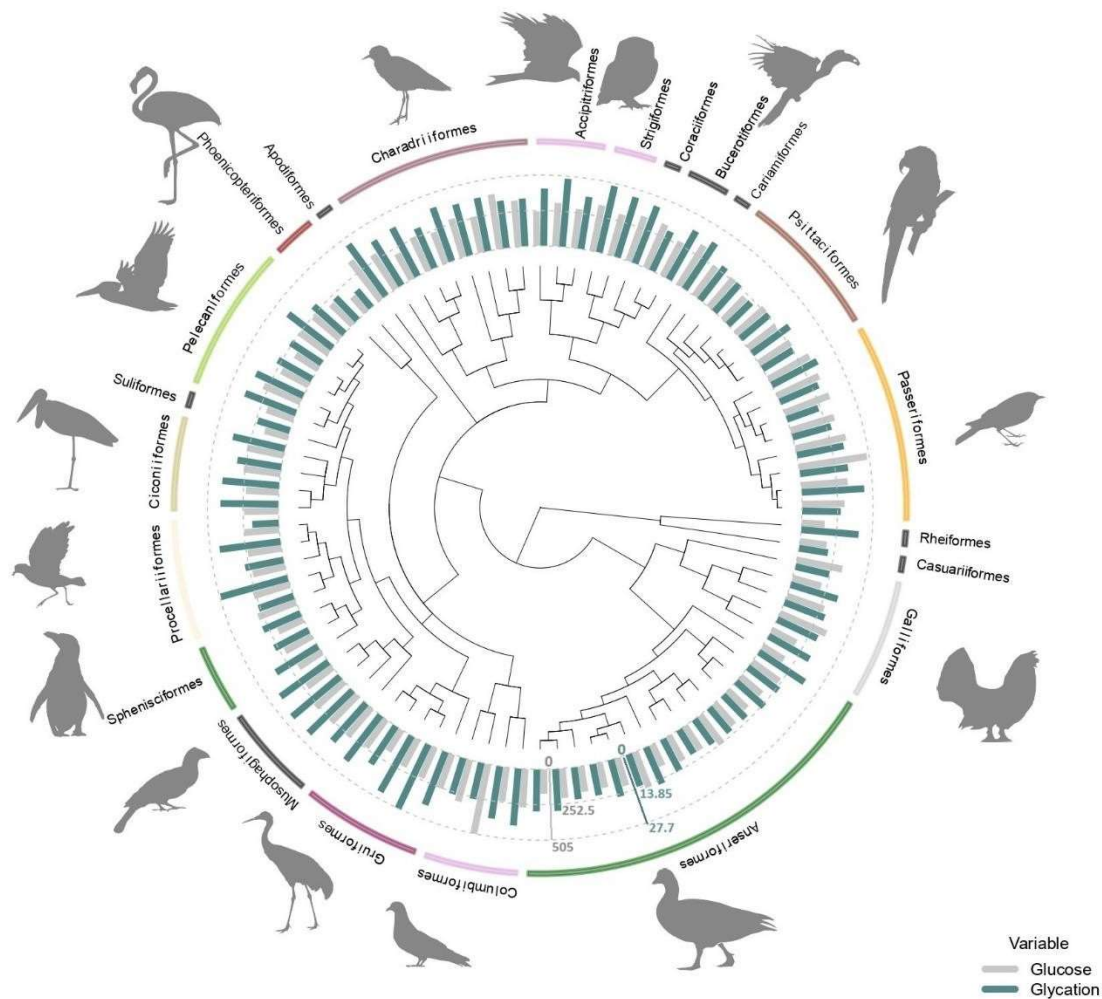
225 Plasma glucose and albumin glycation values varied considerably across species (**Figure 1**).  
226 Considerable within species repeatability (see ESM6) was observed (Glucose:  $R = 0.716$ ,  $SE =$   
227  $0.043$ ,  $CI_{95} = [0.621, 0.786]$ ,  $P\text{-value} < 0.0001$ ; Glycation:  $R = 0.703$ ,  $SE = 0.039$ ,  $CI_{95} = [0.621,$   
228  $0.766]$ ,  $P\text{-value} < 0.0001$ ).

229 We found significant differences between some orders for both glucose and albumin glycation.  
230 ESM1 shows the raw outcomes of the models, showing such significant differences to the  
231 intercept and the Confidence Intervals to perform the pairwise comparisons across all groups.  
232 To explore some of the details, we can see for example how Apodiformes showed the highest  
233 average glucose values (species average model: Estimated mean=351.8 mg/dL,  $CI_{95}[238.9,$   
234  $514.2]$ ; model with individuals: Estimated mean=349.8 mg/dL,  $CI_{95}[251.8, 476.7]$ ,  $n=39$   
235 individuals, 1 species), followed by Passeriformes (species average model: Estimated  
236 mean=349.2 mg/dL,  $CI_{95}[279.6, 438.6]$ ; model with individuals: Estimated mean=337.6 mg/dL,  
237  $CI_{95}[274.5, 414.5]$ ,  $n=84$  individuals, 8 species), while the Suliformes (species average model:  
238 Estimated mean=172 mg/dL,  $CI_{95}[117.6, 252.9]$ ; model with individuals: Estimated mean=169.5  
239 mg/dL,  $CI_{95}[120.9, 239.7]$ ,  $n=5$  individuals, 1 species), Rheiformes (species average model:  
240 Estimated mean=173.7 mg/dL,  $CI_{95}[118, 254.7]$ ; model with individuals: Estimated mean=172  
241 mg/dL,  $CI_{95}[123.7, 239.8]$ ,  $n=9$  individuals, 1 species) and Phoenicopteriformes (species average  
242 model: Estimated mean=176 mg/dL,  $CI_{95}[130, 241.1]$ ; model with individuals: Estimated  
243 mean=141.2 mg/dL,  $CI_{95}[100.4, 198.2]$ ,  $n=5$  individuals, 1 species) show the lowest average

244 values (for the species average models, the pattern is  
245 Suliformes<Rheiformes<Phoenicopteriformes, while for the individual models is  
246 Phoenicopteriformes<Suliformes<Rheiformes).

247 For glycation, Strigiformes (species average model: Estimated mean=25.6 %, CI<sub>95</sub>[20, 31]; model  
248 with individuals: Estimated mean=25.3 %, CI<sub>95</sub>[19.1, 31.5], n=7 individuals, 2 species),  
249 Apodiformes (species average model: Estimated mean=25.5 %, CI<sub>95</sub>[18.6, 32.4]; model with  
250 individuals: Estimated mean=24.8 %, CI<sub>95</sub>[17.9, 32], n=40 individuals, 1 species) and  
251 Coraciiformes (species average model: Estimated mean=24.5 %, CI<sub>95</sub>[17.9, 31.6]; model with  
252 individuals: Estimated mean=23.9 %, CI<sub>95</sub>[15.8, 32], n=2 individuals, 1 species), all terrestrial  
253 carnivores as by our sampled species (see discussion below), had the highest average.

254 On the other hand, Casuariiformes (species average model: Estimated mean=10.8 %, CI<sub>95</sub>[3.7,  
255 18.1]; model with individuals: Estimated mean=11.4 %, CI<sub>95</sub>[2.7, 20.6], n=1 individual, 1  
256 species), Phoenicopteriformes (species average model: Estimated mean=11.2 %, CI<sub>95</sub>[5.6, 16.7];  
257 model with individuals: Estimated mean=10.5 %, CI<sub>95</sub>[4.6, 16.3], n=22 individuals, 2 species) and  
258 Suliformes (species average model: Estimated mean=13.8 %, CI<sub>95</sub>[7, 20.7]; model with  
259 individuals: Estimated mean=13.2 %, CI<sub>95</sub>[5.6, 20.5], n=5 individuals, 1 species) had the lowest  
260 average levels for the species average models, the pattern is  
261 Casuariiformes<Phoenicopteriformes<Suliformes, while for the individual models is  
262 Phoenicopteriformes<Casuariiformes<Suliformes). Graphs with raw data on species average  
263 and individual glucose and glycation values by order are shown on ESM2 (**Figure ESM2.3**).  
264 Estimates of phylogeny effects on glucose and glycation values differ if we consider the models  
265 with intraspecific variability or without it, but not so much between the models with and without  
266 life history traits (within the previous categories). In the case of species averages, the effect of  
267 the tree on glucose is lower and the estimation is less precise than for glycation, while for the  
268 models considering individual values, it is glycation what shows lower levels of tree-related  
269 variance than glucose (see ESM1).



270

271 **Figure 1.** Average plasma glucose values in mg/dL (in grey) and average albumin glycation rate as a  
 272 percentage of total albumin (in blue) from all the species used in this study (some of them with glucose  
 273 values coming from ZIMs database; see methods) with the orders they belong to. Glucose and glycation  
 274 values are standardized in order to be compared, with the dotted lines representing half the maximum  
 275 and maximum values for each variable (as indicated by the axes in their corresponding colours), from  
 276 inside out. Tree from a consensus on 10,000 trees obtained from "Hackett All species" on Birdtree.org,  
 277 including 88 species from 22 orders (see methods).

278 **Bird glycemia appears related to body mass and maximum lifespan**

279 After controlling for intraspecific variation in our analyses, we found that variation in glucose  
 280 levels were significantly explained by variations in body mass and both the linear and quadratic  
 281 component of mass-adjusted maximum lifespan, but not by variations in diet (**Figure 4.A** with  
 282 predictions from the model without life history traits and **Figure ESM2.2.A** with raw individual  
 283 data from the same dataset), clutch mass and developmental time (see **Table 1** for the model

284 including life-history trait variables). Heavier species had lower glucose levels (see **Figure 2.A**,  
 285 drawn with the estimates from the model without life history traits, which includes more  
 286 species). Glucose levels increase with increasing mass-adjusted lifespan until reaching a plateau  
 287 (**Figure 2.B**). Models that did not consider intraspecific variability show no significant effect of  
 288 any of the aforementioned variables on glucose levels (see ESM1).

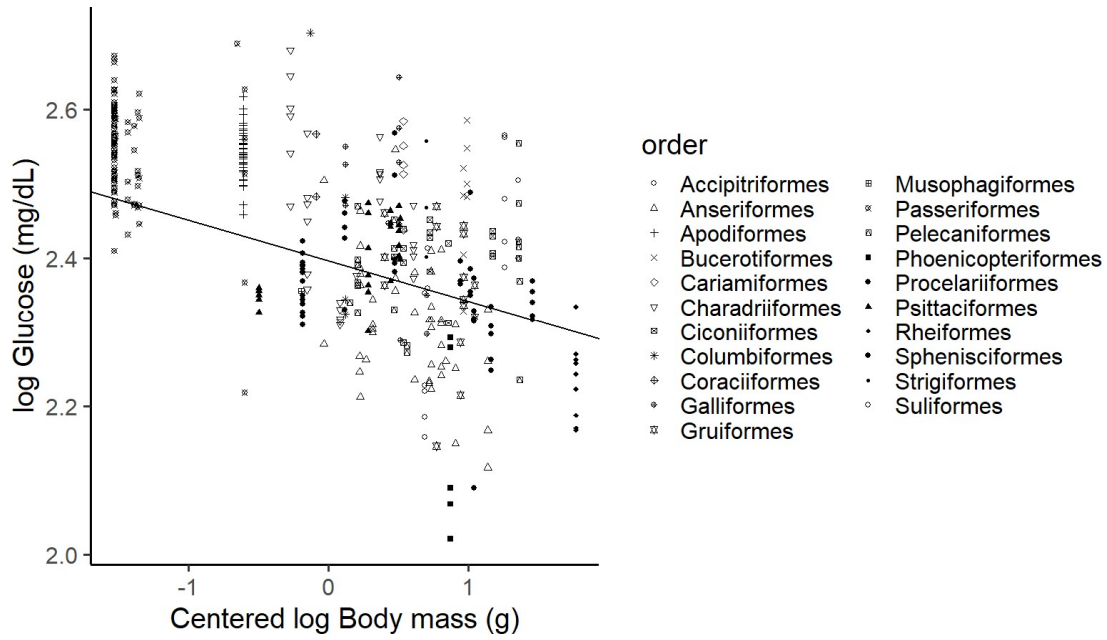
289 **Table 1.** Final glucose model with diet, body mass, and life history traits as explanatory variables, including  
 290 the significant quadratic effect of maximum lifespan. Posterior means, CI<sub>95</sub> and pMCMC from a  
 291 phylogenetic MCMC GLMM model including n=326 individuals of 58 species. Both glucose and body mass  
 292 are log<sub>10</sub> transformed and life history traits are residuals from a pGLS model of log<sub>10</sub> body mass and log<sub>10</sub>  
 293 of the trait in question (see ESM6). Body mass was also centred to better explain the intercept, as 0 body  
 294 mass would make no biological sense. The intercept corresponds to the Omnivore diet, being used as the  
 295 reference as it is considered the most diverse and “neutral” group for this purpose. Significant predictors  
 296 are indicated in bold.

	Estimates	Lower 95% CI	Upper 95% CI	Sampling effort	pMCMC
Intercept (Omnivore)	2.407181	2.309	2.506	59900	<0.001
Diet: Carnivore terrestrial	0.025121	-0.047	0.095	59900	0.475
Diet: Aquatic predator	0.028388	-0.044	0.0989	59900	0.424
Diet: Herbivore	-0.054	-0.167	0.058	60726	0.328
Diet: Frugivore/granivore	-0.01	-0.158	0.14	57618	0.89
Centred Log <sub>10</sub> Body mass	-0.051	-0.087	-0.014	59178	0.009
Maximum lifespan	0.13	0.002	0.253	59900	0.042
Maximum lifespan <sup>2</sup>	-0.627	-1.122	-0.141	59900	0.014
Clutch mass	-0.079	-0.233	0.066	59900	0.292
Developmental time	-0.012	-0.191	0.167	59041	0.891

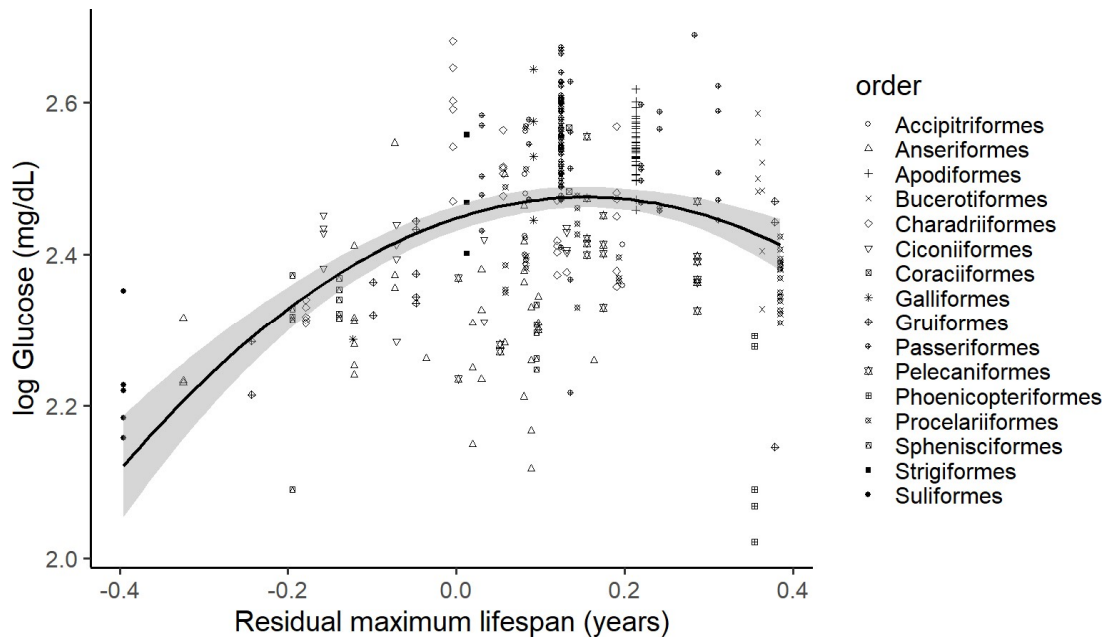
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300 **Figure 2.** Plasma glucose levels (in mg/dL) variation as a function of **A** species mean centred body mass  
 301 and **B** residual maximum lifespan. Both glucose and body mass are log transformed. Maximum lifespan  
 302 (in years) is given as the residues of a pGLS model with body mass (in grams), both log<sub>10</sub> transformed, so  
 303 the effects of body mass on longevity are factored out (see ESM6). Different bird orders, are indicated by  
 304 symbols, as specified in the legends at the right side of the graphs. **Figure 2.A** uses the values and  
 305 estimates from the glucose model without life history traits (n=389 individuals from 75 species), while  
 306 **Figure 2.B** uses only the data points employed on the complete model (n=326 individuals of 58 species).

307 **Bird albumin glycation is related to glycemia and diet**

308 After controlling for intraspecific variability, we found that diet was affecting variation in  
309 albumin glycation rates, with terrestrial carnivorous species having higher glycation levels than  
310 omnivorous species (**Table 2** for complete model; see **Figure 4.B** with predictions of the model  
311 without life history traits and **Figure ESM2.2.B** with raw individual data from the same dataset).  
312 However, for the models for species averages, there was only a trend on this pattern in the one  
313 including life history traits, and no effect in the other (see ESM1). The relation between glycation  
314 and glucose levels was positive and significant in all but the model that included life history traits  
315 but not intraspecific variation (see **Table 2** for the outcome of the model with individual values  
316 and life history traits and ESM1 for the rest; see **Figure 3** with estimates from the model including  
317 individual variation but no life history traits, as it contains more species and the estimates are  
318 similar). Given that plasma glucose is logarithm transformed, this implies that birds with higher  
319 glucose levels have relatively lower albumin glycation rates for their glucose, fact that we  
320 would be referring as higher glycation resistance.

321 Additional analyses looking at the number of exposed lysines in the albumin sequence of a  
322 species show no effect of this variable on albumin glycation rates ( $\alpha=10.39$ :  $CI_{95}[-3.713, 24.993]$ ,  
323  $\beta=0.246$ :  $CI_{95}[-0.144, 0.629]$ ,  $P_{MCMC}=0.196$ ; ESM1).

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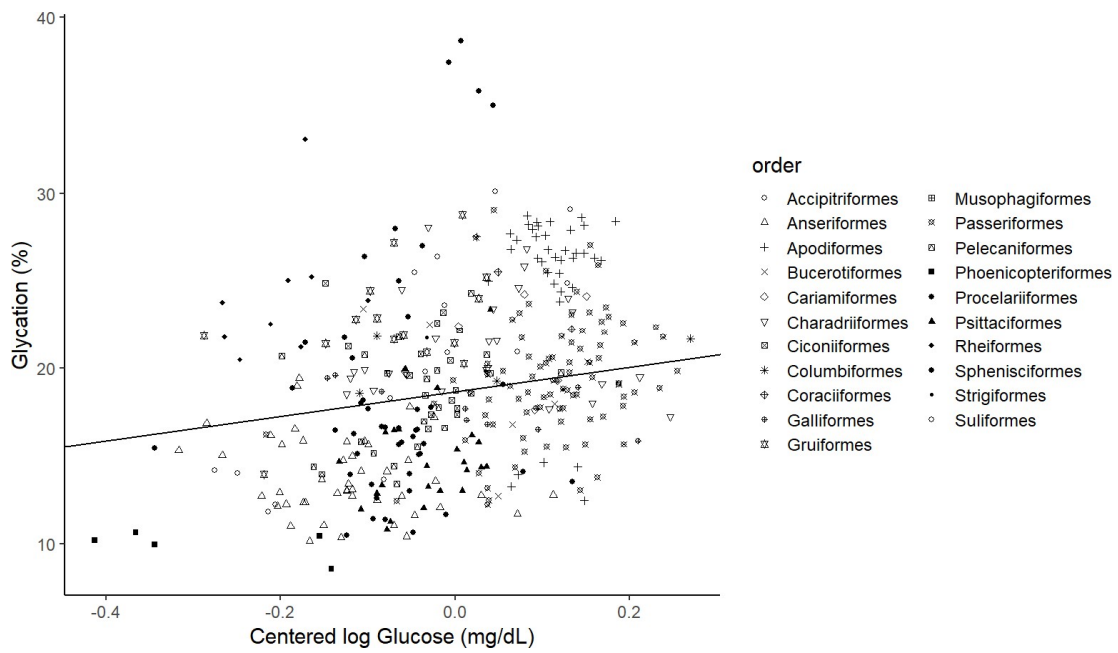
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334

335 **Table 2.** Final glycation model with diet, body mass, glucose and life history traits as explanatory variables.  
 336 Posterior means, CI<sub>95</sub> and pMCMC from a phylogenetic MCMC GLMM model including n=316 individuals  
 337 of 58 species. Both glucose and body mass are log<sub>10</sub> transformed and life history traits are residuals from  
 338 a linear model of log<sub>10</sub> body mass and log<sub>10</sub> of the trait in question (see ESM6). Body mass and glucose  
 339 were also centred to better explain the intercept. The intercept corresponds to the Omnivore diet, being  
 340 used as the reference as it is considered the most diverse and “neutral” group for this purpose. Significant  
 341 predictors are indicated in bold and the confidence intervals are considered for making pairwise  
 342 comparisons between the groups.

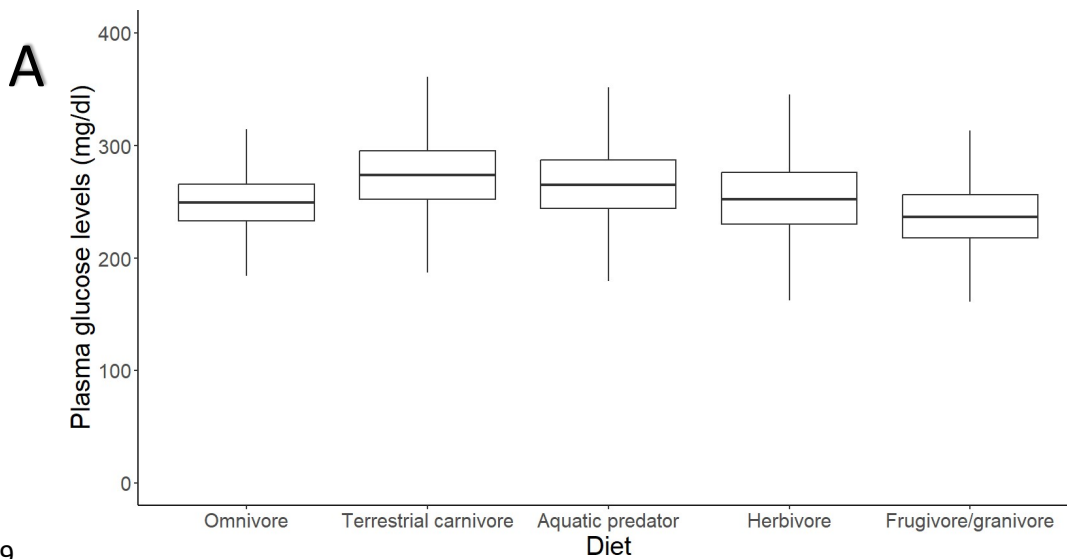
	Estimates	Lower 95% CI	Upper 95% CI	Sampling effort	pMCMC
Intercept	18.066	13.743	22.38274	59900	<0.001
Diet: Carnivore terrestrial	4.18	0.673	7.82346	59900	0.023
Diet: Aquatic predator	1.037	-2.704	4.645	59557	0.577
Diet: Herbivore	-1.301	-6.798	4.266	60677	0.634
Diet: Frugivore/granivore	2.77	-5.88	11.189	59900	0.523
Centred log <sub>10</sub> Body mass	0.015	-1.954	1.908	62211	0.985
Log <sub>10</sub> Glucose	6.498	1.111	11.8	59900	0.017
Maximum lifespan	1.564	-5.5	8.391	59900	0.657
Clutch mass	5.064	-2.84	13	59900	0.203
Developmental time	3.551	-5.935	13.226	60780	0.455

343

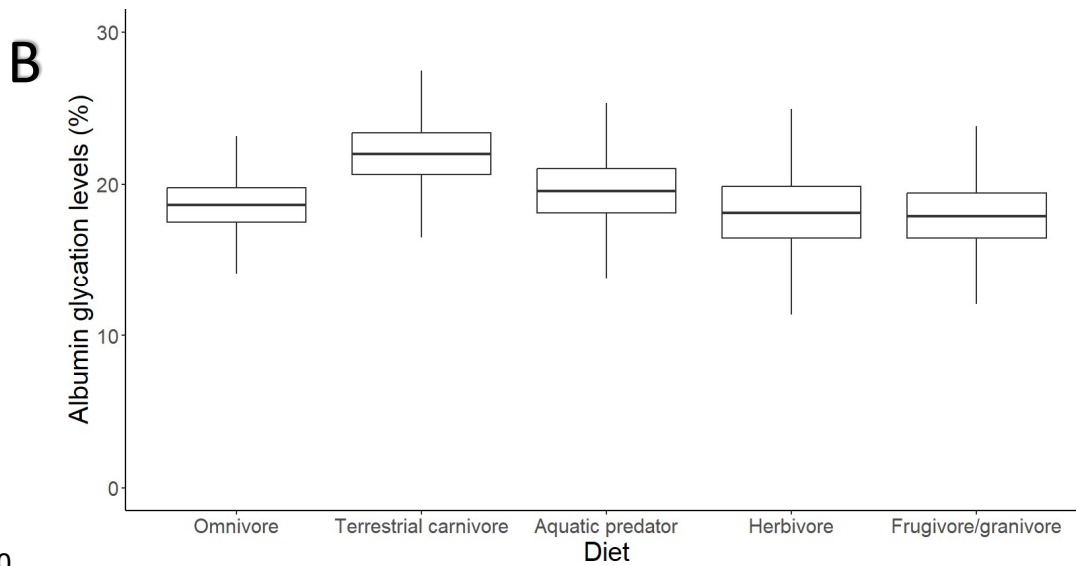


344

345 **Figure 3.** Albumin glycation rates (as a percentage of total albumin) variation as a function of individual  
 346 plasma glucose values (mg/dL). Different bird orders are indicated by symbols, as specified on the legends  
 347 at the right side of the graphs. The values and estimates used are from the glycation model without life  
 348 history traits (n=379 individuals from 75 species).



349



350

351 **Figure 4.** Outcomes of the models on individual data on effects of diet on **(A)** plasma glucose levels and  
 352 **(B)** albumin glycation in birds. Glucose levels are given in mg/dL, while glycation levels are a percentage  
 353 of total plasma albumin which is found to be glycated. Terrestrial carnivores showed significantly higher  
 354 glycation levels than omnivores (Estimate=22.01 %, CI<sub>95</sub>[18.77, 25.15], p<sub>MCMC</sub>=0.039). Models without life  
 355 history traits, including more individuals, are represented, but the models with life history traits do not  
 356 show differences in their qualitative predictions (i.e. higher albumin glycation in terrestrial carnivores than  
 357 in omnivores; see ESM1).

358

359 We observed no significant effect of age relative to maximum lifespan nor sex on either  
 360 glycaemia or glycation (see ESM1).

## 361 **DISCUSSION**

### 362 **Plasma glucose level and albumin glycation rate (co)variation patterns suggests** 363 **resistance mechanisms**

364 Our findings show that glucose levels vary widely among different bird groups. Interspecific  
 365 differences are partly explained by the allometric relationship of glycaemia with body mass  
 366 **(Figure 2.A)**, which has already been reported in previous studies [56, 63, 34, 13, 14]. Indeed,  
 367 Passeriformes, Apodiformes and some Columbiformes (e.g. *Nesoenas mayeri* holds the highest  
 368 value in our dataset) are found at the higher end of the glucose level continuum, in accordance  
 369 with their relatively small body mass and powered flight, while groups of larger birds such as  
 370 Phoenicopteriformes, Anseriformes, Rheiformes and Suliformes tend to show low glycaemia

371 levels. This pattern is similar for glycation, with some groups of large birds (such as  
372 Phoenicopteriformes, Anseriformes and Suliformes) showing the lowest levels of glycated  
373 albumin, while small birds (such as Apodiformes) had the highest values. Nevertheless, glycation  
374 remains high in some birds in relation to their glucose levels, as in Rheiformes, or low as in  
375 Psittaciformes or Passeriformes. The case of Procellariiformes, which are typically long-lived  
376 birds, is particularly striking, with some species exhibiting some of the highest glycation levels  
377 (*Calonectris diomedea* and *Macronectes giganteus*) and others some of the lowest (*Procellaria*  
378 *aequinoctialis*). This suggests that, if birds are protected against the deleterious effects of high  
379 glycaemia, certain species may have evolved mechanisms to efficiently prevent proteins to be  
380 glycated at high rates while others may have evolved mechanisms to resist the consequences of  
381 protein glycation. We should also bear in mind that some taxonomic groups may be  
382 underrepresented in our study, or biases in species selection due to availability contingencies  
383 (e.g. species common in zoos or present in European countries) may exist, so further studies  
384 should target this underrepresented groups in order to confirm our predictions.

385 As expected for a non-enzymatic reaction, just by the law of mass action, bird albumin glycation  
386 rates increase with plasma glucose concentration. However, the logarithmic nature of the  
387 relationship, suggests that species with higher plasma glucose levels exhibit relatively greater  
388 resistance to glycation. This finding aligns with previous research indicating that *in vitro* glycation  
389 levels of chicken albumin increase at a slower rate than those of human albumin when glucose  
390 concentration is elevated [82]. Moreover, these levels are consistently lower than those of  
391 bovine serum albumin regardless of glucose concentration and exposition time [37]. As  
392 discussed in previous studies comparing chicken to bovine serum albumin [37], or zebra finch to  
393 human haemoglobin [53], the lower glycation rates observed in bird proteins may result from a  
394 lower number of glycatable amino acids (e.g. lysines) in their sequence and/or their lesser  
395 exposure at the protein surface.

396 Our analyses do not succeed in indicating a significant positive relationship between average  
397 glycation levels and the number of glycatable lysines in the albumin sequence. This may be  
398 attributed to the limited number of species employed or the weak variation in the number of  
399 glycatable lysine residues, which are mostly ranging from 33 to 39. An interesting exception are  
400 the 18 glycatable lysine residues of flamingos (*Phoenicopterus ruber*), which also shows very low  
401 glycation levels (mean=10.1%). However, the exceptions of 44 in zebra finches and 20 in godwits  
402 (*Limosa lapponica*, used in place of *Limosa limosa*) are nevertheless associated with very similar  
403 average glycation levels of 20.2% and 20.7% respectively.

404 **Plasma glucose relates with longevity and may be influenced by reproductive**  
405 **strategies**

406 Our results were only in minor agreement with predictions from POLS theory: it holds for body  
407 mass, but not for the other life history variables tested. The only previous comparative study of  
408 glycaemia and life history traits to our knowledge [14] shows no relationship with mass-adjusted  
409 maximum lifespan in passerines. However, our study over 88 bird species on 22 orders revealed  
410 an increase in glucose with mass-adjusted longevity up to a plateau (see **Figure 2.B**). Thus, the  
411 relationship between glucose and maximum lifespan may depend on differences between bird  
412 orders or be tied to specific species particularities not explored in our study. Such species  
413 particularities might involve additional undetermined ecological factors that modify the  
414 relationship of glycaemia with longevity. Further exploration of glucose metabolism in relation  
415 with lifestyle will bring further light on species-specific life-history adaptations concerning  
416 glucose. For example, the species with the lower mass-adjusted maximum lifespan here was a  
417 cormorant (*Leucocarbo verrucosus*), which have quite low glucose values for birds.

418 Regarding reproductive investment (i.e. clutch mass), our results show no relationship with  
419 glycation (see **Table 2**), while previous studies reported positive relationships with glycaemia in  
420 passerines [14, 83]. Interestingly, most of the species with high clutch mass included in our study  
421 belong to the Anseriformes (**Figure ESM2.1**). While these species exhibit very low glycaemia and  
422 albumin glycation rates, they are also characterized by a particular reproductive strategy  
423 compared to passerines, for whom clutch mass does not imply the same in terms of parental  
424 investment. For instance, Anseriformes, unlike Passeriformes, are precocial and their offspring  
425 are not very dependent on parental care. Furthermore, they are capital breeders, accumulating  
426 the energetic resources required for reproduction in advance rather than collecting them during  
427 reproduction. These species typically have large clutch sizes and incubation is usually carried out  
428 by females, who store internal fat resources to support this main part of the parental  
429 investment. In addition, ducklings are born highly developed, reducing the amount of care  
430 required post-hatching (see e.g. [84-85]). Consequently, their dependence on glucose as a rapid  
431 energy source for reproduction may be lower, with investment in this activity likely more closely  
432 linked to lipid accumulation. This could explain why we did not detect previously reported  
433 effects of clutch mass on glucose levels.

434

435 **Terrestrial carnivores show a paradoxically increased albumin glycation rate without**  
436 **increased plasma glucose levels**

437 Contrary to our expectation of finding differences across dietary groups, plasma glucose did not  
438 significantly vary with species diet. This aligns with results previously reported by [14] for  
439 Passeriformes or by [55] for 160 species of vertebrates among which 48 were birds, but not with  
440 [15], that showed glycaemia to be increased with the proportion of fruits/seeds in the diet, or  
441 with [56], that showed higher (mass-adjusted) glucose levels for terrestrial carnivores and  
442 insectivores (those included in our terrestrial carnivores' category) and lower for  
443 frugivorous/nectarivorous. On the other hand, intraspecific data indicates that changes in diet  
444 composition hardly affects glycaemia in birds [86]. These studies suggest that glycaemia is tightly  
445 regulated independently of dietary composition within species, while it probably varies across  
446 species depending on the diet they are adapted to, although depending on the bird groups  
447 included in the analyses and the way of assessing it.

448 Although terrestrial carnivore species did not have significantly higher glycaemia levels in our  
449 study, they nonetheless demonstrated significantly higher albumin glycation rates, suggesting a  
450 potential susceptibility to protein glycation. Besides unique structural features, this  
451 phenomenon could be due to lower albumin turnover rates in terrestrial carnivores. Studies in  
452 humans have linked higher oxidative damage to albumin with lower turnover rates (reviewed in  
453 [87]), which may extend to other species and post-translational modifications of albumin, such  
454 as bird's albumin glycation. However, the contrary outcome would be anticipated if protein  
455 intake was high as seen in carnivorous species [87-88].

456 Interestingly, the contrast between terrestrial and aquatic predators, which did not show such  
457 high glycation rates, suggests the beneficial influence of a substantial difference in food  
458 composition or the pre-eminence of another ecological factor, yet to be determined, which may  
459 explain lower glycation levels in aquatic predators. In terms of food composition, the proportion  
460 of essential n-3 PUFA (polyunsaturated fatty acids), particularly long-chain ones such as DHA and  
461 EPA compared with n-6 PUFA and short-chain n-3 PUFA, is different in aquatic and terrestrial  
462 environments [89] and therefore in the diet of predators in each environment (e.g. [90]). For  
463 instance, low n-6 PUFA/n3- PUFA ratio have been shown to decrease insulin levels and improve  
464 insulin resistance in humans [91]. On the other hand, while some studies report that an increase  
465 in dietary n3-PUFA reduces the levels of glycated haemoglobin (HbA1c) in humans, this effect  
466 was not detected in many other studies and therefore remains inconclusive (reviewed in [92]).



467 The detailed study of the fat composition of the diets of terrestrial and aquatic predators, in  
468 particular the ratio between different types of PUFA, merits more attention. In addition,  
469 micronutrient content, such as circulating antioxidant defences, should also be taken into  
470 consideration. Indeed, a positive relationship between oxidative stress and glycated levels of  
471 bovine serum albumin has been reported [93]. One hypothesis is that terrestrial predators have  
472 higher systemic oxidative stress levels compared to other species, which may be explained by  
473 defects in their antioxidant defences. Uric acid is one of the main non-enzymatic antioxidants in  
474 birds [94-96], and uric acid levels are especially high in carnivore species that have a rich protein  
475 diet [95, 97-100]. We should therefore take into account other important antioxidants such as  
476 vitamin E, vitamin A and carotenoids, which may be less abundant in the diets of terrestrial  
477 carnivores (e.g. [101], but see [52]). The question of whether the diet of aquatic carnivores  
478 provides a better intake of antioxidants would therefore requires a more detailed description of  
479 dietary habits. The herbivorous diet, meanwhile, despite the expected possibility contributing  
480 to lower glycaemia and glycation levels due to higher levels of PUFA compared to SFA (saturated  
481 fatty acids) (effects reviewed in [92] for humans) and higher fibre content (see [102]), did not  
482 lead to significantly lower levels of either of these two parameters. However, the relatively low  
483 sample size in that group or the presence of outliers as *Nesoenas mayeri*, with the highest  
484 glucose levels of this dataset, makes the interpretation of the obtained results somehow limited.  
485 Therefore, further research should be carried out on these species to determine if the expected  
486 pattern would emerge with a better sampling.

487 Finally, differences between captive and wild populations could be considered as a source of  
488 variation in glucose or glycation levels, due to a more sedentary lifestyle in captivity, with lower  
489 activity levels and a higher food intake likely to lead to increased circulating glucose levels in  
490 captive individuals. Additionally, differences in nutrient intake, such as antioxidants, specific  
491 fatty acids or amino acids, between captive and wild populations could contribute to variations  
492 in glycation levels, as we saw above. Nevertheless, we think that this factor is unlikely to  
493 significantly affect our findings regarding diet categories, because our study encompasses  
494 species from both captive and wild populations across various diet groups, particularly those  
495 exhibiting significant differences (e.g., omnivores versus terrestrial carnivores).

#### 496 **CONCLUSION AND PERSPECTIVES**

497 In conclusion, the avian plasma glucose levels measured here are generally higher or in the high  
498 range of values recorded for mammalian species [28]. Our study also concludes that there is

499 considerable variation in plasma glucose levels and albumin glycation rates between bird  
500 species, with those with the highest glucose levels showing greater resistance to glycation. The  
501 correlation between plasma glucose and life history traits are primarily influenced by its inverse  
502 association with body mass, along with non-linear, mass-independent effects of longevity.  
503 Finally, although diet does not explain plasma glucose levels in our study, terrestrial carnivores  
504 have higher albumin glycation rates than omnivores. Whether these intriguing results are  
505 explained by specific effects of certain dietary components, such as PUFAs and/or antioxidants,  
506 remains to be determined. Differences in plasma glucose levels and albumin glycation rates  
507 across bird species do not seem consistent with predictions according to the POL hypothesis,  
508 except for body mass. Further investigation is needed to elucidate the correlation between  
509 these traits and specific life conditions, such as reproductive strategy, migration patterns, flight  
510 mode or more detailed diet composition. In addition, more in-depth exploration of glycation  
511 levels within high glycaemic groups such as the Passeriformes and other small birds, which make  
512 up a significant proportion of avian species, could provide valuable new insights. Similarly,  
513 investigating groups such as the Phoenicopteriformes or Anseriformes, which are at the other  
514 end of the glycaemic-glycation spectrum, could shed light on the origin of differences between  
515 avian orders. Furthermore, notable variations were observed between species within orders, as  
516 in the Procellariiformes, which with their high mass-adjusted longevity, and particularly given  
517 that some of the age known individuals in our dataset (indeed, two specimens of snow petrels,  
518 *Pagodroma nivea*, showed ages higher than the maximum lifespan reported in the sources we  
519 explored, determining a new record) have still relatively low levels of glycation, may suggest that  
520 some species (the ones that do show higher glycation levels) are not intrinsically resistant to  
521 glycation, but rather to its adverse consequences for health. As the main limitations from this  
522 study, the usage of individuals from both wild and captive populations, sampled at different  
523 periods of the year, and the low sample size for some species, due to logistic constraints, may  
524 have introduced noise in the values reported that should be addressed in future studies by  
525 implementing a stricter sampling protocol. Also, a more thorough and accurate report and  
526 compilation of life history traits from multiple species would allow to increase the number of  
527 species included in this kind of analyses. Future research should also focus on specific species to  
528 unravel the physiological mechanisms mediating the effects of blood glucose and protein  
529 glycation on life-history trade-offs, in particular mechanisms that may vary between taxa and  
530 contribute to characteristic adaptations in birds to mitigate the potentially negative effects of  
531 comparatively high glycaemia.

532 **CONFLICTS OF INTEREST**

533 None declared.

534 **DATA ACCESSIBILITY**

535 All data used, including references for the values taken from bibliography or databases, will be  
536 available as Electronic Supplementary Material (ESM5).

537 **ETHICS STATEMENT**

538 This study followed all the legal considerations, with the ethic authorisations from the French  
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## 575 **AUTHORS' CONTRIBUTIONS**

576 F. Criscuolo and F. Bertile conceived the idea, directed most of the sample collection and logistics  
577 and contributed significantly to the writing. A. Moreno-Borralló contributed to the development  
578 of the questions, gathered the diet and life-history data, performed the statistical analyses, and  
579 led the writing. S. Jaramillo Ortiz and C. Schaeffer performed the mass spectrometry analyses  
580 for protein glycation measurements, and S. Jaramillo Ortiz contributed to the glucose measures  
581 and commented on the manuscript. T. Boulinier. and V. A. Viblanc coordinated the ECONERGY  
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583 commented on the manuscript. Olivier Chastel collaborated collecting marine bird samples and  
584 commented on the manuscript. B. Rey contributed with the collection of samples from Parc des  
585 Oiseaux (Villars-les-Dombes, France) and commented on the manuscript. P. Bize leads the  
586 monitoring of Alpine swifts' populations from which the samples were obtained, he helped  
587 collecting them and commented on the manuscript. J. S Gutiérrez and J. A. Masero contributed  
588 with samples from Spain and part of the statistic scripts and commented on the manuscript. B.

589 Quintard leads the health monitoring of Mulhouse zoo bird collection, organized and realized  
590 most of Mulhouse zoo samplings and commented on the manuscript. All authors gave their  
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978 <http://dx.doi.org/10.1016/j.bcdf.2017.07.005>

# Electronic Supplementary Material 1 (ESM1)

## Models with species averages

### Glucose model

Iterations = 10001:5999901  
Thinning interval = 100  
Sample size = 59900

DIC: -105.767

G-structure: ~animal

	post.mean	l-95% CI	u-95% CI	eff.samp
animal	0.00778	0.0001963	0.02792	25022

~Method\_Glu

	post.mean	l-95% CI	u-95% CI	eff.samp
Method_Glu	0.02048	0.0002138	0.04069	59900

R-structure: ~units

	post.mean	l-95% CI	u-95% CI	eff.samp
units	0.01655	0.0009857	0.05317	52896

Location effects: logGlucose ~ Diet + Centered\_logBM

	post.mean	l-95% CI	u-95% CI	eff.samp	pMCMC
(Intercept)	2.399647	1.675274	3.079457	60867	0.000167
***					
DietTerrestrial carnivore	0.023422	-1.073377	1.102045	60857	0.963907
DietAquatic predator	0.008556	-1.242851	1.294827	59900	0.984040
DietHerbivore	-0.017745	-1.164199	1.100292	59900	0.976394
DietFrugivore/granivore	-0.035835	-1.305620	1.276010	59900	0.957295
Centered_logBM	-0.107740	-0.782927	0.570726	59900	0.755492
---					

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

### Glucose model with life history traits

Iterations = 10001:5999901  
Thinning interval = 100  
Sample size = 59900

DIC: -75.09374

G-structure: ~animal

	post.mean	l-95% CI	u-95% CI	eff.samp
animal	0.009398	0.000227	0.03334	30571

~Method\_Glu

	post.mean	l-95% CI	u-95% CI	eff.samp
Method_Glu	0.01874	0.0002223	0.04551	59900

R-structure: ~units

```

      post.mean l-95% CI u-95% CI eff.samp
units    0.02032 0.001109 0.06938    55501

```

Location effects: logGlucose ~ Diet + Centered\_logBM + poly(ML, 2, raw = TRUE) + CM + DT

```

      post.mean l-95% CI u-95% CI eff.samp pMCMC
(Intercept)      2.361    1.186    3.592    59900 0.000401 ***
DietTerrestrial carnivore 7.182e-04 -1.505    1.518    59317 0.998497
DietAquatic predator  -1.556e-02 -1.564    1.539    59900 0.982671
DietHerbivore      -3.319e-02 -1.731    1.644    59553 0.969349
DietFrugivore/granivore -4.398e-03 -7.328    7.710    59900 0.999599
Centered_logBM     -7.183e-02 -0.8751   0.7647   59900 0.862270
poly(ML, 2, raw = TRUE)1 1.812e-01 -3.085    3.535    59900 0.917963
poly(ML, 2, raw = TRUE)2 -4.190e-01 -15.21   13.73    59900 0.960100
CM                 -1.592e-01 -3.090    2.784    59900 0.916494
DT                 -4.562e-02 -3.911    3.873    59900 0.981068
---
```

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

### Glycation model

```

Iterations = 10001:5999901
Thinning interval = 100
Sample size = 59900

```

DIC: 452.168

G-structure: ~animal

```

      post.mean l-95% CI u-95% CI eff.samp
animal      11.81    1.526    25.15    54976

```

~Method\_Glu

```

      post.mean l-95% CI u-95% CI eff.samp
Method_Glu    8.687    0.4069    23.68    59900

```

R-structure: ~units

```

      post.mean l-95% CI u-95% CI eff.samp
units    6.463    2.74    10.63    57174

```

Location effects: Glycation ~ Diet + Centered\_logBM + Centered\_logGlucose

```

      post.mean l-95% CI u-95% CI eff.samp pMCMC
(Intercept)      18.2758 12.5663 23.7957 59900 0.000801 ***
DietTerrestrial carnivore 1.3137 -1.5652 4.2967 59900 0.375960
DietAquatic predator  -1.0042 -4.1989 2.3750 60681 0.541002
DietHerbivore      0.6997 -2.8760 4.2590 58771 0.703773
DietFrugivore/granivore -1.1994 -4.0201 1.6324 59900 0.398164
Centered_logBM     0.4628 -1.1913 2.0645 60789 0.573289
Centered_logGlucose 11.9011 1.3035 22.4421 59900 0.027212 *
---
```

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

### Glycation model with life history traits

```

Iterations = 10001:5999901
Thinning interval = 100
Sample size = 59900

```

DIC: 343.8778

```

G-structure: ~animal
      post.mean l-95% CI u-95% CI eff.samp
animal      10.93   0.7711   25.35   54187

      ~Method_Glu
      post.mean l-95% CI u-95% CI eff.samp
Method_Glu   8.874   0.4055   26.68   59900

R-structure: ~units
      post.mean l-95% CI u-95% CI eff.samp
units        7.044   2.62    12.18   57970

Location effects: Glycation ~ Diet + Centered_logBM + Centered_logGlu
cose + ML + CM + DT

      post.mean l-95% CI u-95% CI eff.samp pMCMC
(Intercept)    17.78879 11.72845 23.91039 60459 0.000768 ***
DietTerrestrial carnivore 3.00954 -0.33015 6.27856 59900 0.074925 .
DietAquatic predator  0.01218 -3.60722 3.64812 59900 0.999199
DietHerbivore      0.13111 -4.18837 4.57657 58894 0.965943
DietFrugivore/granivore -2.68295 -8.09751 2.83629 59900 0.329850
Centered_logBM     0.38664 -1.48866 2.25679 59900 0.672922
Centered_logGlucose 10.82714 -2.87500 23.96327 59900 0.112688
ML                 1.44492 -4.73215 7.44321 59210 0.637529
CM                 2.50125 -4.40552 9.50268 58700 0.487312
DT                -0.57544 -8.81798 7.45233 59715 0.884808
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

```

## Models with individuals

### Glucose model

```

Iterations = 10001:5999901
Thinning interval = 100
Sample size = 59900

DIC: -990.7597

G-structure: ~animal
      post.mean l-95% CI u-95% CI eff.samp
animal      0.007075 0.0009546 0.01447   56957

      ~species
      post.mean l-95% CI u-95% CI eff.samp
species      0.002697 0.0006719 0.00501   58026

R-structure: ~units
      post.mean l-95% CI u-95% CI eff.samp
units      0.003975 0.003367 0.004615   59900

Location effects: logGlucose ~ Diet + Centered_logBM

      post.mean l-95% CI u-95% CI eff.samp pMCMC
(Intercept)    2.395863 2.306440 2.485317 58376 <2e-05 ***
DietTerrestrial carnivore 0.039472 -0.033565 0.108562 59900 0.27396
DietAquatic predator  0.026457 -0.052130 0.103220 60792 0.48648
DietHerbivore     0.006858 -0.090177 0.104693 59900 0.90040

```

```

DietFrugivore/granivore -0.022637 -0.090174 0.044136 59900 0.50204
Centered_logBM -0.054737 -0.089817 -0.019702 59900 0.00381 *
*
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

```

## Glucose model with life history traits

```

Iterations = 10001:5999901
Thinning interval = 100
Sample size = 59900

DIC: -821.5912

G-structure: ~animal

      post.mean 1-95% CI u-95% CI eff.samp
animal 0.006536 0.0006673 0.01443 58050

      ~species

      post.mean 1-95% CI u-95% CI eff.samp
species 0.002476 0.0005766 0.004708 58439

R-structure: ~units

      post.mean 1-95% CI u-95% CI eff.samp
units 0.004139 0.003458 0.00485 59900

Location effects: logGlucose ~ Diet + Centered_logBM + poly(ML, 2, raw
w = TRUE) + CM + DT

      post.mean 1-95% CI u-95% CI eff.samp pMCMC
(Intercept) 2.407181 2.309403 2.506404 59900 <2e-05 ***
DietTerrestrial carnivore 0.025121 -0.047437 0.095140 59900 0.47536
DietAquatic predator 0.028388 -0.043758 0.098887 59900 0.42401
DietHerbivore -0.054147 -0.167348 0.058349 60726 0.32755
DietFrugivore/granivore -0.010416 -0.157940 0.140411 57618 0.88975
Centered_logBM -0.050724 -0.086839 -0.013901 59178 0.00855 **
poly(ML, 2, raw = TRUE)1 0.129818 0.002193 0.253196 59900 0.04227 *
poly(ML, 2, raw = TRUE)2 -0.627202 -1.122011 -0.141017 59900 0.01429 *
CM -0.079069 -0.233308 0.066397 59900 0.29202
DT -0.011816 -0.191142 0.166694 59041 0.89068
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

```

## Glycation model

```

Iterations = 10001:5999901
Thinning interval = 100
Sample size = 59900

DIC: 1940.901

G-structure: ~animal

      post.mean 1-95% CI u-95% CI eff.samp
animal 9.386 1.251 20.63 55994

      ~species

      post.mean 1-95% CI u-95% CI eff.samp
species 9.49 4.557 15.07 59900

R-structure: ~units

```

```

      post.mean 1-95% CI u-95% CI eff.samp
units      8.373   7.078   9.701   59900

```

Location effects: Glycation ~ Diet + Centered\_logBM + Centered\_logGlucose

```

      post.mean 1-95% CI u-95% CI eff.samp pMCMC
(Intercept)      18.6602 15.1706 22.2037 59900 <2e-05 ***
DietTerrestrial carnivore  3.3544  0.1133  6.4909 60994 0.03940 *
DietAquatic predator    0.9421 -2.5193  4.4585 59900 0.59279
DietHerbivore          -0.4545 -4.7686  3.7936 59900 0.81593
DietFrugivore/granivore -0.6950 -3.8847  2.5971 58701 0.67245
Centered_logBM         0.1418 -1.4452  1.7423 56802 0.85546
Centered_logGlucose     6.9925  2.2903 11.8247 59900 0.00424 **
---

```

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

## Glycation model with life history traits

```

Iterations = 10001:5999901
Thinning interval = 100
Sample size = 59900

```

DIC: 1636.277

G-structure: ~animal

```

      post.mean 1-95% CI u-95% CI eff.samp
animal      10.16   0.895   24.75   55580

```

~species

```

      post.mean 1-95% CI u-95% CI eff.samp
species      11.55   5.403   18.64   59900

```

R-structure: ~units

```

      post.mean 1-95% CI u-95% CI eff.samp
units      8.943   7.449   10.52   59900

```

Location effects: Glycation ~ Diet + Centered\_logBM + Centered\_logGlucose + ML + CM + DT

```

      post.mean 1-95% CI u-95% CI eff.samp pMCMC
(Intercept)      18.06617 13.74317 22.38274 59900 <2e-05 ***
DietTerrestrial carnivore  4.17994  0.67282  7.82346 59900 0.0228 *
DietAquatic predator    1.03705 -2.70441  4.64476 59557 0.5771
DietHerbivore          -1.30139 -6.79815  4.26616 60677 0.6343
DietFrugivore/granivore  2.72689 -5.88016 11.18913 59900 0.5230
Centered_logBM         0.01477 -1.95368  1.90777 62211 0.9852
Centered_logGlucose     6.49824  1.11151 11.79978 59900 0.0167 *
ML                     1.56442 -5.50000  8.39154 59900 0.6573
CM                     5.06375 -2.83984 13.00020 59900 0.2030
DT                     3.55134 -5.93463 13.22610 60780 0.4555
---

```

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

## Age & Sex models

### Glucose

```

Iterations = 10001:5999901
Thinning interval = 100
Sample size = 59900

```

DIC: -635.2492

G-structure: ~animal

	post.mean	l-95% CI	u-95% CI	eff.samp
animal	0.008703	0.001214	0.01909	59900

~species

	post.mean	l-95% CI	u-95% CI	eff.samp
species	0.002923	0.0006328	0.005658	59900

R-structure: ~units

	post.mean	l-95% CI	u-95% CI	eff.samp
units	0.003799	0.003072	0.004595	59900

Location effects: logGlucose ~ Centered\_logBM + poly(logit(Age\_relative, FALSE), 2, raw = TRUE) + Sex

	post.mean	l-95% CI	u-95% CI
(Intercept)	2.391844	2.278127	
Centered_logBM	-0.052561	-0.097117	
poly(logit(Age_relative, FALSE), 2, raw = TRUE)1	0.001298	-0.013674	
poly(logit(Age_relative, FALSE), 2, raw = TRUE)2	-0.001157	-0.004889	
SexM	0.005705	-0.012474	

	eff.samp	pMCMC
(Intercept)	59186	<2e-05 ***
Centered_logBM	59101	0.0275 *
poly(logit(Age_relative, FALSE), 2, raw = TRUE)1	59900	0.8645
poly(logit(Age_relative, FALSE), 2, raw = TRUE)2	59900	0.5414
SexM	60869	0.5336

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

## Glycation

Iterations = 10001:5999901  
Thinning interval = 100  
Sample size = 59900

DIC: 1145.016

G-structure: ~animal

	post.mean	l-95% CI	u-95% CI	eff.samp
animal	19.3	3.365	38.51	59900

~species

	post.mean	l-95% CI	u-95% CI	eff.samp
species	5.82	1.536	10.92	59900

R-structure: ~units

	post.mean	l-95% CI	u-95% CI	eff.samp
units	6.026	4.861	7.266	59742

Location effects: Glycation ~ Centered\_logBM + Centered\_logGlucose + poly(logit(Age\_relative, FALSE), 2, raw = TRUE) + Sex

	post.mean	l-95% CI	u-95% CI	eff.samp
--	-----------	----------	----------	----------

```

(Intercept) 19.26559 14.02977 24
.55452 59900
Centered_logBM 1.12330 -0.88744 3
.12563 63546
Centered_logGlucose 9.74032 4.27174 15
.04954 59900
poly(logit(Age_relative, FALSE), 2, raw = TRUE)1 -0.07541 -0.66195 0
.53149 59900
poly(logit(Age_relative, FALSE), 2, raw = TRUE)2 -0.05413 -0.20650 0
.09595 60681
SexM 0.44836 -0.28507 1
.20100 59900

pMCMC
(Intercept) < 2e-05 ***
Centered_logBM 0.269850
Centered_logGlucose 0.000267 ***
poly(logit(Age_relative, FALSE), 2, raw = TRUE)1 0.806010
poly(logit(Age_relative, FALSE), 2, raw = TRUE)2 0.482771
SexM 0.233790
---
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

```

## Lysines model

```

Iterations = 10001:5999951
Thinning interval = 50
Sample size = 119800

DIC: 111.8866

G-structure: ~animal

      post.mean l-95% CI u-95% CI eff.samp
animal  14.21    0.5989   45.07   111860

R-structure: ~units

      post.mean l-95% CI u-95% CI eff.samp
units  18.31    4.944   35.13   116731

Location effects: Glycation ~ Lysines

      post.mean l-95% CI u-95% CI eff.samp pMCMC
(Intercept)  10.3914  -3.7127  24.9929  119800 0.144
Lysines      0.2462  -0.1438   0.6290  119800 0.196

```

## Orders models

### Glucose averages

```

Iterations = 10001:5999901
Thinning interval = 100
Sample size = 59900

DIC: -209.2166

G-structure: ~species

      post.mean l-95% CI u-95% CI eff.samp
species 0.001705 0.0003464 0.003511 59900

~Method_Glu

```



```

      post.mean 1-95% CI u-95% CI eff.samp
Method_Glu 0.004672 0.0001915 0.01309 59900

```

R-structure: ~units

```

      post.mean 1-95% CI u-95% CI eff.samp
units 0.003713 0.001608 0.005851 59900

```

Location effects: logGlucose ~ order

```

      post.mean 1-95% CI u-95% CI eff.samp pMCMC
(Intercept) 2.442097 2.317939 2.562344 59900 <2e-05 ***
orderAnseriformes -0.136101 -0.229771 -0.045736 59900 0.00367 **
orderApodiiformes 0.104208 -0.063956 0.269026 59900 0.21910
orderBucerotiformes 0.045225 -0.085697 0.177252 59900 0.49646
orderCariamiformes 0.084108 -0.078716 0.255211 59900 0.31706
orderCasuariiformes -0.163069 -0.337731 0.011609 60636 0.06614 .
orderCharadriiformes 0.005304 -0.095223 0.101372 59900 0.91780
orderCiconiiformes -0.039864 -0.150798 0.069105 59900 0.47349
orderColumbiformes 0.062913 -0.047089 0.173563 59900 0.26331
orderCoraciiformes 0.086470 -0.080365 0.253869 59900 0.30067
orderGalliformes -0.014638 -0.122414 0.088507 59900 0.78227
orderGruiformes -0.086754 -0.191388 0.019906 60809 0.10554
orderMusophagiformes -0.032880 -0.149817 0.083853 59900 0.57723
orderPasseriformes 0.100976 0.004481 0.199953 59900 0.04374 *
orderPelecaniformes -0.078898 -0.182340 0.022862 59900 0.12725
orderPhoenicopteriformes -0.196479 -0.328292 -0.059938 59900 0.00491 *
*
orderProcellariiformes -0.033725 -0.138918 0.071199 59900 0.52568
orderPsittaciformes -0.033701 -0.136006 0.070392 59900 0.51730
orderRheiformes -0.202339 -0.370220 -0.036105 59900 0.01930 *
orderSphenisciformes -0.131626 -0.249327 -0.012622 58358 0.02945 *
orderStrigiformes 0.058533 -0.073821 0.193863 59900 0.38818
orderSuliformes -0.206606 -0.371552 -0.039227 60514 0.01603 *
---

```

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

## Glycation averages

```

Iterations = 10001:5999901
Thinning interval = 100
Sample size = 59900

```

DIC: 444.2672

G-structure: ~species

```

      post.mean 1-95% CI u-95% CI eff.samp
species 2.873 0.6036 5.908 59900

```

~Method\_Glu

```

      post.mean 1-95% CI u-95% CI eff.samp
Method_Glu 8.967 0.3913 25.25 59900

```

R-structure: ~units

```

      post.mean 1-95% CI u-95% CI eff.samp
units 6.234 2.745 9.778 59900

```

Location effects: Glycation ~ order

```

      post.mean 1-95% CI u-95% CI eff.samp pMCMC
(Intercept) 23.8174 18.6864 29.0157 59085 0.000267 ***
orderAnseriformes -9.0998 -12.8132 -5.3161 59900 < 2e-05 ***
orderApodiiformes 1.6941 -5.1762 8.5942 59900 0.625309
orderBucerotiformes -3.8560 -9.1864 1.6821 59900 0.161536
orderCariamiformes -1.6412 -8.5153 5.1824 59900 0.638765
orderCasuariiformes -13.0636 -20.0693 -5.6892 59900 0.000401 ***

```

```

orderCharadriiformes      -3.4750  -7.4881  0.5848  59900  0.091386  .
orderCiconiiformes       -2.3910  -6.9647  2.0819  60604  0.299299  .
orderColumbiformes      -3.5363  -8.0847  1.0178  59900  0.127579  .
orderCoraciiformes       0.7004  -5.8813  7.7817  59900  0.841002  .
orderGalliformes        -5.2965  -9.6262 -0.9362  59900  0.018030  **
orderGruiformes         -0.2166  -4.5451  4.1320  58975  0.917262  .
orderMusophagiformes    -1.3584  -6.2273  3.3938  58473  0.573823  .
orderPasseriformes      -4.5115  -8.4707 -0.4609  59900  0.028815  *
orderPelecaniformes     -4.9655  -9.1079 -0.7669  59900  0.020568  *
orderPhoenicopteriformes -12.6524 -18.1743 -7.1537  59900  < 2e-05  ***
orderProcellariiformes  -3.5527  -7.7734  0.8770  59900  0.105275  .
orderPsittaciformes     -8.4290 -12.7042 -4.2682  59900  0.000134  ***
orderRheiformes         0.5901  -6.4606  7.2461  59900  0.868381  .
orderSphenisciformes    -6.4491 -11.4353 -1.7623  59900  0.009683  **
orderStrigiformes       1.7468  -3.7739  7.2189  59900  0.528013  .
orderSuliformes        -10.0020 -16.8119 -3.1082  59900  0.005376  **

```

---  
Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

### Glucose individuals

Iterations = 10001:5999901  
Thinning interval = 100  
Sample size = 59900

DIC: -989.3556

G-structure: ~species

	post.mean	l-95% CI	u-95% CI	eff.samp
species	0.00332	0.00165	0.005173	59900

R-structure: ~units

	post.mean	l-95% CI	u-95% CI	eff.samp
units	0.003992	0.003375	0.004628	59900

Location effects: logGlucose ~ order

	post.mean	l-95% CI	u-95% CI	eff.samp	pMCMC
(Intercept)	2.441807	2.363471	2.518260	59900	<2e-05 ***
orderAnseriformes	-0.134276	-0.218401	-0.046738	59900	0.003272 **
orderApodiformes	0.101579	-0.041054	0.236021	59600	0.144674
orderBucerotiformes	0.040000	-0.080951	0.157990	60680	0.507980
orderCariamiformes	0.080218	-0.066951	0.227669	59900	0.279399
orderCharadriiformes	0.009452	-0.083092	0.105417	59900	0.840534
orderCiconiiformes	-0.040935	-0.142712	0.060512	59900	0.416561
orderColumbiformes	0.065267	-0.066617	0.193642	59036	0.326811
orderCoraciiformes	0.082562	-0.078494	0.248061	61463	0.313222
orderGalliformes	-0.001747	-0.102287	0.099458	58770	0.978030
orderGruiformes	-0.089967	-0.189241	0.007686	59900	0.072287 .
orderMusophagiformes	-0.085619	-0.272281	0.096474	59900	0.365810
orderPasseriformes	0.086554	-0.003241	0.175723	59900	0.057763 .
orderPelecaniformes	-0.074397	-0.171168	0.018949	59900	0.121736 .
orderPhoenicopteriformes	-0.291942	-0.439996	-0.144614	59900	0.000301 ***
orderProcellariiformes	-0.039027	-0.134402	0.057526	60610	0.413523
orderPsittaciformes	-0.038279	-0.136047	0.057135	61190	0.431419
orderRheiformes	-0.206340	-0.349504	-0.061964	59900	0.006511 **
orderSphenisciformes	-0.136905	-0.243893	-0.031810	59900	0.012721 *
orderStrigiformes	0.034768	-0.119034	0.189827	59900	0.656528
orderSuliformes	-0.212732	-0.359269	-0.062097	61467	0.006344 **

---  
Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

## Glycation individuals

Iterations = 10001:5999901  
 Thinning interval = 100  
 Sample size = 59900

DIC: 2391.883

G-structure: ~species

	post.mean	l-95% CI	u-95% CI	eff.samp
species	8.851	5.417	12.71	59900

R-structure: ~units

	post.mean	l-95% CI	u-95% CI	eff.samp
units	8.099	6.994	9.261	59900

Location effects: Glycation ~ order

	post.mean	l-95% CI	u-95% CI	eff.samp	pMCMC	
(Intercept)	23.3396	19.5523	27.2678	58787	< 2e-05	***
orderAnseriformes	-9.4202	-13.6002	-5.1701	59146	< 2e-05	***
orderApodiformes	1.5038	-5.4759	8.6581	59049	0.669516	
orderBucerotiformes	-4.0335	-10.1095	1.8861	59082	0.185543	
orderCariamiformes	-1.8113	-9.1594	5.6689	59900	0.629149	
orderCasuariiformes	-11.9476	-20.6864	-2.7884	59900	0.009182	**
orderCharadriiformes	-2.4454	-7.0388	1.9826	59129	0.285576	
orderCiconiiformes	-3.0298	-8.0497	2.0927	59187	0.237930	
orderColumbiformes	-2.4171	-7.4663	2.7121	59900	0.347112	
orderCoraciiformes	0.5462	-7.5072	8.6354	56042	0.894124	
orderGalliformes	-5.4805	-10.3152	-0.5489	59900	0.029649	*
orderGruiformes	-0.8043	-5.8130	3.9945	61375	0.745643	
orderMusophagiiformes	-1.3513	-6.6584	3.7670	59900	0.604875	
orderPasseriformes	-5.2095	-9.6273	-0.7066	58879	0.023306	*
orderPelecaniformes	-5.7022	-10.3614	-1.0444	58508	0.017596	*
orderPhoenicopteriformes	-12.8877	-18.7001	-7.0605	59900	6.68e-05	***
orderProcellariiformes	-1.9987	-6.7284	2.7765	59146	0.406912	
orderPsittaciiformes	-8.4219	-12.9810	-3.6229	58856	0.000835	***
orderRheiformes	0.4280	-6.8066	7.5473	59900	0.908414	
orderSphenisciformes	-6.6127	-11.8293	-1.2290	59900	0.015559	*
orderStrigiformes	1.9480	-4.2668	8.1967	59900	0.533623	
orderSuliformes	-10.1649	-17.7348	-2.8516	56705	0.007546	**

---  
 Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

## VIF models

### Glucose averages

	GVIF	Df	GVIF <sup>1/(2*Df)</sup>
Diet	1.131518	4	1.015565
Centered_logBM	1.131518	1	1.063729

### Glucose averages life history

	GVIF	Df	GVIF <sup>1/(2*Df)</sup>
Diet	3.069605	4	1.150497
Centered_logBM	1.489277	1	1.220359
poly(ML, 2, raw = TRUE)	1.745451	2	1.149415
CM	2.598470	1	1.611977
DT	1.281634	1	1.132093

### Glycation averages

	GVIF	Df	GVIF <sup>1/(2*Df)</sup>
Diet	1.295990	4	1.032940

Centered_logBM	1.641464	1	1.281196
Centered_logGlucose	1.620827	1	1.273117

### Glycation averages life-history

	GVIF	Df	GVIF <sup>1/(2*Df)</sup>
Diet	3.151870	4	1.154306
Centered_logBM	1.971214	1	1.403999
Centered_logGlucose	2.188302	1	1.479291
ML	1.380756	1	1.175056
CM	2.098269	1	1.448540
DT	1.295233	1	1.138083

### Glucose individuals

	GVIF	Df	GVIF <sup>1/(2*Df)</sup>
Diet	1.470112	4	1.049346
Centered_logBM	1.470112	1	1.212482

### Glucose individuals life-history

	GVIF	Df	GVIF <sup>1/(2*Df)</sup>
Diet	7.225664	4	1.280441
Centered_logBM	2.798296	1	1.672811
poly(ML, 2, raw = TRUE)	1.768094	2	1.153125
CM	3.663550	1	1.914040
DT	1.438711	1	1.199463

### Glycation individuals

	GVIF	Df	GVIF <sup>1/(2*Df)</sup>
Diet	1.643911	4	1.064106
Centered_logBM	2.189558	1	1.479715
Centered_logGlucose	1.946567	1	1.395194

### Glycation individuals life-history

	GVIF	Df	GVIF <sup>1/(2*Df)</sup>
Diet	6.218989	4	1.256652
Centered_logBM	3.413201	1	1.847485
Centered_logGlucose	2.074540	1	1.440326
ML	1.296298	1	1.138551
CM	3.114929	1	1.764916
DT	1.446495	1	1.202703

### Glucose age & sex

	GVIF	Df	GVIF <sup>1/(2*Df)</sup>
Centered_logBM	1.148783	1	1.071813
poly(logit(Age_relative, FALSE), 2, raw = TRUE)	1.146260	2	1.034715
Sex	1.008919	1	1.004450

### Glycation age & sex

	GVIF	Df	GVIF <sup>1/(2*Df)</sup>
Centered_logBM	1.949521	1	1.396252
Centered_logGlucose	2.042604	1	1.429197
poly(logit(Age_relative, FALSE), 2, raw = TRUE)	1.200827	2	1.046815
Sex	1.029088	1	1.014440

## Within species repeatability

### Glucose

Linear mixed model fit by REML. t-tests use Satterthwaite's method [`'merModLmerTest'`]  
 Formula: `logGlucose ~ (1 | species)`

Data: Bird.caracIndGlu

REML criterion at convergence: -875.1

Scaled residuals:

Min	1Q	Median	3Q	Max
-3.7868	-0.5688	-0.0078	0.5683	2.8026

Random effects:

Groups	Name	Variance	Std.Dev.
species	(Intercept)	0.009768	0.09883
Residual		0.003869	0.06220

Number of obs: 389, groups: species, 75

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(> t )
(Intercept)	2.39913	0.01214	71.83263	197.7	<2e-16 ***

Repeatability estimation using the lmm method

Repeatability for species

R = 0.716  
SE = 0.043  
CI = [0.621, 0.786]  
P = 8.57e-80 [LRT]  
NA [Permutation]

## Glycation

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']

Formula: Glycation ~ (1 | species)  
Data: Bird.caracIndGly

REML criterion at convergence: 2521.1

Scaled residuals:

Min	1Q	Median	3Q	Max
-4.3646	-0.4484	-0.0123	0.4749	3.4483

Random effects:

Groups	Name	Variance	Std.Dev.
species	(Intercept)	18.898	4.347
Residual		7.994	2.827

Number of obs: 471, groups: species, 88

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(> t )
(Intercept)	18.4571	0.4921	87.9914	37.51	<2e-16 ***

Repeatability estimation using the lmm method

Repeatability for species

R = 0.703  
SE = 0.039  
CI = [0.621, 0.766]  
P = 3.53e-86 [LRT]  
NA [Permutation]

## Variances explained by the tree

### Glucose averages

0.2282438

```
          lower      upper
var1 6.498843e-06 0.695486
attr(,"Probability")
[1] 0.95
```

### **Glucose averages life history**

```
0.2262482
          lower      upper
var1 6.721386e-05 0.7054011
attr(,"Probability")
[1] 0.95
```

### **Glycation averages**

```
0.4765213
          lower      upper
var1 0.1240947 0.8125361
attr(,"Probability")
[1] 0.95
```

### **Glycation averages life-history**

```
0.4279933
          lower      upper
var1 0.06510515 0.7745717
attr(,"Probability")
[1] 0.95
```

### **Glucose individuals**

```
0.4842442
          lower      upper
var1 0.1855086 0.7712471
attr(,"Probability")
[1] 0.95
```

### **Glucose individuals life-history**

```
0.4595189
          lower      upper
var1 0.1425944 0.7571919
attr(,"Probability")
[1] 0.95
```

### **Glycation individuals**

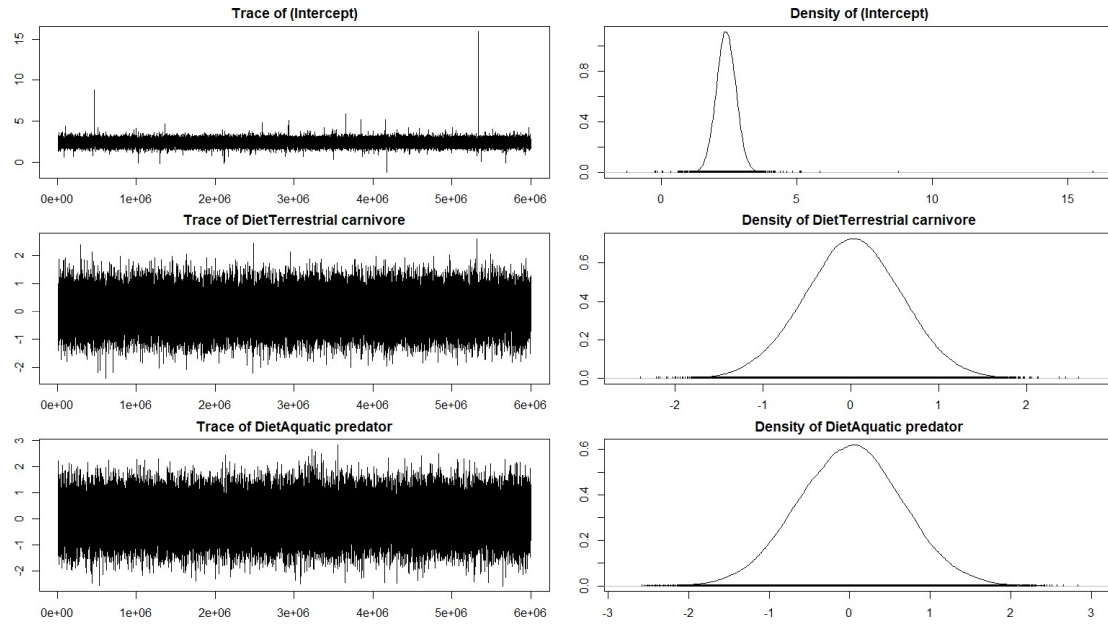
```
0.3255109
          lower      upper
var1 0.07951431 0.5922364
attr(,"Probability")
[1] 0.95
```

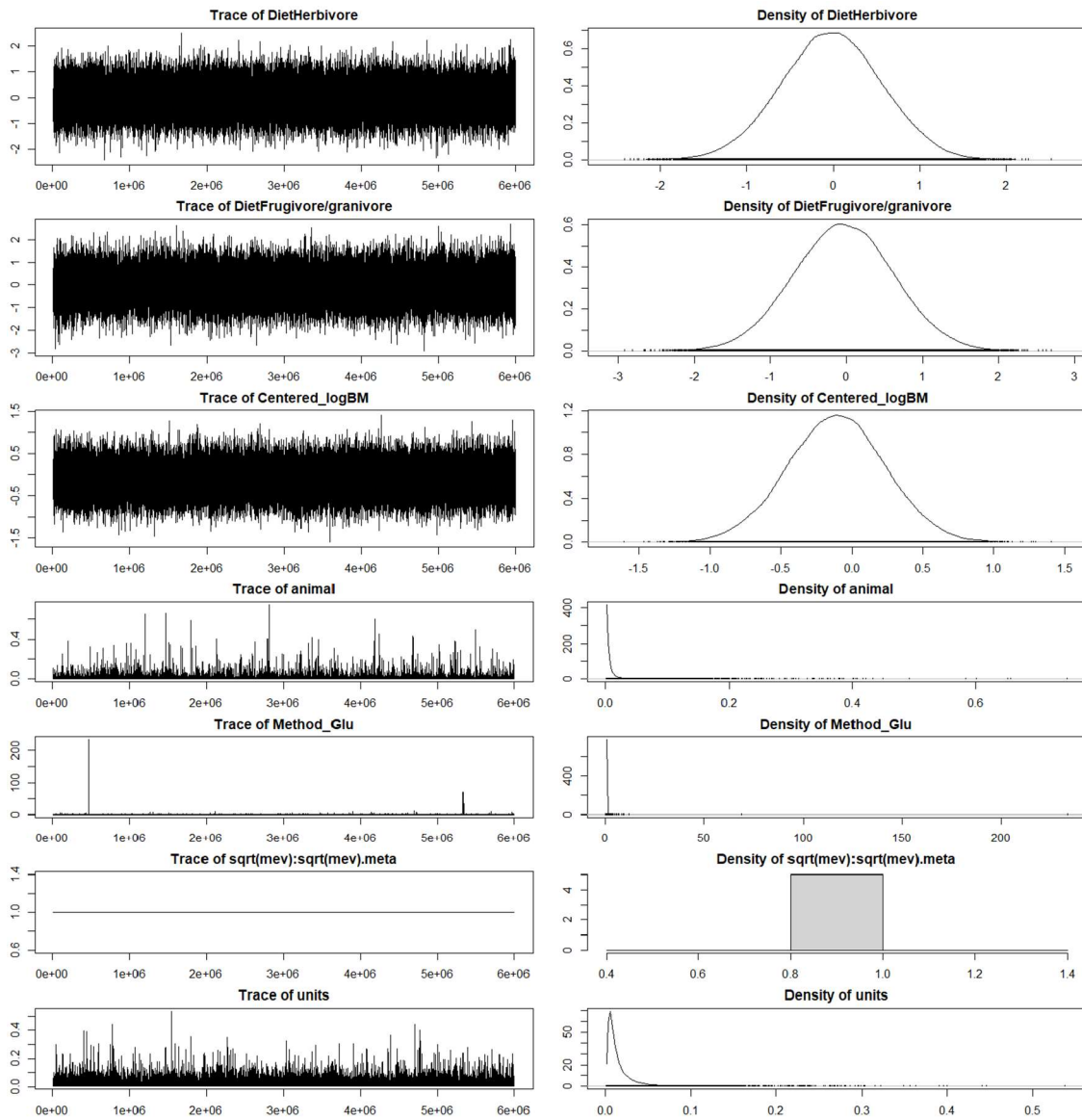
### **Glycation individuals life-history**

```
0.3060218
          lower      upper
var1 0.05274904 0.5917645
attr(,"Probability")
[1] 0.95
```

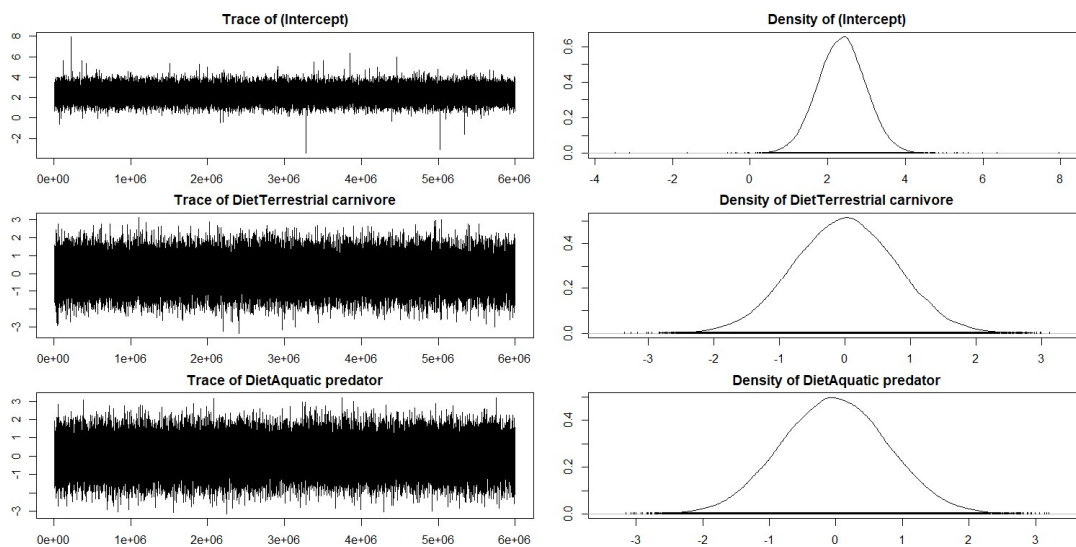
# Traces and posterior distributions

## Glucose averages

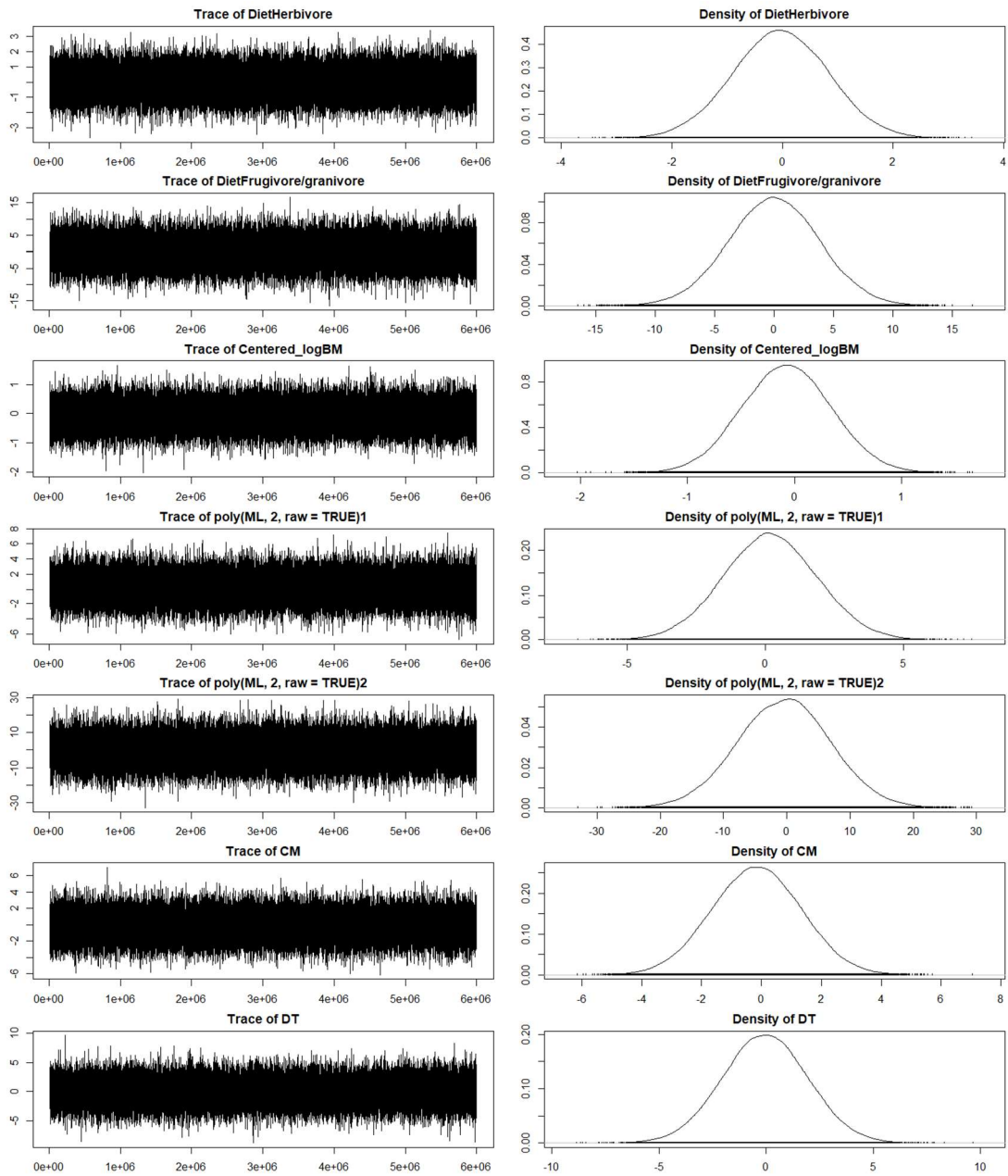


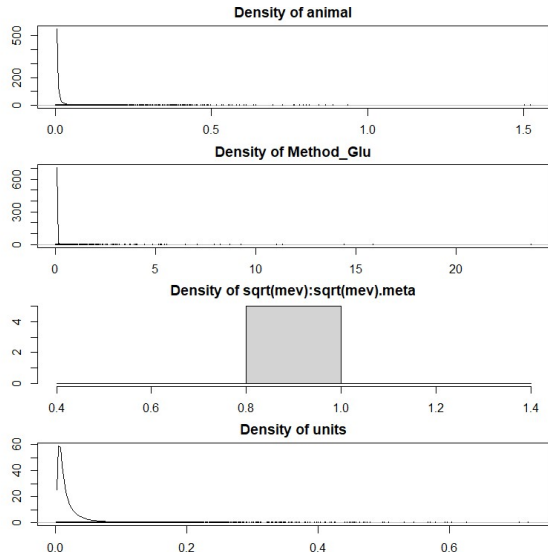
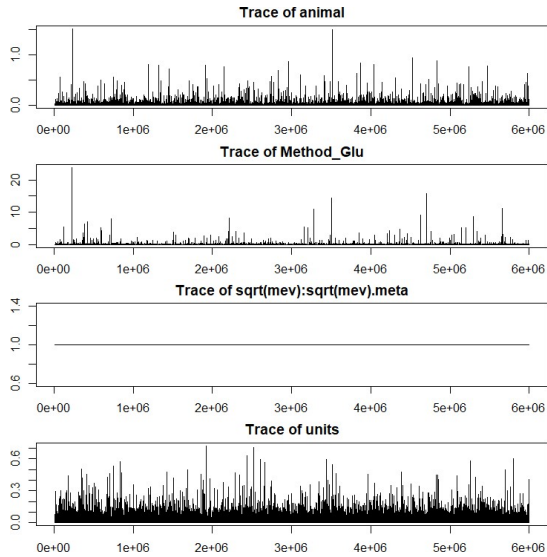


## Glucose averages life history

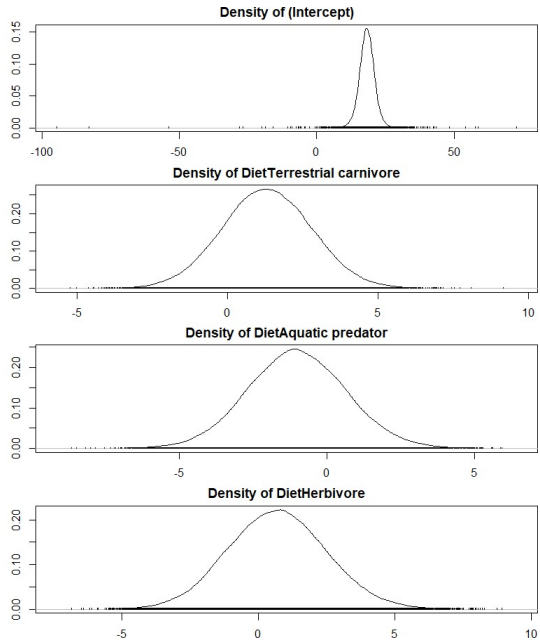
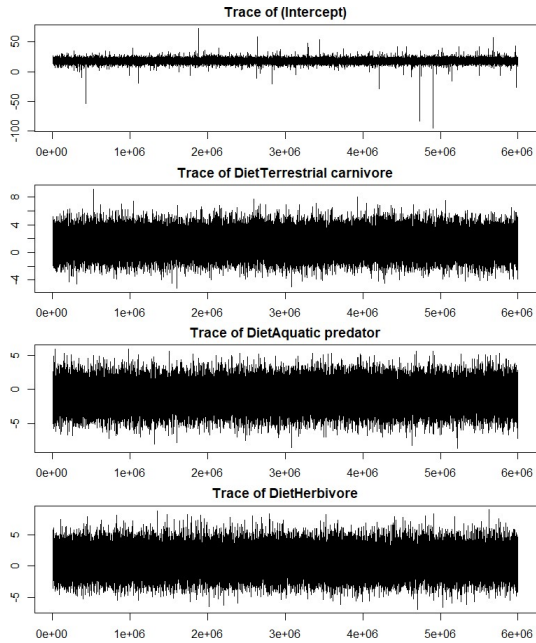


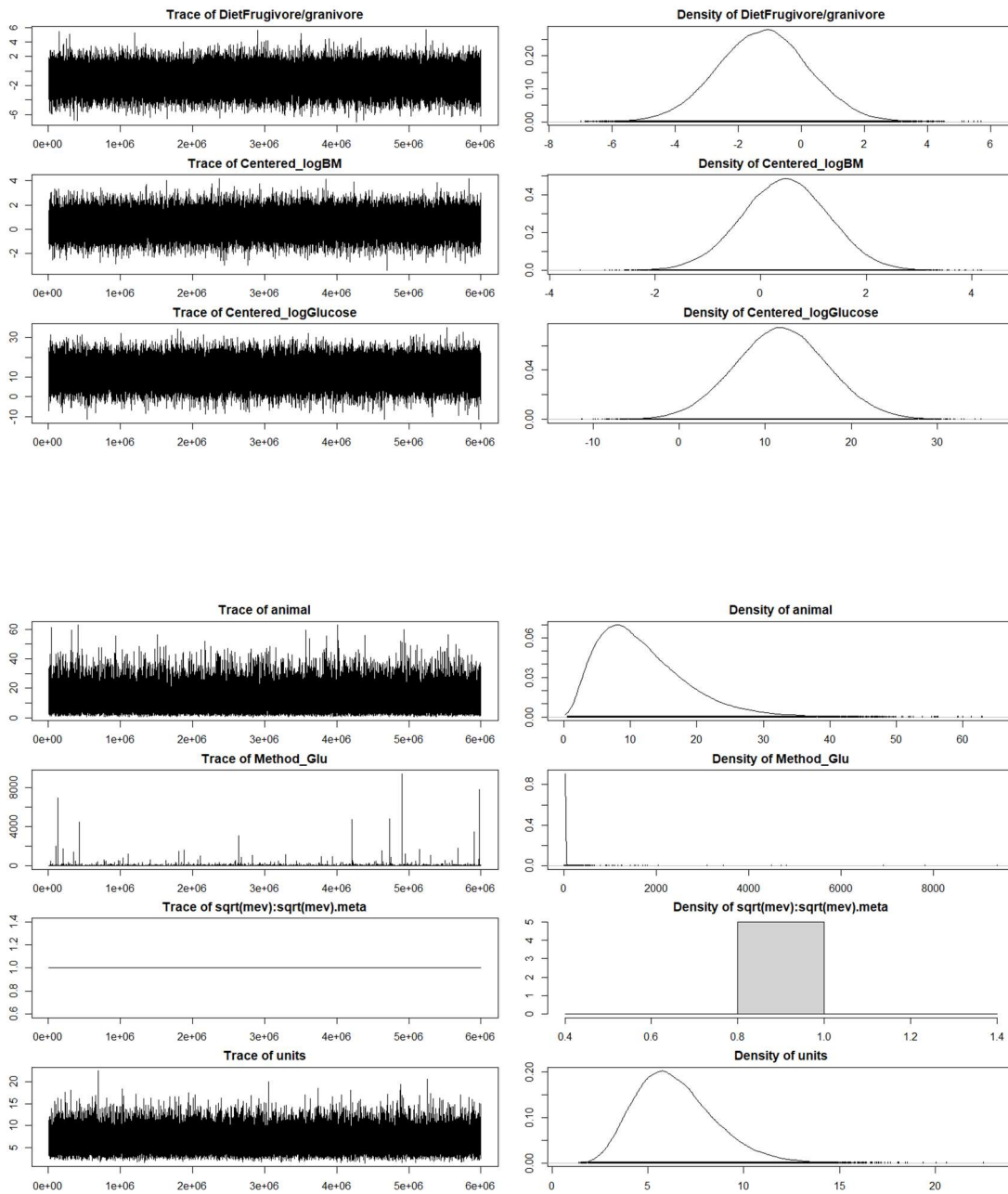




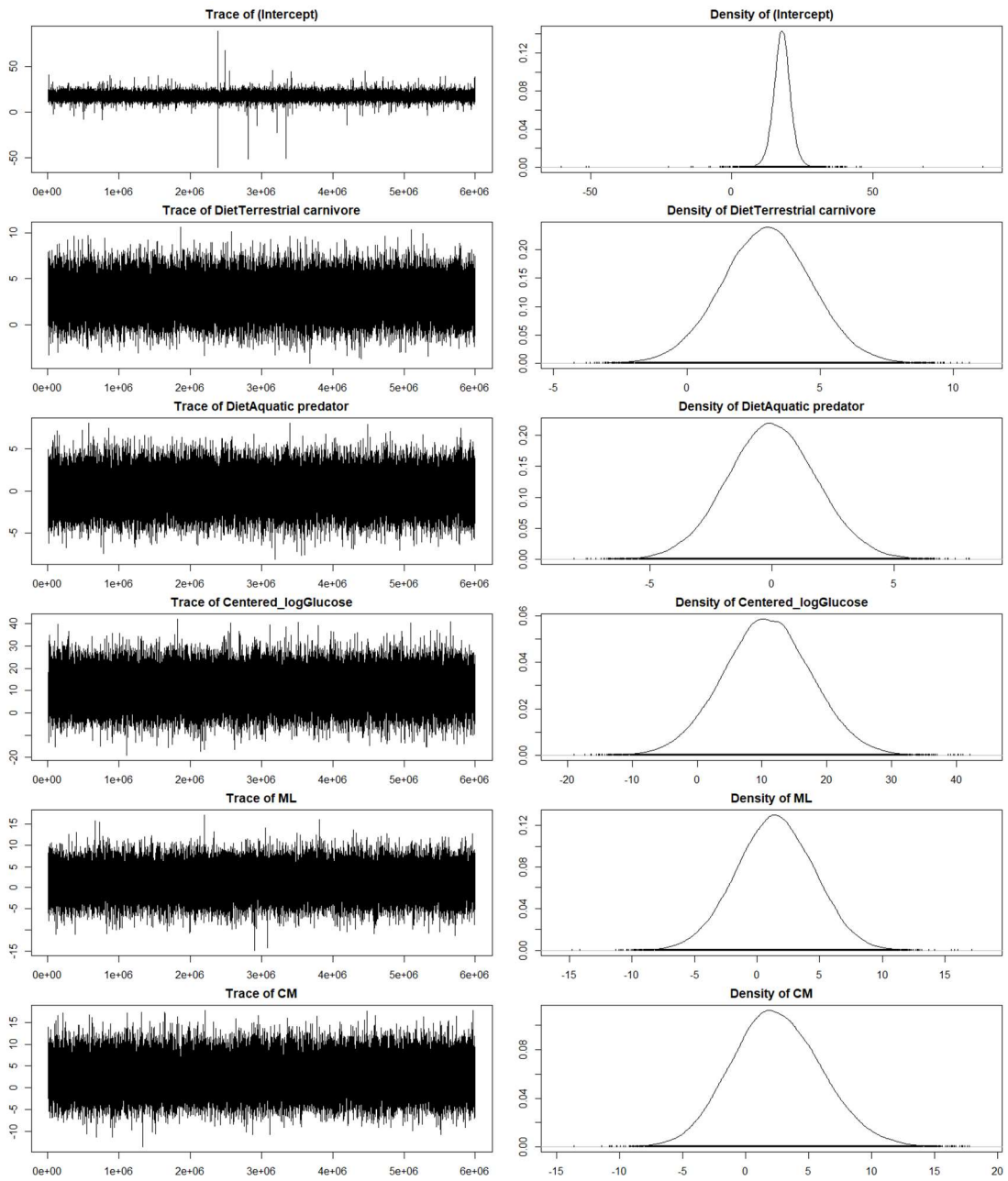


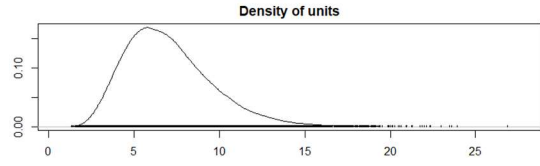
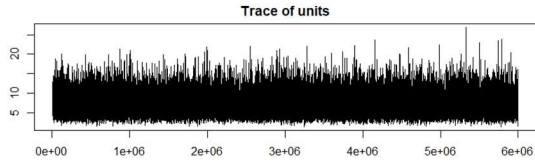
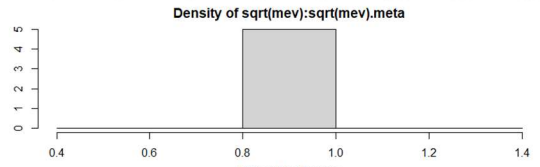
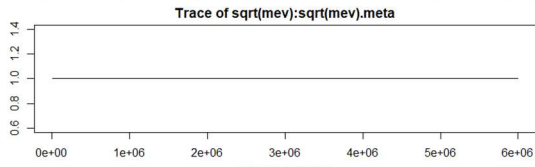
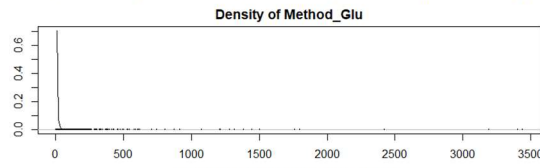
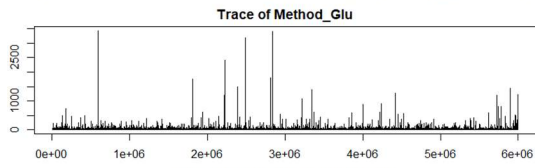
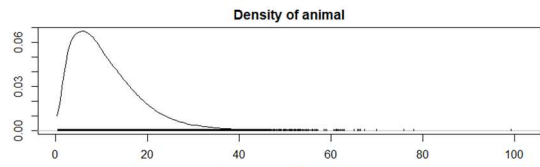
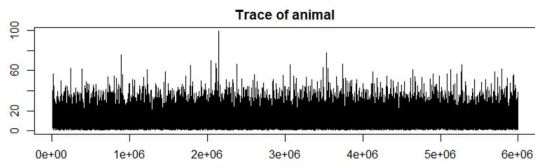
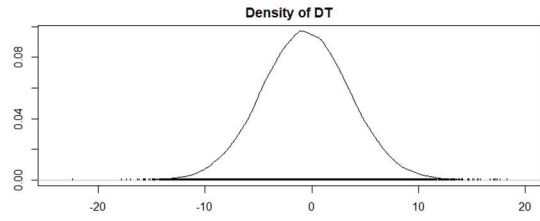
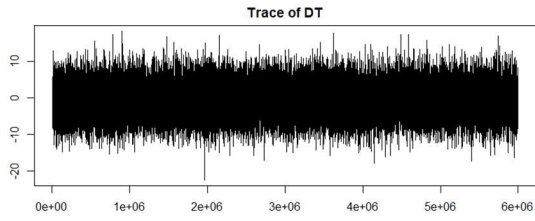
### Glycation averages



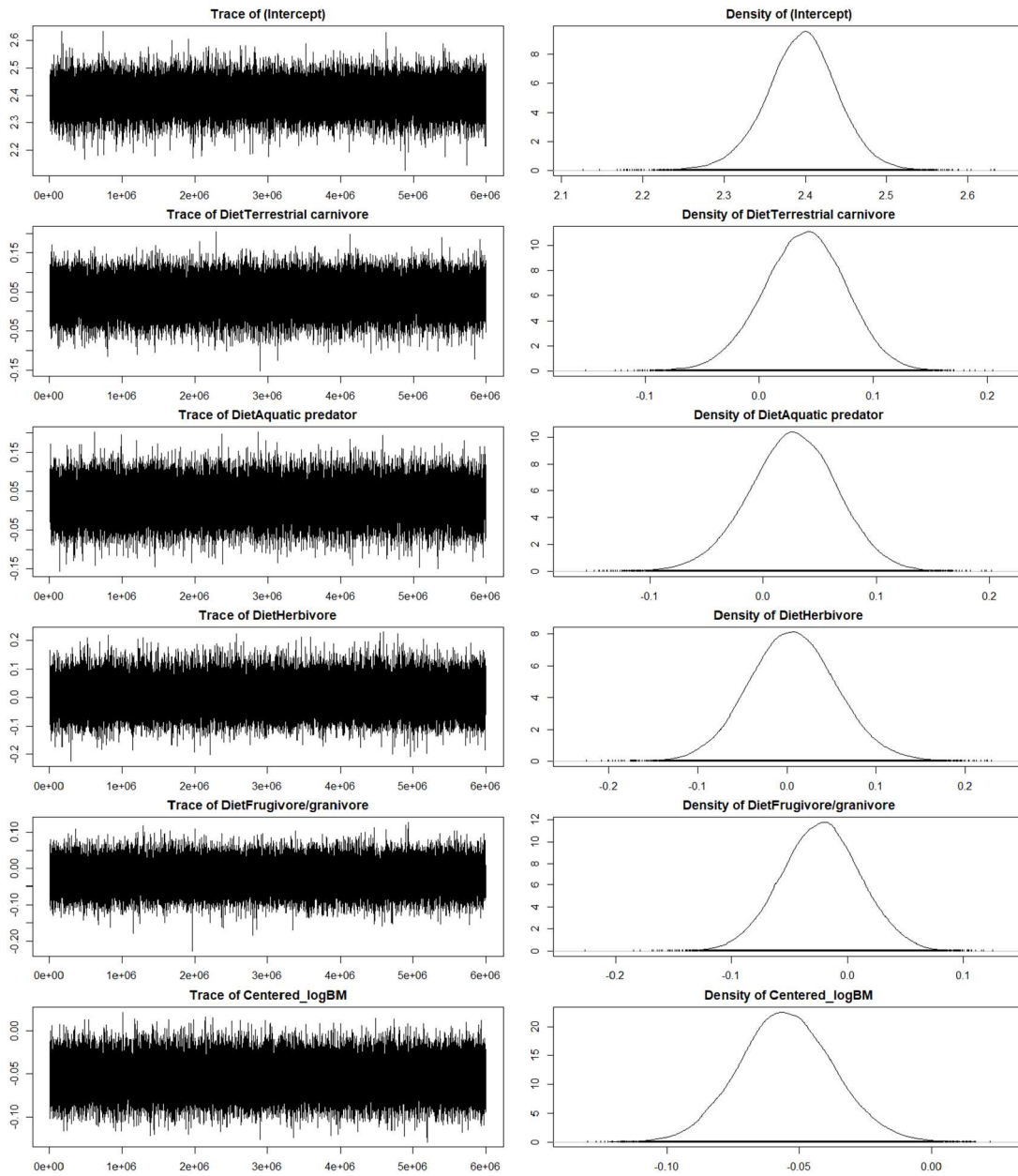


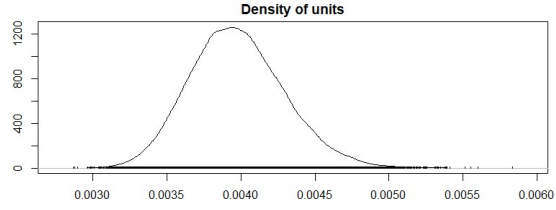
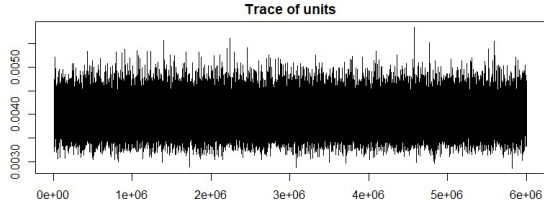
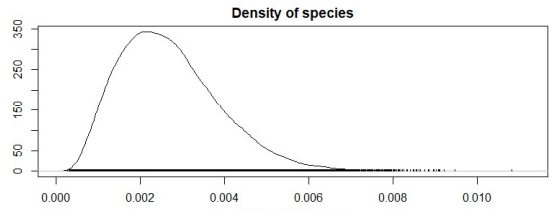
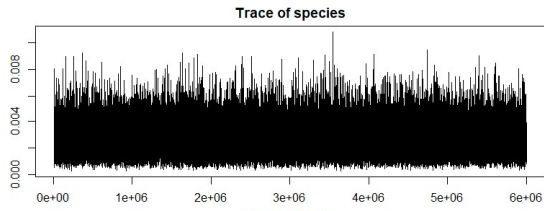
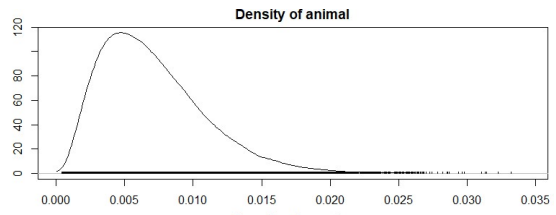
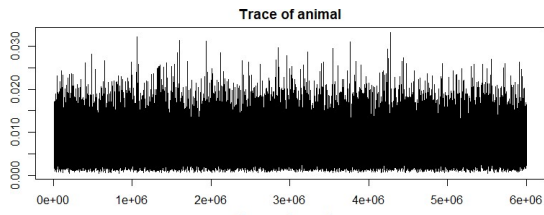
**Glycation averages life-history**



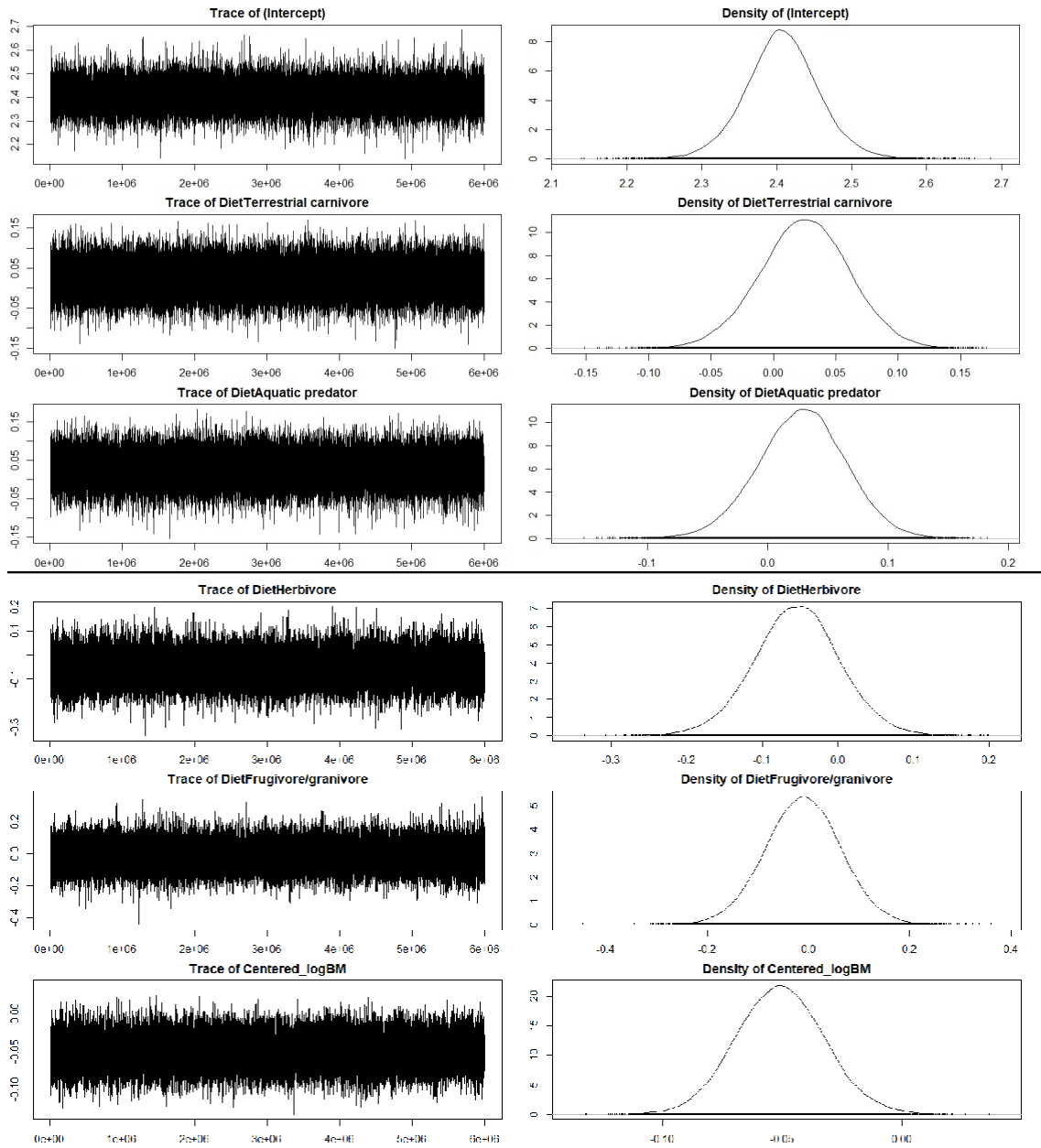


**Glucose individuals**

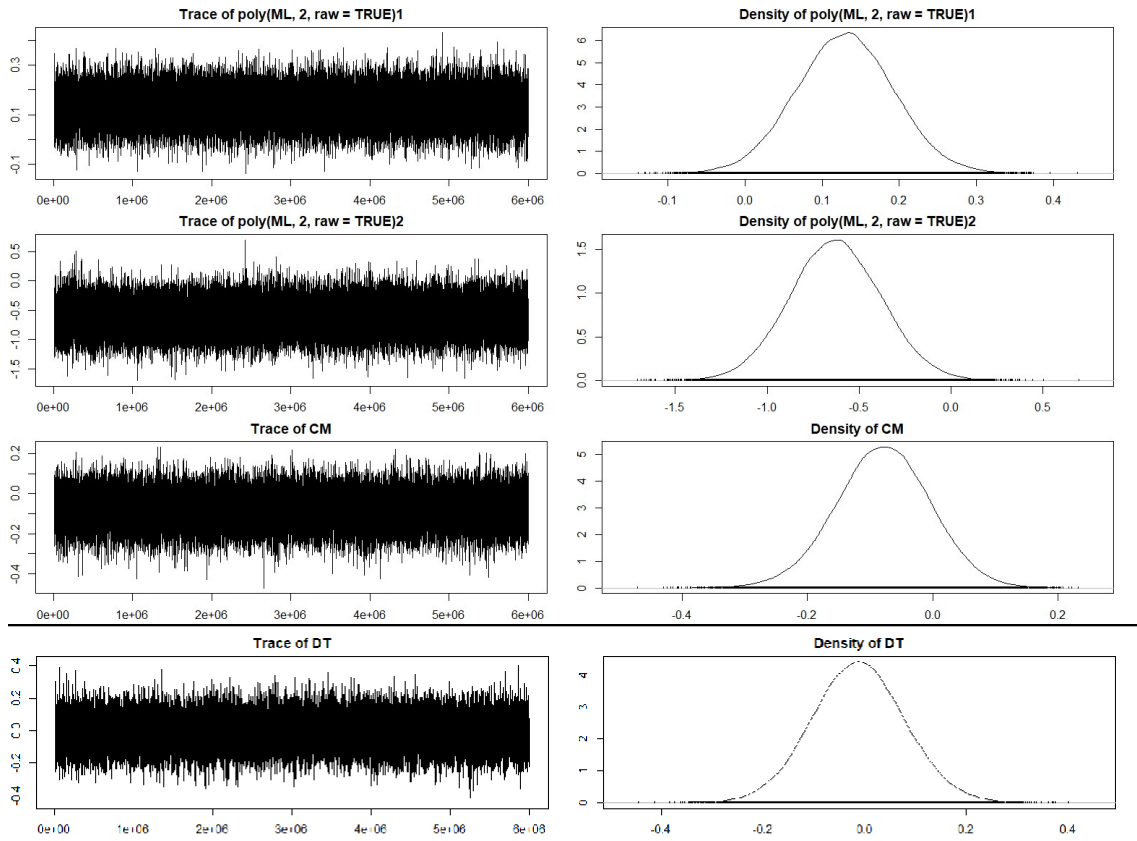


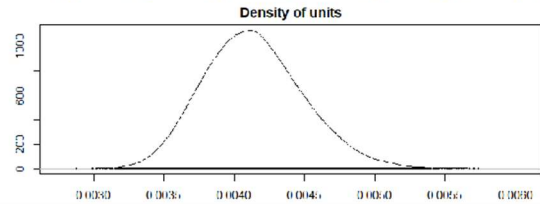
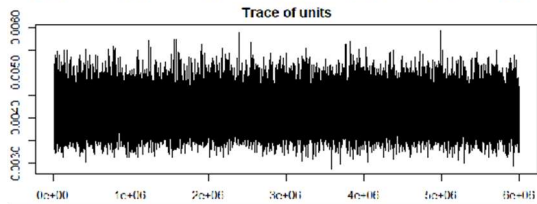
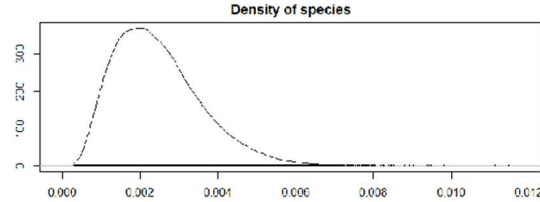
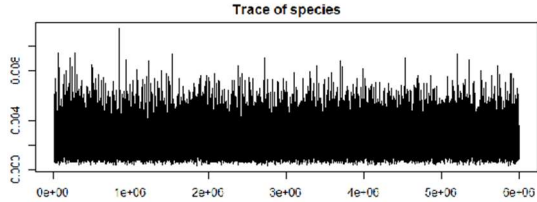
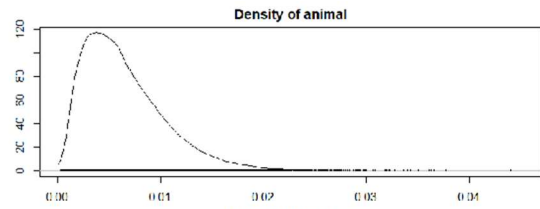
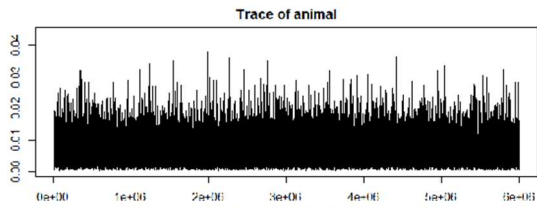


# Glucose individuals life-history

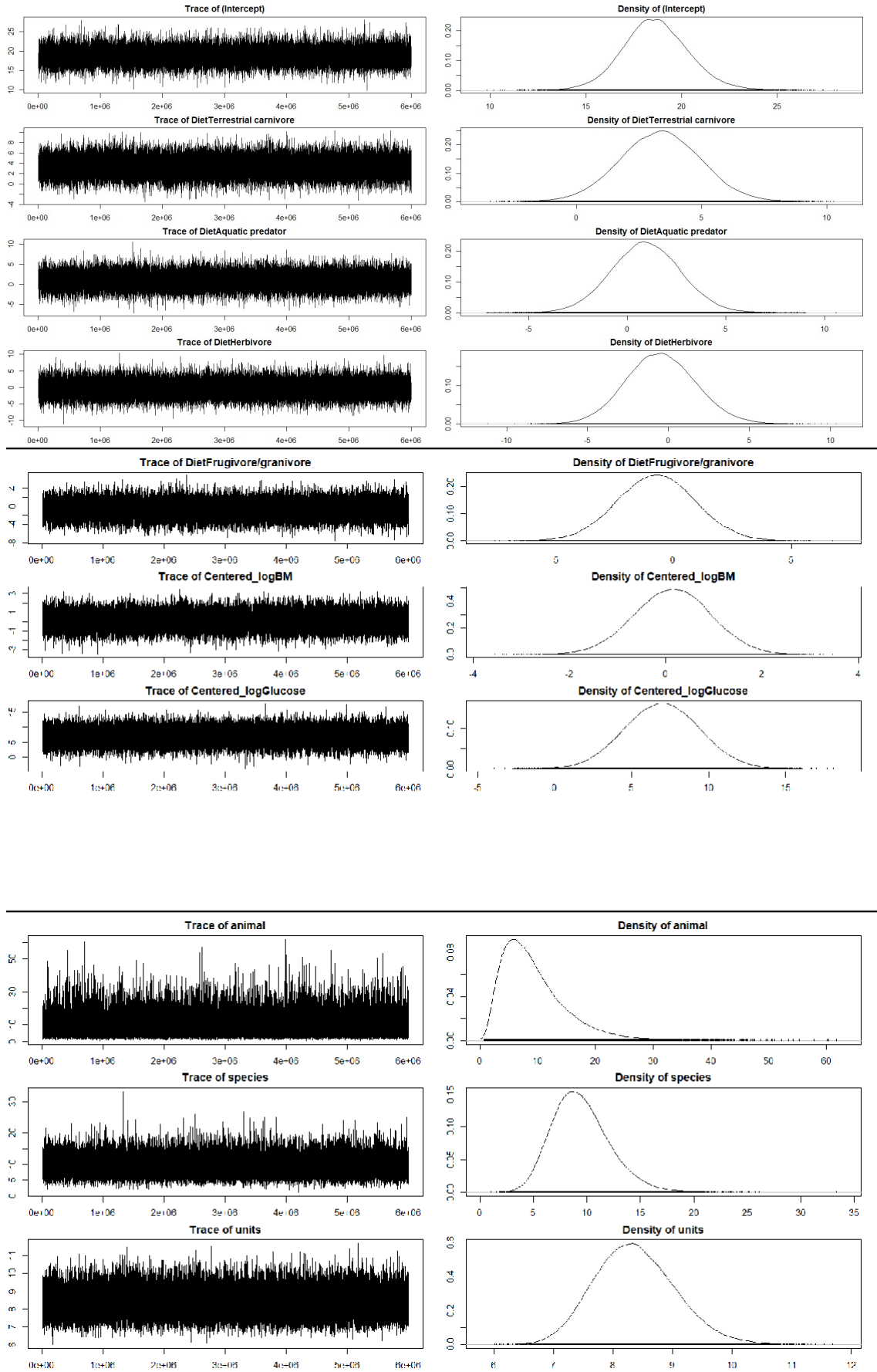




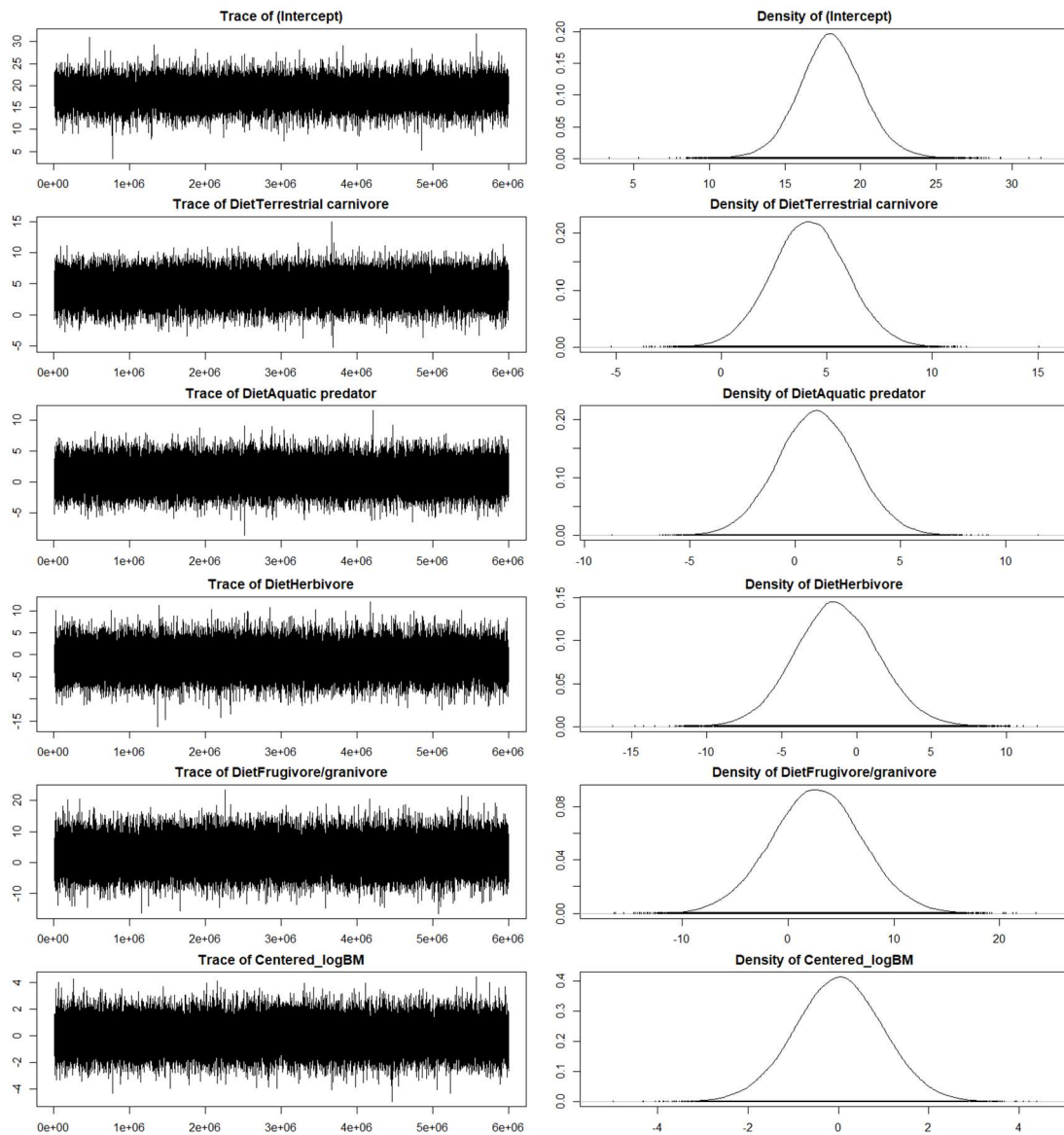




# Glycation individuals



## Glycation individuals life-history



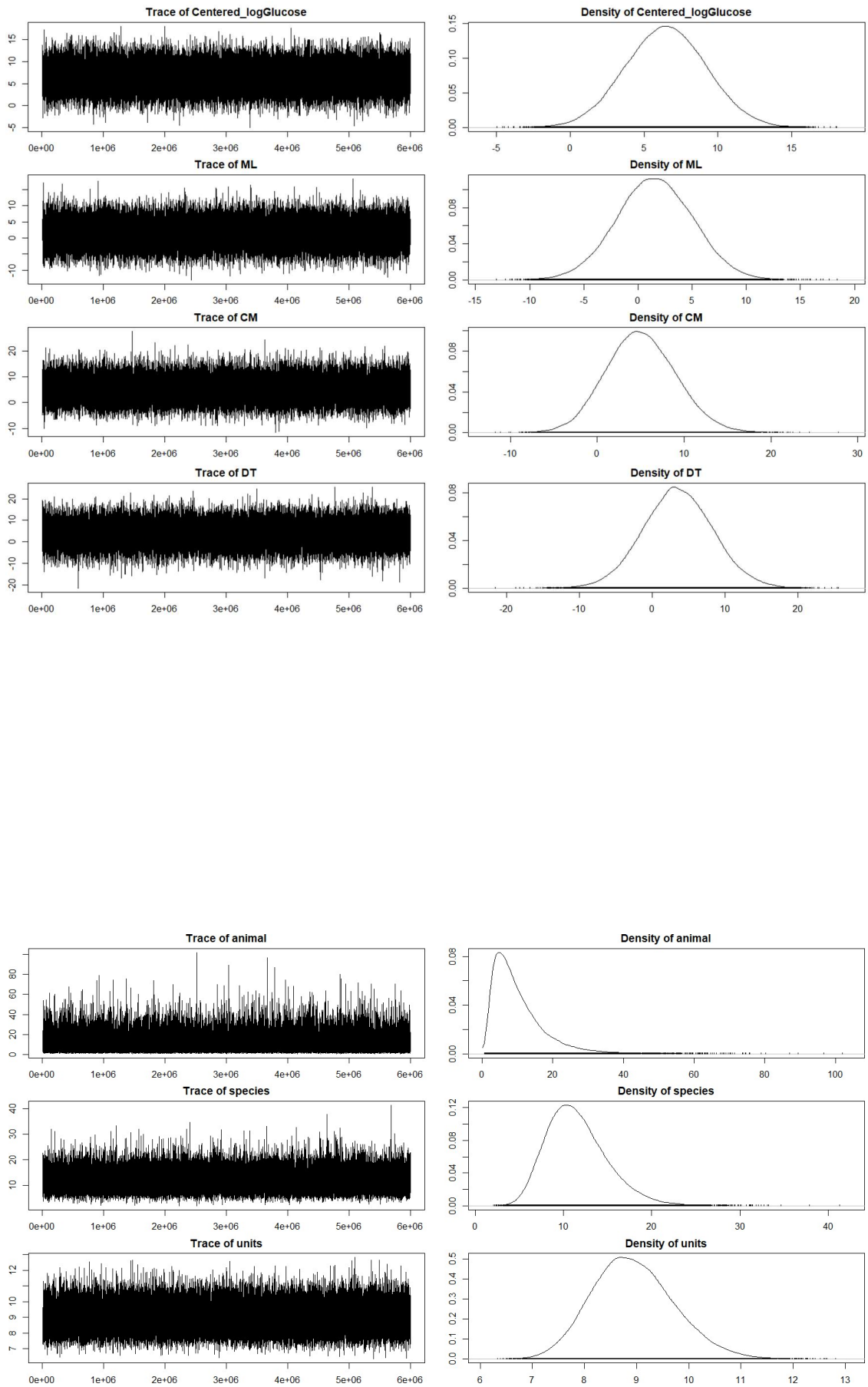


Figure ESM1.1 Trace showing convergence of the models and posterior density plots of the parameters estimated.

## Supplementary analyses of stress on glucose

### Glucose repeatability

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']

Formula: Glucose ~ (1 | SpeciesBirdTree/Individual)

Data: Tomasek2022

REML criterion at convergence: 44335.9

Scaled residuals:

Min	1Q	Median	3Q	Max
-4.6683	-0.5772	0.0246	0.5585	4.0989

Random effects:

Groups	Name	Variance	Std.Dev.
Individual:SpeciesBirdTree	(Intercept)	5.188	2.278
SpeciesBirdTree	(Intercept)	2.222	1.490
Residual		6.204	2.491

Number of obs: 8862, groups: Individual:SpeciesBirdTree, 1705; speciesBirdTree, 158

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(> t )
(Intercept)	13.8677	0.1496	146.9083	92.67	<2e-16 ***

Repeatability estimation using the lmm method

Repeatability for SpeciesBirdTree

R = 0.163

SE = 0.024

CI = [0.117, 0.209]

P = 0 [LRT]

NA [Permutation]

### For individuals within species:

> 5.188/(5.188+2.222+6.204)

[1] 0.3810783

### Glucose repeatability (with stress effects)

Linear mixed model fit by REML. t-tests use Satterthwaite's method ['lmerModLmerTest']

Formula: Glucose ~ Time + (1 | SpeciesBirdTree/Individual)

Data: Tomasek2022

REML criterion at convergence: 40911.9

Scaled residuals:

Min	1Q	Median	3Q	Max
-5.0618	-0.5405	-0.0221	0.5398	4.6158

Random effects:

Groups	Name	Variance	Std.Dev.
Individual:SpeciesBirdTree	(Intercept)	5.499	2.345
SpeciesBirdTree	(Intercept)	2.267	1.506
Residual		3.864	1.966

Number of obs: 8862, groups: Individual:SpeciesBirdTree, 1705; SpeciesBirdTree, 158

Fixed effects:

	Estimate	Std. Error	df	t value	Pr(> t )	
(Intercept)	1.211e+01	1.525e-01	1.573e+02	79.41	<2e-16	***
TimeG15_	2.936e+00	5.458e-02	7.341e+03	53.79	<2e-16	***
TimeG30_	2.905e+00	4.976e-02	7.271e+03	58.37	<2e-16	***

---  
Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Correlation of Fixed Effects:

	(Intr)	TimeG15_
TimeG15_	-0.140	
TimeG30_	-0.153	0.442

Levene's Test for Homogeneity of Variance (center = median)

group	Df	F value	Pr(>F)
	2	455.14	< 2.2e-16 ***
	8859		

Kruskal-wallis rank sum test

data: Glucose by Time

Kruskal-wallis chi-squared = 1347.7, df = 2, p-value < 2.2e-16

Repeatability estimation using the lmm method

Repeatability for SpeciesBirdTree

R = 0.195

SE = 0.027

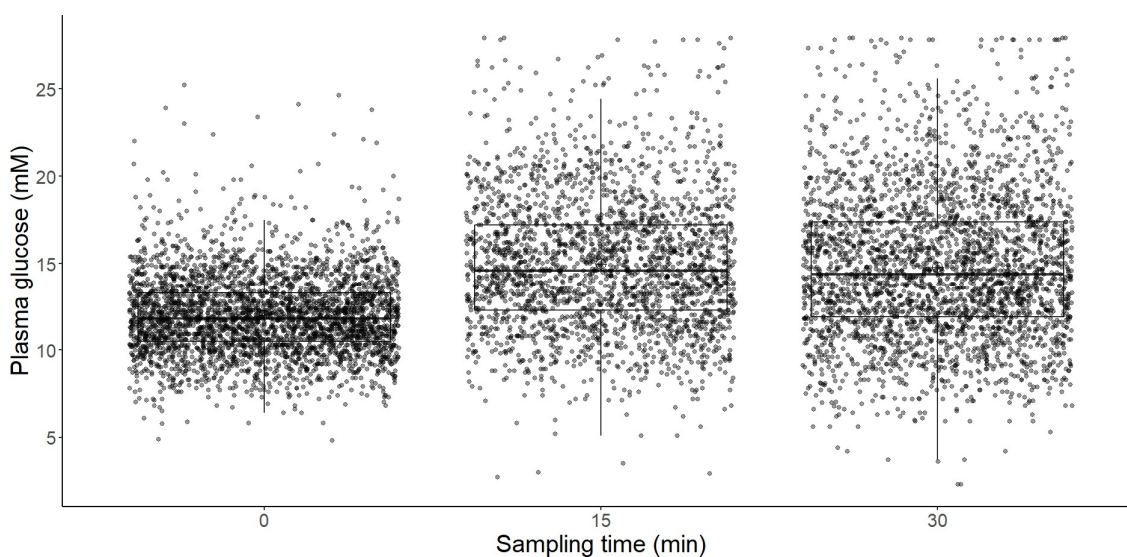
CI = [0.141, 0.248]

P = 0 [LRT]

NA [Permutation]

**For individuals within species:**

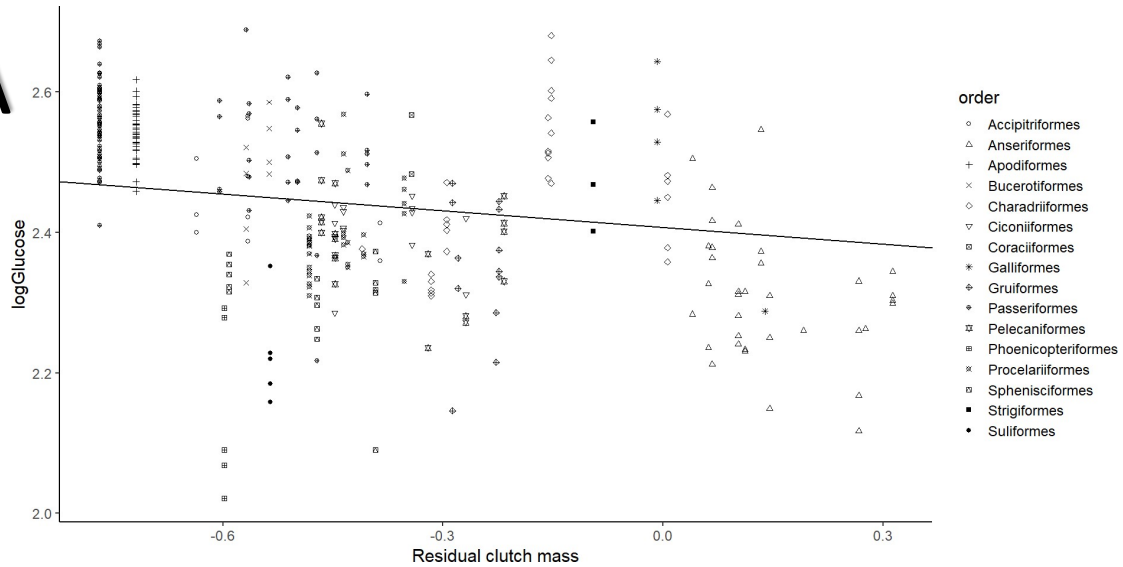
```
> 5.499/(5.499+2.267+3.864)
[1] 0.4728289
```



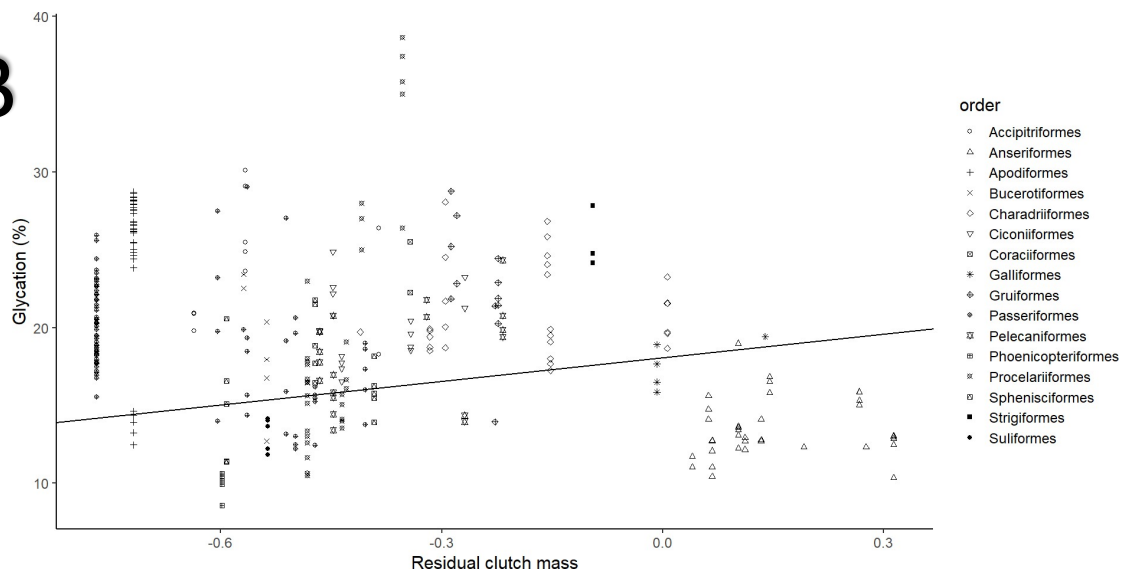
**Figure ESM1.2** Glucose variation (in mM) with sampling time at 0, 15 and 30 min, showing clear heteroskedasticity. Data publicly available from Tomasek et al. 2022 (see ESM6).

# Electronic Supplementary Material 2 (ESM2)

A

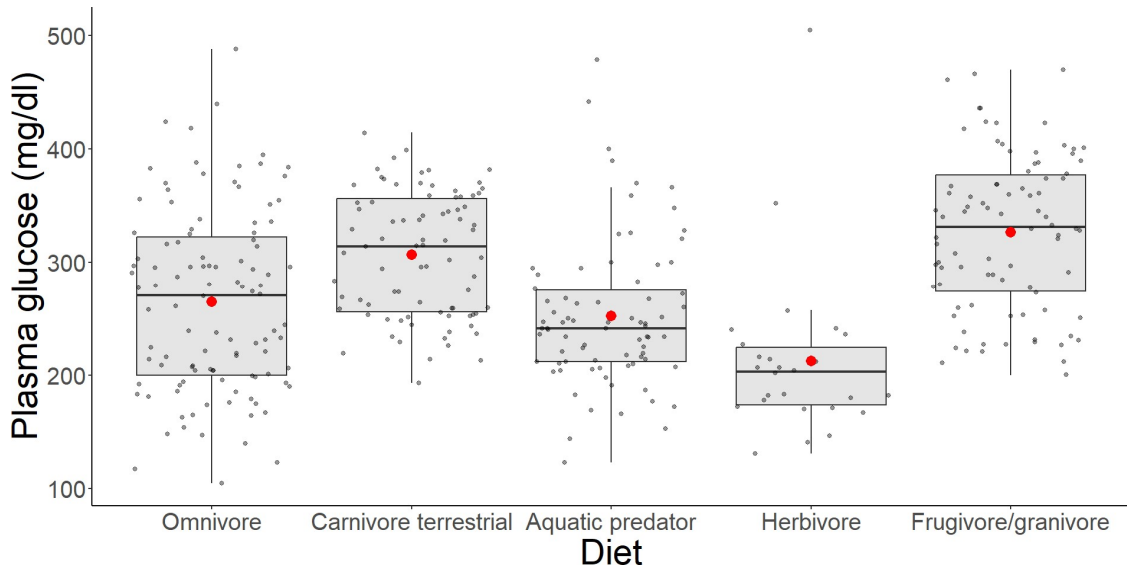


B

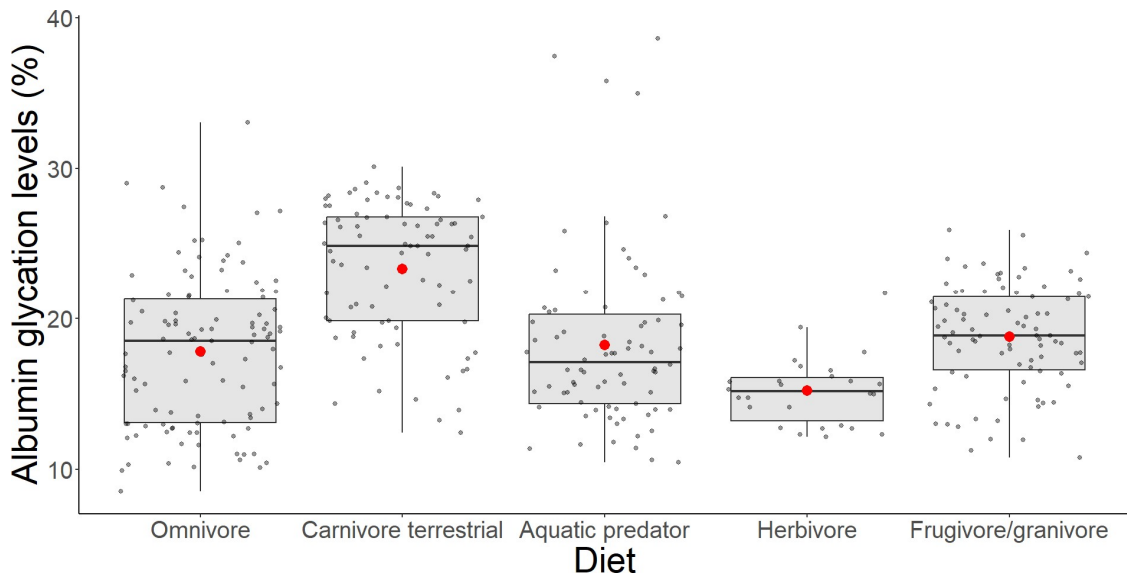


**Figure ESM2.1.** Variation in **A**  $\log_{10}$ (plasma glucose) in mg/dl and **B** albumin glycation levels (%) with respect to residuals of body mass-adjusted clutch mass of the species. This is calculated by multiplying egg mass times clutch size, and then performing a pGLS (see ESM6) of the decimal logarithm of this value against the decimal logarithm of body mass, to finally extract the residuals of such regression. Glycation levels are reported as a percentage of glycated albumin versus total albumin. The legends at right show the different orders to which our species belong.





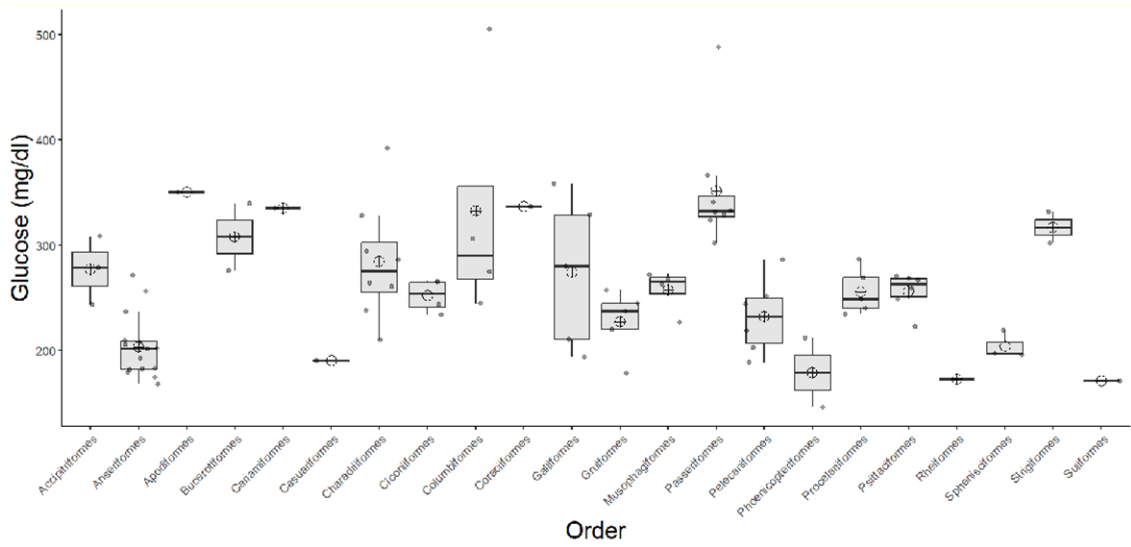
13



14

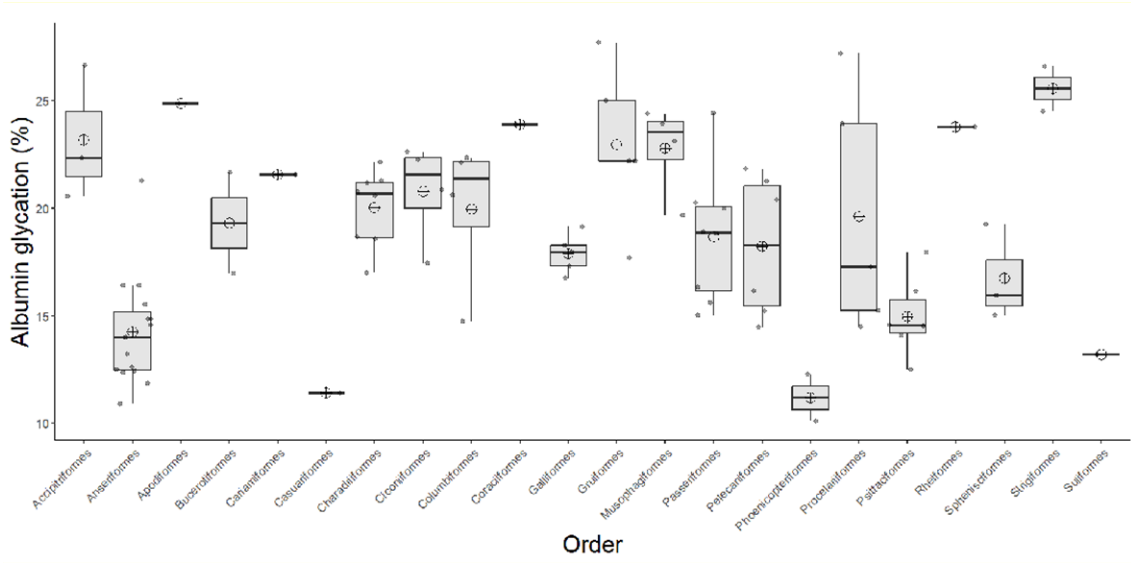
15 **Figure ESM2.2.** Representation of raw individual data on (A) plasma glucose levels and (B) albumin  
 16 glycation in function of birds' diet. Glucose levels are given in mg/dl, while glycation levels are a  
 17 percentage of total plasma albumin which is found to be glycated.

A1



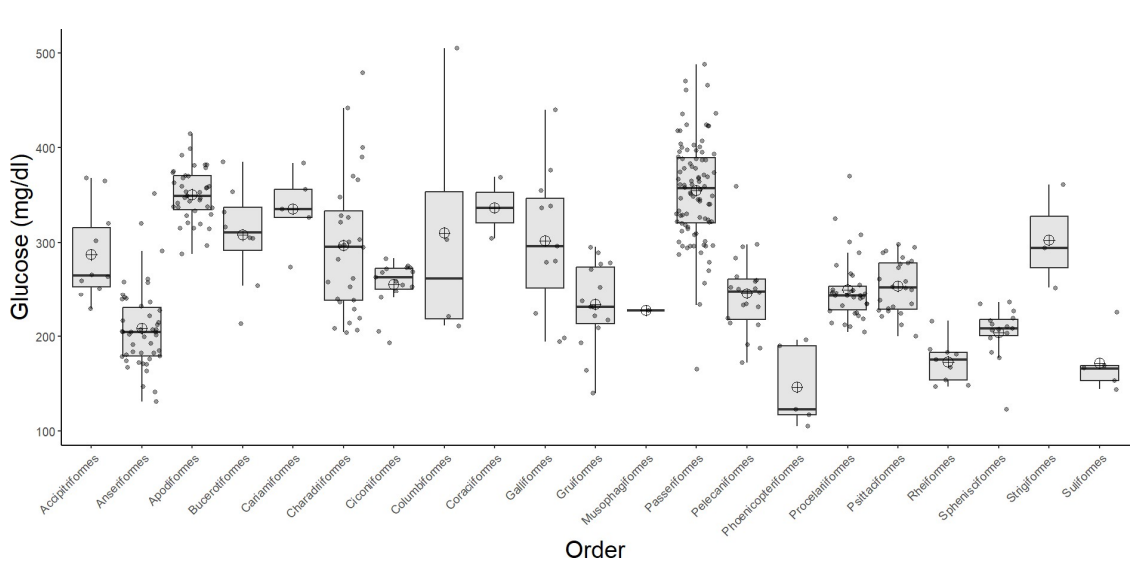
18

A2



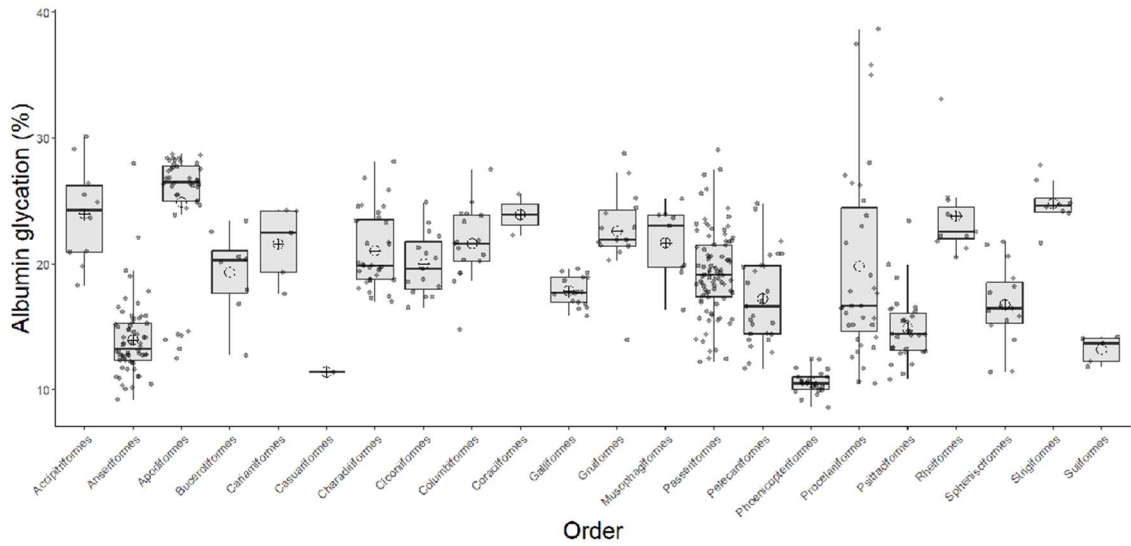
19

B1



20

B2



21

22

23

24

**Figure ESM2.3.** Variation in (A1-B1) plasma glucose levels in mg/dl and (A2-B2) albumin glycation rates across birds' orders included in our study. Panel A represents average species values and B includes individual values.

# **Electronic Supplementary Material 4 (ESM4)**

## **General**

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### **Macronectes hali**

AnAge from ABBBS - Australian Bird and Bat Banding Scheme

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**Larus dominicanus**

- ABBBS - Australian Bird and Bat Banding Scheme

# Electronic Supplementary Material 6 (ESM6)

1

2

## 3 Study species

4 Of all the species on the study, 65 came from captive populations of the Mulhouse zoo  
5 (Mulhouse, France) and the Parc des Oiseaux (Villars-les-Dombes, France). These samples were  
6 collected during annual vaccination campaigns in October 2018 and 2021. Zebra finch  
7 (*Taeniopygia guttata*) samples were taken in August-September 2022 from individuals kept in  
8 captivity in the animal facilities of the DEPE-IPHC (Department of Ecology, Physiology and  
9 Ethology, Institut Pluridisciplinaire Hubert Curien, Strasbourg, France). Alpine swift  
10 (*Tachymarptis melba*) samples were collected in May and August 2023 from wild individuals  
11 breeding in colonies in Switzerland (see e.g. [1-3]). Adult black-tailed godwits (*Limosa limosa*)  
12 were sampled in February 2022 in Extremadura rice fields, southwestern Spain. Adult gull-billed  
13 terns (*Gelochelidon nilotica*) were sampled in May-June 2022 from a breeding colony located at  
14 Villalba de los Barros reservoir in Extremadura, southwestern Spain. All passerine samples,  
15 excluding zebra finches and Bali myna (*Leucopsar rothschildii*), were obtained from wild  
16 individuals captured using mist nets during a migration monitoring project in a wetland at  
17 Sainte-Soline (France) in August 2022. Scopoli's shearwater (*Calonectris diomedea*) samples  
18 were collected in 2011 in the Chafarinas Islands, Spain. The remaining samples from  
19 Procellariiformes species, penguins (Sphenisciformes), cormorants (Suliformes) and all marine  
20 Charadriiformes were collected from wild populations in the French Southern Territories of  
21 Crozet and Kerguelen during 2022. Sample sizes for each species measured by our team went  
22 from 1 to 60 for glucose (mean=5.32;  $\sigma$ =7.07; median=4.5; mode=5) and from 1 to 55 for  
23 glycation (mean=5.19;  $\sigma$ =7.9; median=4; mode=5). Excepting zebra finches (*Taeniopygia*  
24 *guttata*) (60 for glucose, 55 for glycation) and Alpine swifts (*Tachymarptis melba*), (39 for  
25 glucose, 40 for glycation) whose sample sizes are bigger because these measurements were  
26 originally collected on untreated animals for separate analyses (unpublished data), the values  
27 are lower and vary much less in number (1-16 for both glucose and glycation), and are limited  
28 in size by logistical reasons, mainly related to mass-spectrometry operation. The values reported  
29 here for glycation are counting the individuals included in the statistical analyses, not the ones  
30 sampled, as some individuals had glycation values under the limit of detection, that were  
31 eliminated from the analyses, as stated in the main text. However, when considering glucose  
32 values coming from the 13 species from ZIMs database, the sample sizes were bigger 17-387  
33 individuals, (mean=90.46;  $\sigma$ =26.31; median=55; see main text). The validity of the glucose and

34 glycation values from our dataset to represent the species (as sample size per species was often  
35 low) was assessed by performing general linear mixed models (GLMM; *lmer()* function from  
36 *lme4* package in R [4]) with either glucose or glycation as dependent variables and “species” as  
37 a random factor for the intercept only, and then obtaining the repeatability score with  
38 *rptGaussian()* from *rptR* package [5] in order to determine if the variability between species is  
39 higher than within species.

#### 40 **Possible effects of stress**

41 Although glucose levels are known to be affected by the stress response, and thus by handling  
42 time (see e.g. [6-8] for birds), and time between bird capture and blood sampling (‘handling  
43 time’) is not measured here, there are some reasons to consider that our results are still robust.  
44 First, most individuals were sampled shortly after captured, so that the stress response could  
45 not play a big role on glucose variation. Second, we considered that this change in glucose levels  
46 with stress is mostly driven by an increase in variation instead of an increase in average values,  
47 as shown by the clear heteroskedasticity of publicly available data from 160 species of  
48 Passeriformes from [9] across sampling time in min (0, 15 and 30), in which we performed a  
49 GLLM on glucose with sampling time as fixed factor (see **Figure ESM1.2**) and individual nested  
50 within species as random factor, and a model without the fixed factor. This way, we compared  
51 the percentages of variance explained by the species and individual when considering all  
52 reported glucose values (i.e. at all sample times) together (see ESM1), showing a lower species  
53 repeatability than that from our data. This increased variability when sampling time is not  
54 considered means that our study is more conservative, so that the probability of finding  
55 significant results is lower, which makes the patterns of variation that we did find more robust.  
56 Third, preliminary analyses performed by our team on data from Alpine swifts and zebra finches  
57 (unpublished results), in which many individuals were sampled during the same session, and  
58 therefore differences in stress levels across individuals may be present, show no significant  
59 effect of time on glucose variation, particularly when the individuals were overnight fasted, as  
60 it is the case for zebra finches.

#### 61 **Diet data clarifications**

62 In many cases, adjustments were necessary because the criteria used by [10] to classify species  
63 (subsequently used in AVONET) were based on the ecological niche and its relationship with  
64 morphology rather than considering the potential association of dietary composition with  
65 physiological variables. To address this limitation, we used the “Trophic.Niche” variable from

66 AVONET and merged some categories: “Vertivore”, “Invertivore” and “Scavenger” into  
67 “Carnivore terrestrial”, “Frugivore” and “Granivore” into “Frugivore/granivore” and “Herbivore  
68 terrestrial” and “Herbivore aquatic” into “Herbivore”, due to lack of sufficient species on each  
69 separate category (as in “Herbivore terrestrial” and “Herbivore aquatic”) and sometimes on  
70 difficulties to tease apart the diet categories for our species (as in “Frugivore” and “Granivore”,  
71 as most of our species classified within these groups had a 50%-50% or similar distribution in  
72 their food source between both categories, zebra finches being the only exception being  
73 exclusively granivorous). Furthermore, some considered in AVONET as “Omnivores” were  
74 reclassified, and only considered “Omnivores” if diet was widely distributed between animal and  
75 plant sources, and not only within animal or plant categories (e.g. a species that eats  
76 invertebrates, vertebrates and carrion was not considered by us as an “Omnivore”, but as either  
77 an “Aquatic predator” or a “Carnivore terrestrial”, depending on its main feeding source).

## 78 **Statistics**

### 79 **Life history traits**

80 Given that all the 3 life history parameters (maximum lifespan, clutch mass and developmental  
81 time) used in our analyses are widely recognized to be strongly correlated with body mass, and  
82 interpretations of the variation in life history traits can change if body mass is not adjusted for  
83 [11] we chose to adjust the life history traits by body mass prior to run our analyses, as done by  
84 [12], the most similar study on glucose levels in birds in function of life history, so that our results  
85 are easily comparable. This was done by  $\log_{10}$  transforming all variables and performing a  
86 phylogenetically controlled Generalized Least Squares model (pGLS, *gls* function in R; [13])  
87 including a correction for phylogeny (assuming a Brownian model and the same tree as  
88 described in the main text for the MCMC GLMMs), from which the residuals were extracted.  
89 These residuals were the variables used in the main models when referring to “life history traits”,  
90 and they are also the same thing as when referring to mass-adjusted variables across the text.  
91 Maximum lifespan was used here as a proxy for longevity. Despite concerns about its use (e.g.  
92 [14-15]), it remains the most widely available longevity-related data in many databases for a  
93 large number of species. Clutch mass, calculated by multiplying clutch size by egg mass (as in  
94 [12], it was used as an indicator of reproductive investment. Finally, developmental time  
95 (incubation time plus number of days to fledging) was used as a proxy for growth, given that  
96 growth rate was difficult to calculate due to lack of information on fledging mass. These variables  
97 were also chosen as they were among the ones available for a greater number of species from  
98 the sources we used, and for comparative purposes with [12]. Including more variables as e.g.

99 number of clutches per year (one of the other available variables for a considerable number of  
100 species) would have further reduced the sample size without, in our opinion, adding much more  
101 information, as reproductive effort is already accounted for in the clutch mass variable.

## 102 **Main models**

103 Models were performed in the following way (see **Table ESM6. 1**): first, in order to account for  
104 all the species in the dataset (as individual values were not available for all the species, with no  
105 individual at all in some cases; see *Species and sample collection* section from main text  
106 methods) with average glucose and then with average glycation as dependent variables, and  
107 later with the individual values in another set of models. Body mass and diet were always  
108 included as independent variables. All of this was repeated also including life history traits but  
109 with the 66 species for which data were available (only species for which we had all the selected  
110 life-history variables were kept). Glycaemia and body mass were  $\log_{10}$  transformed and centred  
111 in all the models (centring of glucose only occurred when it was a covariable) in order to better  
112 interpret the intercepts [16]. In models predicting glycation, plasma glucose was also added as  
113 a covariate. Quadratic component of maximum longevity was also included in glucose and  
114 glycation models including life-history data, after data exploration suggested such pattern. In  
115 glucose models, the methodology used for determining it (glucometer or kit) was included as a  
116 random factor, in order to determine if this caused a bias in the results. The species was added  
117 as a random factor (given a tree provided within the *pedigree* argument of the *MCMCglmm*  
118 function).

119 Since glucose values were not available for all individuals, our models addressing intraspecific  
120 variability were limited to 379 individuals from 75 species when considering only diet and body  
121 mass predictors (and glucose when modelling glycation). Similarly, only 316 individuals from 58  
122 species were incorporated into the models when life-history traits were introduced. The species  
123 identity was always included as a random factor twice: one to control for the phylogeny and the  
124 other to avoid the pseudo-replication effect of having repeated measures within species. The  
125 method used to determine plasma glucose levels was not included in this case, since here the  
126 glucometer was used in all cases.

127 Gaussian distribution was always assumed for the models, as even if glycation values are, strictly  
128 speaking, proportions, not only they follow a continuous distribution (as their calculation come  
129 from the division of area values that vary in a continuous way; see main text), but they vary  
130 within a limited range (8.57-38.65 %, mean=18.8 %,  $\sigma=5.3$ ) never sufficiently close to 0 or 1 and  
131 also do not represent probabilities given by observations of cases as to be treated as binomial

132 with log link function. Furthermore, we believe that there are some reasons why logit  
133 transformation, as previously also recommended for proportional data [17], is not the best  
134 option in this case: first, logit calculations are suitable for exploring how probabilities of certain  
135 outcomes differ between groups, which is not the case in our analyses; second, exploration of  
136 the data suggested this transformation did not markedly alter the variance structure of the  
137 variable, which was close to a gaussian curve, while making more difficult the interpretation of  
138 the outcomes of the models.

139 Model convergence and lack of autocorrelation was checked by visual inspection of trace of the  
140 model MCMC simulations (see trace and density plots of posteriors in **Figure ESM1.1**).

#### 141 **Models on age and sex, on lysine residues and on orders**

142 We performed supplementary analyses only with relative age (when it was known, in most cases  
143 for birds born in captivity, or belonging to wild populations with demographic monitoring),  
144 including a quadratic term, and sex of the individuals (as provided by the caregivers at the zoos  
145 or the person in charge of organizing the sample collection and known by direct phenotypic  
146 observation when existent sexual dimorphism allows it or by molecular sexing with PCR when  
147 not), as predictive variables of glucose and glycation levels. Relative age was defined as its  
148 proportion to maximum lifespan of the species (i.e. divided by it), and logit transformation was  
149 performed for this ratio to avoid the particular distribution characteristics of proportion data, as  
150 Gaussian distribution was assumed in the models. We included glucose as a covariate for the  
151 glycation models, and body mass in every case, when it was available for the individuals. These  
152 models included a total of 239 individuals from 49 species (see **Table ESM6. 1.**).

153 We performed a model (MCMCglmm with species as a random factor) comparing the number  
154 of lysines exposed in the albumin of each species with the glycation levels measured in our study.  
155 This analysis aimed to assess whether this factor could represent a meaningful mechanism of  
156 resistance to glycation, as outlined in [18]. When the albumin amino acids sequence for a species  
157 we measured was not available on NCBI or UniprotKB (the sources used to determine the  
158 number of lysines of each species' albumin; see below for additional details), we selected a  
159 closely related species, typically from the same genus (see **Table ESM6. 2.** for a list of the species  
160 selected and their correspondence to ours). In total, 19 species were included in this analysis.  
161 Finally, the models on glucose and glycation values controlling for the bird orders included in  
162 the dataset (see ESM5) were also performed as MCMCglmm, with two models considering the  
163 species averages (one for glucose and one for glycation) and two including the intraspecific  
164 values and therefore the species as a random factor to control for pseudo-replication due to



165 multiple sampling of the same species. The species were allocated to the same orders employed  
 166 on the classification given by the most updated version of Birds of the World [19]. The orders  
 167 were organized in the model by alphabetic order, Accipitriformes constituting thus the intercept  
 168 of all of them.

169 **Table ESM6. 1.** Main set of models performed with the number of species and individuals included in  
 170 them. A total of 10 MCMCglmm models were performed, having either plasma glucose or albumin  
 171 glycation levels as response variable and a different number of species and total datapoints depending  
 172 on if the life-history (LH) traits were included (i.e. maximum lifespan, clutch mass and developmental  
 173 time) or only diet and body mass (also glucose in the glycation models). The models considering age and  
 174 sex did not include life history traits nor diet.

Models performed and N for each	Species averages				Intraspecific variation				Age and sex	
	Glucose		Glycation		Glucose		Glycation		Glucose	Glycation
	Non	LH	Non	LH	Non	LH	Non	LH	X	
	LH		LH	LH		LH				
Number of species	88	66	88	66	75	58	75	58	49	49
Number of individuals	X				379	316	379	316	239	239

175

## 176 Quantification of exposed lysine residues

177 The aminoacidic sequences of albumins were searched in UniprotKB and the NCBI database. For  
 178 twenty species, the sequences were submitted to the PHYRE2 server to generate a PDB  
 179 structure. Then, using DEPTH, the accessible surface area (ASA) was calculated for each residue  
 180 in the sequences. We used a custom Python script to determine whether each lysine residue is  
 181 exposed or not. To achieve this, the ASA of the lysine side chain was divided by 205 angstroms  
 182 (the maximum observed accessible surface area for a lysine [20-21]). If it exceeded 0.25, the  
 183 residue was considered exposed. This methodology allowed us to determine the count of  
 184 exposed lysine residues in our avian albumin sequences.

185 PHYRE2: <http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index>

186 DEPTH: <https://bio.tools/depth> ; <http://mspc.bii.a-star.edu.sg/depth> (Depth web server is  
 187 actually dead)

188 **Table ESM6. 2.** List of species used in the models testing the relationship between the number  
 189 of albumin's exposed lysines and its glycation rates. On the left, the list of considered species  
 190 and on the right, the list of species used as references for the number of exposed lysines. The  
 191 ones in bold are those that coincide.

Species in our dataset	Original species
<b>Anas platyrhynchos</b>	<b>Anas platyrhynchos</b>
Anser anser	Anser brachyrhynchus*
Anser indicus	Anser cygnoides*
<b>Aptenodytes patagonicus</b>	<b>Aptenodytes patagonicus</b>
Tachymarptos melba	Apus apus
Aythya baeri	Aythya fuligula
<b>Balearica regulorum</b>	<b>Balearica regulorum</b>
<b>Bubo bubo</b>	<b>Bubo bubo</b>
<b>Cariama cristata</b>	<b>Cariama cristata</b>
<b>Cygnus atratus</b>	<b>Cygnus atratus</b>
<b>Eudypetes chrysolophus</b>	<b>Eudypetes chrysolophus</b>
Limosa limosa	Limosa lapponica
<b>Numida meleagris</b>	<b>Numida meleagris</b>
Leucocarbo verrucosus	Phalacrocorax carbo
<b>Phoenicopterus ruber</b>	<b>Phoenicopterus ruber</b>
<b>Pygoscelis papua</b>	<b>Pygoscelis papua</b>
<b>Rhea pennata</b>	<b>Rhea pennata</b>
<b>Taeniopygia guttata</b>	<b>Taeniopygia guttata</b>
<b>Tauraco erythrolophus</b>	<b>Tauraco erythrolophus</b>
Turdus merula	Turdus rufiventris

192 \* For these species, as they both belong to the same genus, an average was calculated for both  
 193 the number of exposed lysines and the glycation levels, considering them in the analyses as one.  
 194 This way, *Anser anser* and *Anser indicus* were substituted by an *Anser sp.* average glycation value,  
 195 and *Anser brachyrhynchus* and *Anser cygnoides* by an average number of lysine exposed. The  
 196 actual values were very similar in both cases, so this average is likely to reflect realistic values.

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# Chapter 2

Correlational approach: fitness links of glycaemia and glycation in the wild





## Résumé

Le glucose est un composant métabolique vital pour le fonctionnement des organismes, mais il peut également se lier aux biomolécules par des réactions de glycation non enzymatiques qui entraînent une perte de fonctions. Alors que les effets de la glycation sur la santé, et en particulier sur le vieillissement, ont été bien démontrés par la recherche biomédicale, on sait peu de choses sur les effets de la glycation chez les animaux sauvages. Ici, nous avons étudié comment les niveaux de glucose plasmatique et les taux de glycation de l'albumine varient avec l'âge et sont liés au fitness chez le martinet alpin (*Tachymarptis melba*), un oiseau qui est comparativement hyperglycémique et qui vit longtemps pour sa masse corporelle. Nous avons mesuré les niveaux du glucose plasmatique et de glycation de l'albumine avant et après la reproduction chez des femelles adultes d'âge connu (2-14 ans), montrant que, tandis que les niveaux de glucose augmentaient parallèlement à la masse corporelle pendant cet événement reproductif, le taux de glycation de l'albumine diminuaient. La glycation de l'albumine, mais pas le glucose, varie avec l'âge, atteignant son maximum à 5 ans, en accord avec d'autres paramètres liés à l'âge précédemment rapportés chez cette espèce. Il est intéressant de noter que des niveaux de glucose plasmatique plus élevés avant la reproduction sont liés à une augmentation du succès à l'envol jusqu'à un certain seuil. En outre, en termes de dynamique, les femelles qui gagnent plus de masse pendant toute la période de reproduction réduisent plus leurs niveaux de glycation, tandis qu'une mineure prise de masse et une majeure réduction de leurs niveaux de glycation sont associées à des couvées plus grandes. Finalement, une masse corporelle et des niveaux de glucose plasmatique plus élevés après la reproduction prédisent une probabilité de survie plus élevée à la saison suivante, tandis qu'une glycation de l'albumine plus élevée prédit une survie plus faible, bien que de manière dépendante de l'âge. Notre étude met en évidence les niveaux de glucose plasmatique et d'albumine glyquée chez les adultes comme de nouveaux marqueurs potentiels du vieillissement et du fitness qui devraient être plus explorés chez cette espèce.





# **Glycaemia and albumin glycation rates are fitness mediators in the wild: the case of a long-lived bird**

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## **Abstract**

Glucose is a vital metabolic component in the functioning of organisms, but can also bind to biomolecules through non-enzymatic glycation reactions that result in loss of functions. While the effects of glycation on health, and particularly in ageing, have been well demonstrated in biomedical research, little is known about the effects of glycation in wild animals. Here, we studied how plasma glucose levels and albumin glycation rates vary with age and are related to fitness in the Alpine swift (*Tachymarptis melba*), a bird which is comparatively hyperglycaemic and long-lived for its body mass. We measured plasma glucose and albumin glycation levels before and after reproduction in adult females of known age (2-14 years), showing that, while glucose levels increased in parallel with body mass during this reproductive event, albumin glycation rates decreased. Albumin glycation, but not glucose, varies with age, peaking at 5 years, consistently with other age-related parameters previously reported in this species. Interestingly, higher plasma glucose levels before reproduction were related to increased fledging success up to a certain threshold. In addition, in terms of dynamics, females gaining more mass during the entire reproductive period lowered more their glycation levels, while gaining less mass and lowering the more their glycation levels was associated with bigger laid clutches. Finally, higher body mass and plasma glucose levels after reproduction predicted a higher survival probability to the next season, whereas higher albumin glycation predicted lower survival, although in an age-dependent manner. Our study highlights adult plasma glucose and glycated albumin levels as new potential markers of ageing and fitness that should be further explored in this species.

**Keywords:** Glucose, albumin glycation, ageing, swifts, glycaemia, fitness.

## 37 **Introduction**

38 Understanding why some individuals are more successful than others at passing genes to the  
39 next generation is one of the main questions of evolutionary biology. To answer this question,  
40 we need not only to measure fitness *via* various indicators of individual survival  
41 and/or reproduction, but also to understand the underlying physiological mechanisms  
42 regulating organisms' morphology and performance, and in turn fitness. An interesting  
43 physiological mechanism in this respect involves blood glucose and its metabolic by-products.  
44 Although glucose plays an essential role in supplying energy to tissues, its binding to proteins,  
45 lipids and nucleic acids resulting from the non-enzymatic reaction of sugars with nitrogen  
46 compounds such as amino acids like lysine or arginine, namely glycation (Maillard 1912), can  
47 also result in a loss of function of the glycated molecules (see e.g. Bakala et al. 2012; Dinda et al.  
48 2015; Suravajjala et al. 2013).

49 The role of glucose and glycation on the health and performance of organisms is well studied in  
50 medical research on laboratory animals and humans (see e. g. Rabbani and Thornalley 2021).  
51 Most importantly, we know that blood sugar levels well above the normal physiological  
52 values are linked to diabetes, a major human disease in developed countries today (Sun et al.  
53 2022). The morbidity related to diabetes is thought to be mediated by several mechanisms,  
54 including non-enzymatic glycation (Brownlee 1994). This reaction occurs readily under  
55 physiological conditions and can lead to the formation of advanced glycation end-products  
56 (AGEs) (Cerami, et al. 1986). AGEs are toxic compounds whose accumulation rate in tissues  
57 depends on protein turnover (Verziji et al. 2000) and metabolic health (Uruska et al. 2019).  
58 AGEs accumulate in particular in the case of several pathologies such as cardiovascular or  
59 neurodegenerative diseases (see e.g. Poulsen et al. 2013; Chaudhuri et al. 2018; Twarda-  
60 clapa et al. 2022; Khalid, et al. 2022). Furthermore, the relationship between glycaemia and  
61 fitness is supported by studies in captivity showing that high blood glucose levels and poor  
62 glycaemia regulation are linked to mortality rates in zebra finches (Montoya et al. 2018;  
63 2022) and in certain primates, including humans (Palliyaguru et al. 2021), while glucose  
64 supplementation can improve survival and immunity in *Drosophila* (Galenza et al. 2016). In  
65 contrast, very little is known about the consequences of glycaemia and glycation in natural  
66 populations (see Duval et al. 2024 for a review on non-model species).

67 The case of birds is of particular interest given that their glycaemia is the highest within  
68 vertebrates, being in twice as high as that of mammals in average (Polakof et al. 2011).  
69 However, except from rare cases, birds usually do not show the pathologies (e.g. diabetes)  
70 associated with so high glycaemic levels that would be mostly fatal for mammals (partly

71 reviewed in Van de Weyer and Tahas 2024).

72 Protein glycation might nevertheless still constitute an indicator of health status and a  
73 possible fitness predictor. In birds, only three studies have previously investigated the link  
74 between haemoglobin glycation and measures of fitness in the wild. They revealed an U-  
75 shaped variation of haemoglobin glycation levels with age (although in a cross-sectional  
76 study) and a negative link with survival probability (Récapet et al. 2016), as well as a  
77 negative relationship with date of arrival to the breeding grounds from migration (a  
78 parameter negatively associated with reproductive success) and an increased fledgling  
79 production by birds with higher haemoglobin glycation levels at arrival (Andersson and  
80 Gustafsson 1995) in the collared flycatcher (*Ficedula albicollis*). Finally, chick haemoglobin  
81 glycation levels at day 24 have also been found to positively correlate with growth rate in American  
82 kestrels (*Falco sparverius*; Ardia 2006). However, these studies used mostly glycation detection  
83 methodologies that are non-specific, as affinity resins for separating glycated from non-  
84 glycated protein fractions, followed by absorbance measures to quantify protein concentration  
85 in each fraction. In Andersson and Gustafsson (1995), indeed, methods description suggests  
86 they exposed hemolysates to glucose until glycation was detected, which makes unclear how  
87 this would thus represent original glycation levels. Our study, however, used a more  
88 specific technique, i.e. liquid chromatography coupled to mass spectrometry (see  
89 Methods), currently considered as the gold standard for protein glycation  
90 measurements (Priego-Capote et al. 2014).

91 Unlike previous studies of glycation in wild birds (Miksik and Hodny 1992; Rosa 1993; Andersson  
92 and Gustafsson 1995; Beuchat and Chong 1998; Ardia 2006; Récapet et al. 2016; Ling et al. 2020),  
93 we assessed here plasma albumin glycation levels instead of glycated haemoglobin levels in  
94 erythrocytes. Albumin is the most abundant plasma protein and its glycation levels can be  
95 considered as an alternative marker for disease progression monitoring due to its relevance in  
96 regulating processes such as blood pressure and oxidative status (Furusyo and Hayashi 2013;  
97 Kohzuma et al. 2021). Albumin glycation levels are representative of short-term glycaemia levels  
98 (few weeks, instead of several months, as for haemoglobin, in humans), so it provides a  
99 higher resolution tool for glycaemic regulation (Inaba et al. 2007; Kim and Lee 2012).

100 Importantly, albumin is much more exposed than haemoglobin to blood circulating glucose, as,  
101 contrary to the latter, it is free in the plasma, along with most blood glucose (see Bell 1957 for  
102 chicken). Haemoglobin is indeed protected from glucose in erythrocytes given that the  
103 transport of glucose inside mature erythrocytes in birds seems to be extremely low,  
104 and energy production within fully developed erythrocytes depends very little on glycolysis

105 (Johnstone et al. 1998). Accordingly, a previous study shows that, in captive adult zebra  
106 finches, plasma albumin glycation levels are rather high whereas no glycation was observed on  
107 haemoglobin (Brun et al. 2022).

108 In this study, we investigated age-related variations in glycaemia and albumin glycation levels in  
109 a cross-sectional sample of a natural population of a relatively long-lived bird, the Alpine swift  
110 (*Tachymarptis melba*; median and maximum lifespan of 7 and 26 years, respectively; Fransson  
111 et al. 2023). We also sought to determine if glycaemia and albumin glycation levels are  
112 related to fitness, so they can be subjected to natural selection. Finally, we investigated  
113 whether glycaemia and albumin glycation could reflect the cost of reproduction, mediating a  
114 trade-off between reproductive success and survival, and thus potentially underlying part of  
115 the ageing process. As this bird species is long-lived, adults are expected, in line with life  
116 history theory, to favour maintenance and survival over current reproduction, therefore  
117 limiting current effort, as it would be advantageous for maintaining future reproduction  
118 prospects (e.g. Sæther et al. 1993). Adult Alpine swifts weigh about 100 g (Dumas et al.  
119 2024) and feed on aerial insects caught exclusively in flight. Given their relatively small size and  
120 insect-based diet, their glycaemia is expected to be high, even for birds. Indeed, in birds, blood  
121 glucose levels have repeatedly found to correlate negatively with body mass (Kjeld and  
122 Ólafsson 2007; Braun and Sweazea 2008; Tomasek et al. 2019), and carnivorous birds have  
123 been found to show higher glucose levels (Szarka and Lendvai 2024; but see Tomášek et al.  
124 2019; Tomášek et al. 2022 for no difference in fasting glucose levels with diet in  
125 Passeriformes).

126 Animals with high protein intake have constantly high levels of hepatic gluconeogenesis, which  
127 has been shown to be a mechanism maintaining glucose levels relatively high independently of  
128 their feeding status (reviewed in Moon 1988). This could explain why carnivorous birds show  
129 higher fasting glucose levels, as higher gluconeogenesis has been found in some carnivorous  
130 birds in comparison to chickens (Migliorini et al. 1973; Myers and Klasing 1999).

## 131 **Materials and methods**

### 132 **Species and study colonies**

133 The Alpine swift is a socially monogamous bird that breeds in colonies of a few to hundred pairs  
134 in cliffs or in the roof spaces of tall buildings. For this project, data were collected in 2023-2024  
135 in three urban colonies of Alpine swifts located under the roof of clock towers in the Swiss cities  
136 of Biel (Stadkirche, Latitude-longitude: 7°14'46.21"-47°8'29.8"; containing about 60 breeding  
137 pairs), Solothurn (Bieltor, Latitude-longitude: 7°32'1.21"-47°12'33.12"; containing about 40  
138 breeding pairs) and Luzern (Hofkirche, Latitude-longitude: 8°18'50.08"-47°3'20.48";

139 containing about 30 breeding pairs). There is an easy access to the nests in these colonies  
140 (buildings), which have been monitored for over 70 years in Solothurn, 40 years in Biel and 10  
141 years in Luzern. Each year, nests are monitored to record the number of eggs laid and hatched  
142 as well as the number of chicks fledged (see e.g. Bize et al. 2006; 2008; 2014). Female Alpine  
143 swifts produce a single clutch per year of 1 to 4 eggs (modal clutch size is 3). Both parents then  
144 share breeding duties, incubating the eggs for 18 days and feeding their offspring until 50 to 70  
145 days after hatching, at which point they fledge. Chicks are ringed at 15 days of age and, given  
146 that many individuals are locally recruited (Bize et al. 2017), around 70% of the adult birds in  
147 this population have been ringed as chicks and therefore have an exact known age. These long-  
148 distant migrants arrive in Switzerland in mid-April, start laying eggs in May and leave in  
149 September for their wintering grounds in West and Central Africa (Meier et al. 2020).

#### 150 **Sample collection and analyses**

151 Alpine swifts were captured under the legal authorisation of the Swiss Federal Agency for  
152 Environment, Forests and Landscapes (ringing permit #2235). Blood sampling was performed  
153 under a licence of the Veterinary Services of the Cantons Berne, Solothurn and Luzern (National  
154 license #34497).

155 As part of the regular adult monitoring of this population, each year adults are captured  
156 after their arrival from spring migration (late April to early May; i.e. pre-breeding period) and  
157 at the end of the reproductive period (August) before they leave for their wintering grounds.  
158 All the adults in a given colony of the Swiss followed population are captured on the same  
159 day, after dusk, using trap doors that are manually closed after they entered their colony (i.e.  
160 building) to roost for the night. After the traps are closed, all the birds are immediately  
161 captured by hand and kept in bags until they are measured (for a description of the different  
162 measures, see Moullec et al. 2023), marked with a metallic ring whenever needed, and blood  
163 samples are taken from some of them, from which a subsample was used for this study. For  
164 those, the sampling time goes from 21:05 to 1:00 the 1<sup>th</sup>/2<sup>th</sup> of May, 22:15 to 00:38 the 3<sup>th</sup>/4<sup>th</sup>  
165 of May, 21:50 to 23:30 the 9<sup>th</sup> of May, 22:00 to 22:30 the 8<sup>th</sup> of August, 22:00 to 23:00 the 9<sup>th</sup>  
166 of August and 22:00 to 23:30 the 11<sup>th</sup> of August. This is considered in order to control for  
167 possible stress effects on plasma glucose levels, by assigning to each bird a value in hours  
168 counting from the first bird sampled (from those here reported), which is given a 0 (see  
169 Statistics section). Blood (ca. 150µl) is collected by piercing a blood vessel from a toe with a  
170 syringe needle and then using a heparinized Microvette®(Sarstedt). They are kept on ice  
171 before being transported to the laboratory and centrifuged (3500 rpm, 4°C, 10 min), and  
172 then the plasma is aliquoted and frozen at -20°C. This processing of blood samples (i.e.

173 centrifugation and -20°C storage) is carried out within 4 hours of collection.

174 In 2023, the pre- and post-breeding captures of adults took place between May 1 and 4 and  
175 between August 9 and 12, respectively, with thus a period of about 100 days separating the two  
176 sessions of capture. Given that the number of samples that could be analysed in the laboratory  
177 for glycation levels was restricted due to existent limitations on time and spectrometry devices  
178 (see below) availability, we selected samples from 36 females (12 from Biel, 19 from  
179 Solothurn, and 5 from Luzern). We restricted our sampling design to one sex to minimize the  
180 variance associated with sex, and in turn increase the statistical power associated with  
181 analyses on small sample sizes. Birds are identified with metallic rings and had previously  
182 been sexed by molecular techniques, as described elsewhere (Bize et al. 2005). We chose  
183 females because we aimed to study the links with reproduction, and we expected stronger  
184 links in females than males (Bize et al 2008). To investigate the links with age, we took care  
185 to have a widespread age range, also without significant differences on it between colonies  
186 (see **Figure ESM1.3** and **Table ESM1.4**).

187 Glycation levels were determined at the IPHC (Institut Pluridisciplinaire Hubert Curien) in  
188 Strasbourg, using a liquid chromatography coupled to mass spectrometry-based method, as  
189 previously described in Brun et al. (2022). Our measures of albumin glycation rate  
190 correspond to the percentage of glycated albumin relative to total plasma albumin.  
191 Glucose levels were determined by a Contour Plus (Ascensia® diabetes care) portable  
192 glucometer. The validity of these devices has previously been reported for its usage in birds'  
193 studies (Mohsenzadeh et al. 2015; Morales et al. 2020), with several examples of it in the  
194 literature (e.g. Downs et al. 2010; Breuner et al. 2013; Tomasek et al. 2019; McGraw et al.  
195 2020). We also performed an assay to validate it with 46 samples coming from 9 species of  
196 birds (including Alpine swifts) distributed  
197 across a wide range of glucose values (3 “low”, 3 “medium” and 3 “high”, from both captive and  
198 wild populations), confirming a positive linear correlation ( $R^2_{\text{marginal}}=0.66$ ;  $R^2_{\text{conditional}}=0.84$ , for a  
199 model including the species as random factor) of this device with Randox GLUC-PAP  
200 colourimetry kits (P-value<0.001; mean CV: glucometer =9.86%, kit=8.84%) (Moreno Borrallo  
201 et al., unpublished data).

202 We estimated individual annual survival from 2023 to 2024 by looking the presence/absence of  
203 the birds in their colony between May and July 2024 following capture sessions at night in spring  
204 and during daytime during reproduction. Birds that are not re-seen in 2024 are supposed dead  
205 since breeders show no breeding dispersal (Bize et al. 2017), and the probability to recapture a  
206 bird that is still alive is virtually 1 in our colonies (Bize et al. 2006).

## 207 **Statistics**

208 First, we investigated sources of variation in blood glucose levels, albumin glycation levels and  
209 body mass using general linear mixed models where, as fixed explanatory variables (for random  
210 factors, see below), we entered the sampling period (2 factor levels: pre- versus post-  
211 breeding), body mass on the night of capture (except for the model on mass itself), and  
212 chronological age. When analysing the sources of variation in glucose levels, we also tested  
213 for stress response effects by including the sampling time in our model. When analysing the  
214 sources of variation in albumin glycation levels, we included the measures of glucose levels  
215 as explanatory variable to test whether females with higher glycaemia had higher albumin  
216 glycation levels.

217 Besides, we investigated whether pre-breeding glycaemia and glycation levels explained female  
218 reproductive performance in the same year by performing a set of models testing the effects of  
219 pre-breeding glucose and glycation levels, as well as pre-breeding body mass and chronological  
220 age on a series of reproductive traits used as proxies of fitness. In this sense, we analysed, in  
221 four different models, effects of the above explanatory variables on: (i) clutch size, (ii) brood size  
222 at fledging, (iii) hatching success (proportion of eggs that hatched), (iv) and fledging success  
223 (proportion of hatchlings that fledged). This approach allowed testing whether pre-breeding  
224 physiological traits may constrain female reproductive performance (see e.g. Metcalfe and  
225 Alonso-Álvarez 2010; Stier et al. 2012 for similar discussions with oxidative stress). To test for  
226 possible costs of reproduction on the state of physiological traits after breeding (see e.g. Rose  
227 and Bradley 1998; Harshman and Zera 2006), we then investigated how post-breeding measures  
228 of glycemia and albumin glycation were affected by either clutch size or brood size at fledging  
229 (tested in two separated models for each physiological variable, i.e. a total of four models) after  
230 controlling for post-breeding mass, chronological age, pre-breeding values of the variable in  
231 question (either glucose or glycation) and post-breeding glucose values in the model explaining  
232 glycation.

233 Furthermore, we also investigated how changes in glucose or albumin glycation levels during  
234 the breeding seasons (computed as the post-values minus the pre-values) are related to  
235 reproductive effort (either clutch size or number of chicks fledged, in two separated models).

236 We also controlled for age and changes in body mass in these models, and for changes in glucose  
237 levels in the model testing how changes in glycation levels affected reproduction. In all the  
238 models, we entered the colony identity, as well as female identity (nested within colony) in  
239 models with repeated measures from the same females, as random intercept to control  
240 for pseudo-replication. To account for possible non-linear effects of age, body mass, glucose



241 levels and sampling time on the response variables, we included both linear and  
242 quadratic effects in our starting models using raw polynomials. In models with significant  
243 quadratic effects, segmented analyses were performed *a posteriori* to explore the possible  
244 existence of a breakpoint (i.e. threshold) separating different linear relationships on either side  
245 of this breakpoint. Afterwards, a model including a variable, called “pre\_break”, with an  
246 assigned value of 0 after the breakpoint and 1 before, and the interaction of such variable with  
247 the variable for which the breakpoint was calculated, was fitted. This was repeated swapping  
248 the values of the “pre\_break” variable to 0 before and 1 after the breakpoint such that both  
249 slopes could be estimated. Finally, we performed generalized linear mixed models with a  
250 binomial error structure and a logit link function testing the effects of both post-reproductive  
251 (August) plasma glucose and albumin glycation, controlling for post-reproductive body mass and  
252 age, and the dynamics over the reproductive period (differences between before and after  
253 reproduction, i.e. May and August) on survival probability (as the recapture or not of the  
254 individual in May 2024). For the models testing the dynamics, body mass differences were  
255 included instead of post-reproductive body mass. All the variables included quadratic terms in  
256 the initial full models.

257 In models where glycaemia, glycation or body mass values were entered as covariates, these  
258 were mean centred in order to better interpret their intercepts (Schielzeth 2010),  
259 unless they included quadratic terms (in which case the mean centring would distort the  
260 squared values). A stepwise backwards procedure eliminating the quadratic components,  
261 the hour and/or the mass when these were not significant was used to simplify the models,  
262 comparing the AICs and BICs reported by the *anova()* function of R, and selecting the ones  
263 with the lowest values. For two individuals sampled in August, we were unable to detect  
264 any glycated form of albumin, making them outliers. As it is not possible to know if this is a  
265 technical issue or a biological reality, these two measures were excluded from the final  
266 models on glycated albumin. Three additional samples from May (before reproduction)  
267 were found to be missing and thus those samples (but not the whole individual record)  
268 were also excluded. We used general linear mixed models with appropriate distribution for  
269 our response variables: Gaussian for glucose and glycation levels, Gaussian with a log link for  
270 clutch size and brood size at fledging, and binomial with a logit link for the proportion of eggs  
271 that hatched (hatching success) and the proportion of hatched chicks that fledged (fledging  
272 success). Models were ran using the *lmer()* function of the *lme4* R package, plus *lmerTest()*  
273 for obtaining p-values (Bates et al. 2015; Kuznetsova et al. 2017). P-values  $\leq 0.05$  were  
274 reported as significant, while when  $0.1 \geq P\text{-value} > 0.05$ , this result was reported as a trend.

275 Shapiro and Levene test are performed to test the normality of the models' residual  
276 distribution and the homoskedasticity of the variance across groups (when models include  
277 qualitative variables), respectively.

## 278 **Results**

### 279 **Variation of glycaemia and albumin glycation levels before and after reproduction and with** 280 **age**

281 Glucose levels were significantly higher after reproduction than before reproduction (mean  $\pm$  SE  
282 glucose levels in mg/dL before versus after reproduction:  $328.8 \pm 12.3$  versus  $374.8 \pm 7.7$ ;  
283 **Table 1; Figure 1A**). Glucose levels were not significantly related to body mass or  
284 chronological age (**Table 1A**), nor affected by measurement time. Albumin glycation levels  
285 were significantly higher before reproduction when compared to after reproduction (mean  $\pm$   
286 SE albumin glycation levels before versus after reproduction:  $25.39 \% \pm 1.19 \%$  versus  $24.48 \%$   
287  $\pm 0.38 \%$ ; **Table 1B; Figure 1B**). Albumin glycation levels linearly decreased with increasing body  
288 mass (**Table 1B; Figure 2A**). Albumin glycation levels varied non-linearly with age (**Table 1B**;  
289 **Figure ESM1** and **Figure 2B**). Follow-up analyses using a segmented approach indicated a  
290 breakpoint between 5 and 6 years of age, with albumin glycation levels being significantly  
291 lower between 2 and 5 years of age than between 6 and 14 (estimate  $\pm$  SE: 2-5 years  $25.22 \% \pm$   
292  $1.54 \%$ ; 6-14 years:  $28.89 \% \pm 1.14 \%$ ;  $P = 0.024$ ). The albumin glycation levels tended to  
293 decrease with age after the breakpoint (slope  $\pm$  SE:  $-0.156 \pm 0.09$ ;  $P = 0.095$ ) and this  
294 slope tended to be different to the one from before the breakpoint as showed by the outcome  
295 of the interaction term (estimate  $\pm$  SE:  $0.646 \pm 0.318$ ;  $P = 0.051$ ), while no significant trend was  
296 found for the slope calculated before the break point (slope  $\pm$  SE:  $0.223 \pm 0.14$ ;  $P = 0.123$ ).  
297 Variation in glycation levels was not explained by variation in plasma glucose levels (**Table 1B**).  
298 Finally, body mass was higher after reproduction (estimate  $\pm$  SE: before =  $100.6 \text{ g} \pm$   
299  $2.3$ ; after =  $250 105.41 \text{ g} \pm 0.99$ ;  $t = 4.87$ ,  $P < 0.001$ ; **Figure ESM1.4**).

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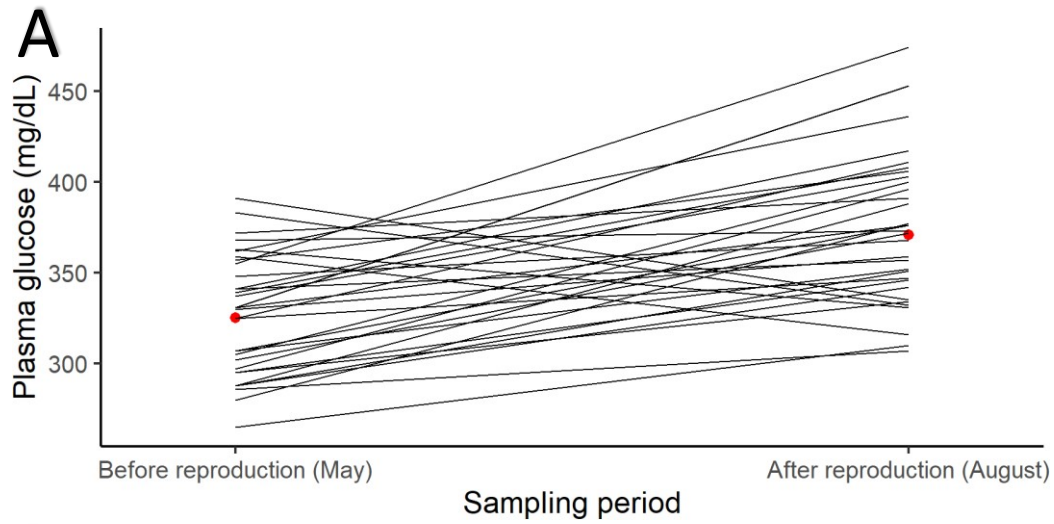
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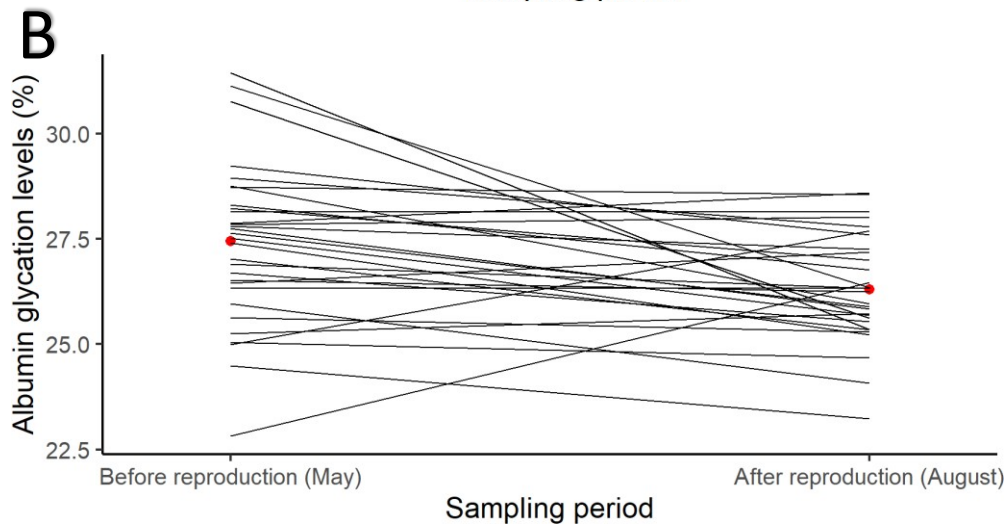
309 **Table 1.** Results of a general linear mixed model showing the effects on glucose (A) and albumin glycation (as a  
 310 percentage of total albumin) (B) of the sampling period (before and after reproduction), body mass, age (using a 2-  
 311 level polynomial approach), and plasma glucose in adult female Alpine swifts. Both glucose and mass are centred to  
 312 better interpret the intercept. The reference level of the sampling period is before reproduction. Significant predictors  
 313 are indicated in bold. Bird identity and sampling colony were entered as random effects.

	Estimates	Standard errors	<i>df</i>	t-values	p-values
<b>(A) Glucose levels</b>					
Random effects: $V_{ID} = 313.85$ (35 individuals), $V_{colony} = 57.47$ (3 colonies), $V_{residuals} = 1008.6$ (69 observations)					
<b>Intercept</b>	<b>328.821</b>	<b>12.271</b>	<b>19.076</b>	<b>26.796</b>	<b>&lt; 0.001</b>
<b>Sampling period (August)</b>	<b>45.972</b>	<b>7.690</b>	<b>35.482</b>	<b>5.978</b>	<b>&lt;0.001</b>
Age (years)	-0.318	1.441	33.258	-0.221	0.827
<b>(B) Albumin glycation levels</b>					
Random effects: $V_{ID} = 0.24$ (35 individuals), $V_{colony} = 1.54$ (3 colonies), $V_{residuals} = 1.42$ (67 observations)					
<b>Intercept</b>	<b>25.392</b>	<b>1.185</b>	<b>10.82</b>	<b>21.423</b>	<b>&lt; 0.001</b>
<b>Sampling period (August)</b>	<b>-0.944</b>	<b>0.384</b>	<b>46.865</b>	<b>-2.460</b>	<b>0.0177</b>
<b>Body mass (grams)</b>	<b>-0.105</b>	<b>0.033</b>	<b>56.927</b>	<b>-3.213</b>	<b>0.002</b>
<b>Age (years)</b>	<b>0.580</b>	<b>0.265</b>	<b>31.224</b>	<b>2.193</b>	<b>0.036</b>
<b>Age (years)<sup>2</sup></b>	<b>-0.036</b>	<b>0.016</b>	<b>30.627</b>	<b>-2.294</b>	<b>0.029</b>
Plasma glucose (mg/dL)	0.007	0.005	59.118	1.458	0.150

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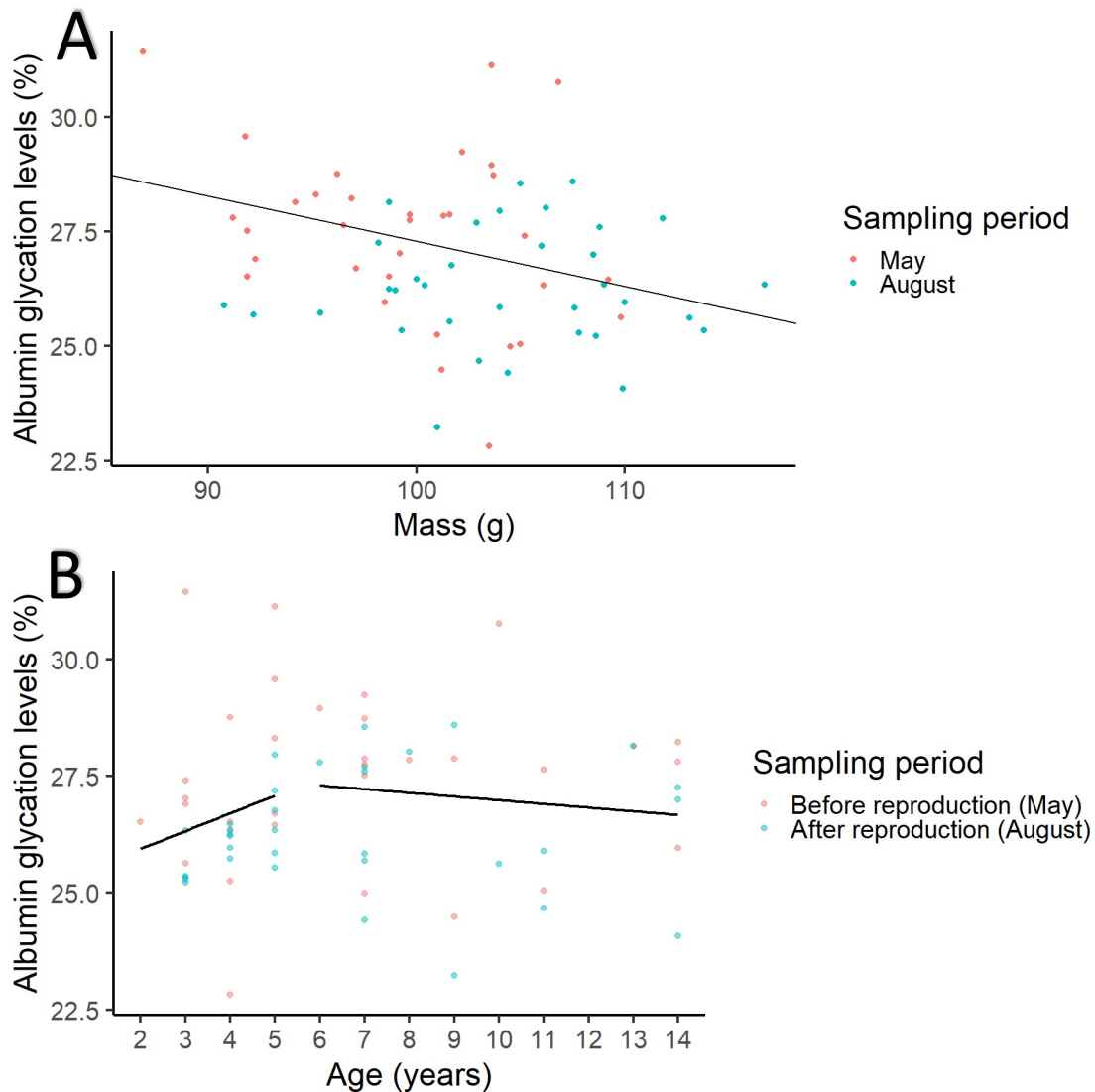


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320 **Figure 1.** Mean (red points) (A) plasma glucose levels and (B) albumin glycation levels measured before (May) and  
 321 after (August) reproduction in adult female Alpine swifts. Lines are describing the individual changes between May  
 322 and August.



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**Figure 2.** Variation of albumin glycation levels (measured as a percentage of total albumin) across the whole study in relation to (A) body mass (May=before reproduction, August=after reproduction) and (B) chronological age in adult female Alpine swifts (following the predictions of a segmented model). Both panels include repeated measures on individuals from before and after reproduction.

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### **Glycaemia levels are related to fledging success, but not albumin glycation rates**

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Plasma glucose and glycation levels measured before reproduction did not significantly explain how many eggs females laid (**Table 2A**) nor how many chicks fledged (**Table 2B**). However, glucose levels, but not albumin glycation, showed a negative trend on hatching success (**Table 2C**), and plasma glucose levels, but not albumin glycation rates, measured before reproduction (May), significantly explained the proportion of chicks fledged from a brood, following positive linear and negative quadratic effects (see **Table 2D** and **Figure 3**). Segmented analyses showed a breakpoint at 307 mg/dL with fledging success showing a trend to be lower below the breakpoint ( $P = 0.095$ ). The fledging success tended to increase with glucose before the breakpoint (estimate  $\pm$  SE:  $0.017 \pm 0.009$ ;  $P = 0.074$ ) and this slope tended to be different from

339 that observed after the breakpoint, as shown by the outcome of the interaction term  
340 (estimate  $\pm$  SE:  $-0.017 \pm 0.01$ ;  $P = 0.088$ ). No significant trend was found after the breakpoint  
341 (slope  $\pm$  SE:  $-0.0008 \pm 0.005$ ;  $P = 0.881$ ). Overall, this suggests that there is a limit to plasma  
342 glucose levels beyond which fledging success no longer increases as plasma glucose levels  
343 rise. No effect of clutch or brood size at fledging (i.e., reproductive effort) was found on  
344 plasma glucose or albumin glycation values measured at the end of the reproduction period (in  
345 August), after controlling for glucose/glycation levels measured at the start of the season (in  
346 May) (see **Table ESM1.1**).

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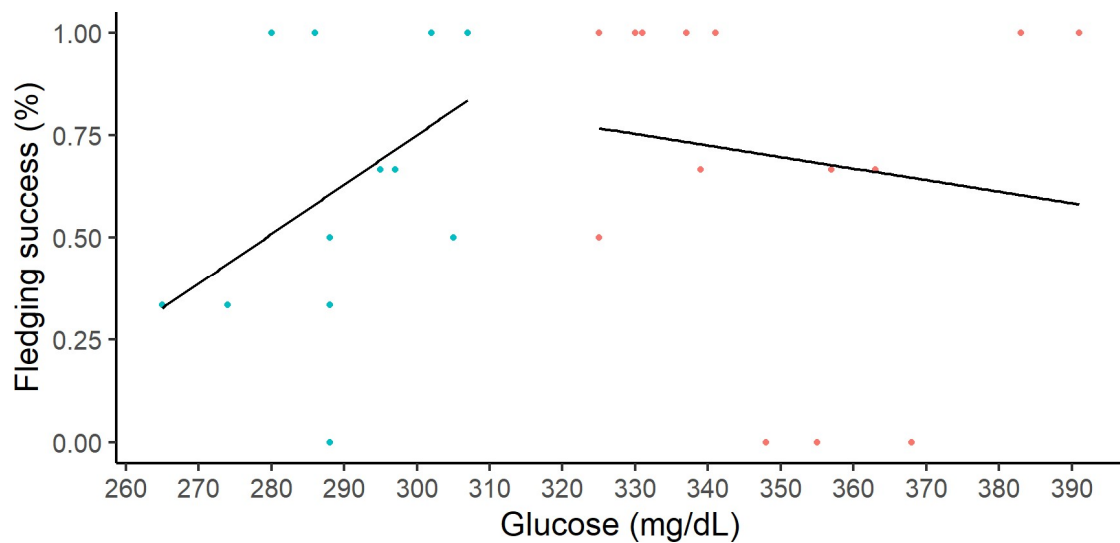
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374 **Table 2.** Results of a generalized linear mixed model on (A) clutch size, (B) brood size at fledging, (C) hatching  
 375 success, i.e. proportion of eggs that hatched, and (D) fledging success, i.e. proportion of hatchlings that fledged. Clutch  
 376 size and brood size at fledging modelled with a Gaussian distribution and log link function and logit link function on  
 377 binomial distribution is employed for hatching and fledging success. Age, mass and plasma glucose in May (before  
 378 reproduction) are included in the model with quadratic effects, and albumin glycation in may only as a linear  
 379 predictor. Albumin glycation is measured as a proportion of glycated vs total albumin. Significant predictors are  
 380 indicated in bold.

	Estimates	Standard Errors	z-values	p-values
<i>(A) Clutch size</i>				
Random effects: $V_{\text{colony}} = 0.007$ (3 colonies), $V_{\text{residuals}} = 0.201$ (33 observations)				
<b>Intercept</b>	<b>0.99</b>	<b>0.085</b>	<b>11.61</b>	<b>&lt;0.001</b>
Glucose before reproduction (mg/dL)	-0.0006	0.0009	-0.655	0.512
Albumin glycation before reproduction (%)	-0.001	0.02	-0.064	0.949
<i>(B) Brood size at fledging</i>				
Random effects: $V_{\text{colony}} = 0$ (3 colonies), $V_{\text{residuals}} = 1.18$ (33 observations)				
<b>Intercept</b>	<b>1.424</b>	<b>0.189</b>	<b>7.53</b>	<b>&lt;0.001</b>
Glucose before reproduction (mg/dL)	-0.0007	0.006	-0.126	0.9
Albumin glycation before reproduction (%)	0.074	0.103	0.716	0.479
<i>(C) Hatching success</i>				
Random effects: $V_{\text{colony}} = 2.185 \times 10^{-10}$ (3 colonies), $V_{\text{residuals}} = \text{NA}$ (33 observations)				
Intercept	373.55	236.87	1.58	0.115
Glucose before reproduction (mg/dL)	-0.041	0.023	-1.78	0.075
Albumin glycation before reproduction (%)	-0.249	0.385	-0.645	0.519
Mass (g)	-7.653	4.831	-1.584	0.113
Mass <sup>2</sup> (g)	0.039	0.025	1.589	0.112
Age (years)	0.587	0.333	1.762	0.078
<i>(D) Fledging success</i>				
Random effects: $V_{\text{colony}} = 0.00008$ (3 colonies), $V_{\text{residuals}} = \text{NA}$ (28 observations)				
<b>Intercept</b>	<b>-499.04</b>	<b>4.085</b>	<b>-12.005</b>	<b>&lt;0.001</b>
<b>Glucose before reproduction (mg/dL)</b>	<b>0.29</b>	<b>0.013</b>	<b>22.623</b>	<b>&lt;0.001</b>
<b>Glucose<sup>2</sup> before reproduction(mg/dL)</b>	<b>-0.0004</b>	<b>0.0000</b> <b>07</b>	<b>-60.522</b>	<b>&lt;0.001</b>
Albumin glycation before reproduction (%)	0.172	0.221	0.777	0.437

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**Figure 3.** Variation in fledging success measured as the proportion of hatchlings that fledged in function of plasma glucose levels (in mg/dL) measured in May, before reproduction (following the predictions of a segmented model).

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### 385 **Changes in albumin glycation rates are related to those in body mass and to clutch size**

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Albumin glycation rate was found to decrease between the start (May) and end (August) of the breeding season, while glucose and body mass increased in parallel over the same period in adult female Alpine swifts (see above). Regarding the dynamics (i.e. differences in the parameters between May and August), we found a significant negative effect of body mass difference on glycation difference (Estimate  $\pm$  SE =  $-0.219 \pm 0.055$ ;  $P < 0.001$ , with brood size at fledging as a covariate). In other words, females whose body mass increased the least during the breeding season had a smaller decrease in glycation levels. A similar relationship was obtained when using clutch size as a covariate (estimate  $\pm$  SE =  $-0.243 \pm 0.061$ ;  $P = 0.0142$ , see **Table ESM1.2**). When testing the influence of glycation dynamics on clutch size, we found a significant negative statistical effect of body mass and glycation difference on clutch size (**Table 3**). In other words, females with the least reduction in glycation levels laid fewer eggs. Changes in plasma glucose levels were not statistically influenced by any of the breeding output variables (**Table ESM1.1**), nor did they in turn explain them (**Table 3**).



400 **Table 3.** Results of generalized linear mixed models on (A) clutch size, (B) brood size at fledging, (C) hatching success,  
 401 i.e. proportion of eggs that hatched, and (D) fledging success, i.e. proportion of hatchlings that fledged. Clutch size  
 402 and brood size at fledging modelled with a Gaussian distribution and log link function and logit link function on binomial  
 403 distribution is employed for hatching and fledging success. The fixed predictors are the difference between before  
 404 and after reproduction of plasma glucose in mg/dL, albumin glycation as a percentage of total albumin and mass in  
 405 grams. The model also includes age in years with a quadratic component. Significant estimates are indicated in bold.

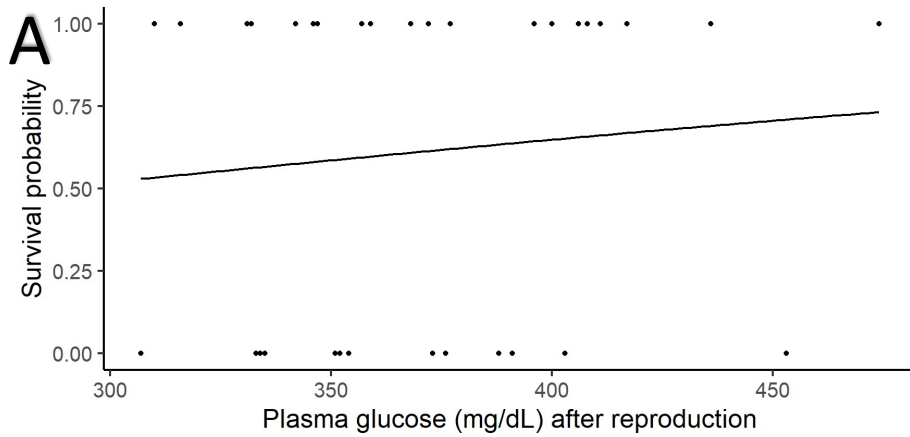
	Estimates	Standard Errors	t-values	p-values
<b>(A) Clutch size</b>				
Random effects: $V_{\text{colony}} = 0.013$ (3 colonies), $V_{\text{residuals}} = 0.156$ (31 observations)				
<b>Intercept</b>	<b>1.17</b>	<b>0.18</b>	<b>6.58</b>	<b>&lt;0.001</b>
Glucose difference	0.0005	0.0006	0.97	0.334
<b>Glycation difference</b>	<b>-0.04</b>	<b>0.02</b>	<b>-2.3</b>	<b>0.022</b>
<b>Mass difference</b>	<b>-0.03</b>	<b>0.01</b>	<b>-3.46</b>	<b>0.001</b>
Age (years)	-0.06	0.04	-1.57	0.116
Age <sup>2</sup> (years)	0.004	0.002	1.83	0.067
<b>(B) Brood size at fledging</b>				
Random effects: $V_{\text{colony}} = 0.511$ (3 colonies), $V_{\text{residuals}} = 1.04$ (31 observations)				
Intercept	1.66	0.51	3.25	0.065
Glucose difference	-0.001	0.004	-0.23	0.816
Glycation difference	-0.191	0.314	-0.44	0.542
Mass difference	-0.081	0.053	-1.53	0.139
<b>(C) Hatching success</b>				
Random effects: $V_{\text{colony}} = 1.652 \times 10^{-10}$ (3 colonies), $V_{\text{residuals}} = \text{NA}$ (31 observations)				
Intercept	-0.966	1.703	-0.57	0.571
Glucose difference	0.011	0.013	0.85	0.395
Glycation difference	0.039	0.422	0.092	0.927
Mass difference	0.005	0.155	0.034	0.973
Age (years)	0.447	0.317	1.41	0.158
<b>(D) Fledging success</b>				
Random effects: $V_{\text{colony}} = 1.652 \times 10^{-10}$ (3 colonies), $V_{\text{residuals}} = \text{NA}$ (31 observations)				
Glucose difference	-0.017	0.012	-1.34	0.179
Glycation difference	-0.191	0.314	-0.61	0.542
Mass difference	-0.084	0.123	-0.68	0.496
Age (years)	-1.725	0.929	-1.86	0.063
Age <sup>2</sup> (years)	0.095	0.053	1.8	0.072

406 The effects on survival probability of plasma glucose, albumin glycation and body mass after  
 407 reproduction (August), controlling for age, are shown in **Table 4**. While plasma glucose and body  
 408 mass have linear and quadratic effects on survival (**Figure 4 A, B**) independently of age control,  
 409 albumin glycation levels only have an effect on survival when age is controlled for (**Figure 4C**).

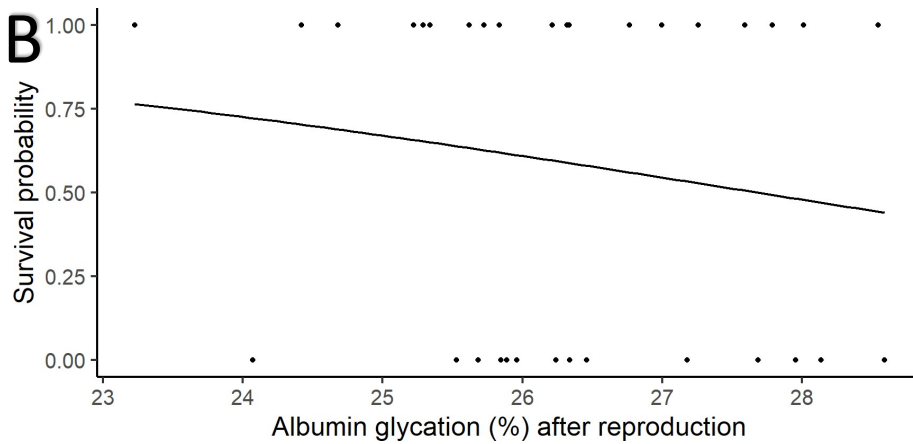
410 **Table 4.** Results of generalized linear mixed models on survival to the next season (August 2023 to May 2024)  
 411 determined by recapture in the colony. Both the complete (A) and the best model by AIC and BIC (B) are shown, to  
 412 discuss the mediation of age on albumin glycation effects. Significant estimates are indicated in bold.

	Estimate	Standard Error	z value	P-value
<i>(A) Complete model</i>				
Random effects: Vcolony = 1.336*10 <sup>-17</sup> (3 colonies), Vresiduals = NA (34 observations)				
Intercept	0.19	0.199	0.953	0.34063
<b>Glucose after (mg/dL)</b>	<b>0.046</b>	<b>0.011</b>	<b>4.36</b>	<b>&lt;0.001</b>
<b>Glucose<sup>2</sup> after (mg/dL)</b>	<b>-0.00005</b>	<b>0.000005</b>	<b>-9.177</b>	<b>&lt;0.001</b>
<b>Albumin glycation (%)</b>	<b>-3.43</b>	<b>1.325</b>	<b>-2.592</b>	<b>0.00955</b>
<b>Albumin glycation<sup>2</sup> (%)</b>	<b>0.059</b>	<b>0.025</b>	<b>2.394</b>	<b>0.017</b>
<b>Mass (g)</b>	<b>0.313</b>	<b>0.073</b>	<b>4.316</b>	<b>&lt;0.001</b>
<b>Mass<sup>2</sup> (g)</b>	<b>-0.001</b>	<b>0.0001</b>	<b>-8.587</b>	<b>&lt;0.001</b>
Age (years)	-0.276	0.614	-0.45	0.653
Age <sup>2</sup> (years)	0.016	0.036	0.451	0.652
<i>(B) Best model</i>				
Random effects: Vcolony = 0 (3 colonies), Vresiduals = NA (34 observations)				
Intercept	-12.57	19.91	-0.631	0.528
<b>Glucose after (mg/dL)</b>	<b>0.041</b>	<b>0.0102</b>	<b>4.03</b>	<b>&lt;0.001</b>
<b>Glucose<sup>2</sup> after (mg/dL)</b>	<b>-0.00004</b>	<b>0.000005</b>	<b>-8.15</b>	<b>&lt;0.001</b>
Albumin glycation (%)	-1.54	1.325	-1.16	0.244
Albumin glycation <sup>2</sup> (%)	0.022	0.025	0.912	0.362
<b>Mass (g)</b>	<b>0.455</b>	<b>0.071</b>	<b>6.37</b>	<b>&lt;0.001</b>
<b>Mass<sup>2</sup> (g)</b>	<b>-0.002</b>	<b>0.0001</b>	<b>-14.3</b>	<b>&lt;0.001</b>

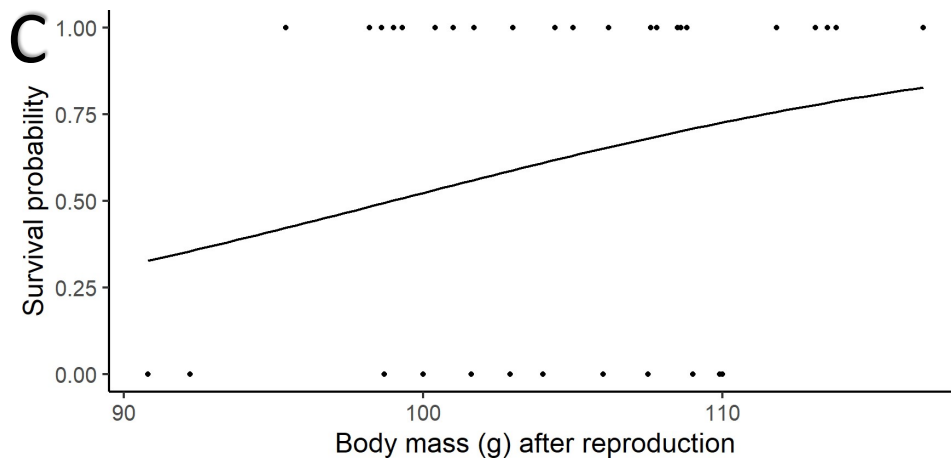
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418 **Figure 4.** Probability of survival from August 2023 to May 2024 depending on post-reproduction (August 2023) values  
 419 of (A) plasma glucose levels, (B) albumin glycation rate and (C) body mass.

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421 There was no significant effect of changes in plasma glucose levels, albumin glycation and body  
 422 mass between pre- and post-breeding stage on the probability of survival (see **Table ESM1.3**).

423 **Discussion**

424 **Body mass, glycaemia and albumin glycation: related signs of body condition?**

425 Female Alpine swifts had lower levels of plasma glucose and higher levels of glycated albumin  
 426 before reproduction (May) than after reproduction (August). Alpine swifts are trans-Saharan

427 migratory birds that arrive in Switzerland in April, reproduce between May and August, and then  
428 leave in September to return to their wintering grounds in Western Africa (Meier et al. 2020).  
429 Hence, blood parameters measured in May could partially mirror a cost of migration, although  
430 measures before the migratory event should be performed to discriminate such an effect. Blood  
431 glucose levels have been found to positively correlate with residual body mass (adjusted for  
432 structural size) in American house finches, *Haemorrhous mexicanus*, (McGraw et al. 2020),  
433 breeding pale-bellied Tyrant-Manakins, *Neopelma pallescens* (Azeredo et al. 2016) and with  
434 body mass in garden warblers (*Sylvia borin*) (Jenni-Eiermann and Jenni 1994) and barn swallows  
435 (*Hirunda rustica*) (Bendová 2020, but see Lill (2011) and Ramage-Healey and Romero  
436 (2000) for contrasted results). Although plasma glucose and body mass were not correlated  
437 in our study, they both increased over the course of the reproductive season. This is in  
438 contrast with the decrease in mass along the season found by Dumas et al. (2024) in the  
439 same Alpine swift population; however, that study focused on body mass measurements  
440 from a big pool of individuals (not necessarily recaptured) during the whole breeding season,  
441 not single pre- and post-breeding measurements as reported in the present analysis. It remains  
442 also possible that the effects we observe are related to particularly good conditions during  
443 2023. An increase in feeding frequency of the chicks from the pre-laying to the fledging  
444 phase could explain the higher glucose levels (Jackson et al. 2023). However, it remains  
445 unclear if the observed increase in body mass across the breeding season depends more  
446 on muscle size (proteins), as it would be expected when activity levels are increased  
447 (Marsh 1984 and discussion in Jackson et al. 2023), or on fat deposition, potentially  
448 derived from sugars. Measures of changes in body composition during breeding, apart from  
449 merely body mass, would also be fruitful for understanding how dynamics of fuel usage and  
450 sparing affect the value of plasma metabolites such as glucose.

451 The concomitant increase in body mass and glucose and reduction in glycation between May  
452 and August may indicate that glycated albumin plasma levels may be a proxy of individual quality  
453 (see Wilson and Nussey 2010) in breeding female swifts. This new individual quality marker  
454 would therefore add to the various blood metabolites already described to date as markers of  
455 quality in birds (Jenni-Eiermann and Jenni 1994; Minias and Kaczmarek 2013; Azeredo et al.  
456 2016; Jackson et al. 2023). Individuals of higher quality may therefore be better protected  
457 against glycation, either by avoiding it or by being more efficient in quickly clearing glycated  
458 proteins from the body. Therefore, restoring an adult physiological status promoting survival  
459 would also limit the deleterious effects of glycation over time, and could therefore promote  
460 longevity. Interestingly, albumin glycation and, more generally, fructosamine levels (a marker of

461 general plasma protein glycation) are negatively related to BMI (Body Mass Index) in humans  
462 (Selvin et al. 2018). A similar result was found here in swifts, i.e. a negative relationship between  
463 albumin glycation levels and body mass. This is the first time this relationship is found in  
464 birds, as other studied that tested it failed to find any significant associations (Andersson and  
465 Gustafsson 1995; Brun et al. 2022; a positive trend is reported by Récapet et al. 2016). Beyond  
466 a simple covariation of body mass and glycation, how body mass may relate to glycation  
467 levels is not yet well-known, but it is hypothesized to be related with an increase in  
468 protein turnover associated to the proinflammatory state induced by an augmented  
469 adiposity (see e.g. Chagnac et al. 2003; Koga408 et al. 2007).

470 To our knowledge, few studies described glycation status in wild breeding birds. The only  
471 publication in this regard found no significant variation in the levels of circulating glucose and  
472 fructosamine after breeding in common eiders (*Somateria mollissima*), despite a decrease in  
473 body mass (Ma et al. 2020). This discrepancy may have several explanations. Common eiders  
474 are capital breeders and they rely mostly on internal lipid stores while fasting during breeding  
475 (Parker and Holm 1990). The relationship between plasma glucose, glycation levels and  
476 reproduction may therefore not be as straightforward as in income breeders like swifts.

477 With regard to body mass change in the incubating sex during breeding, we can observe clear  
478 differences between bird species, depending mainly on their reproductive strategy (Moreno  
479 1989), with capital breeders losing weight during incubation while income breeders maintain or  
480 even increase their body mass, and undergo the main weight loss shortly after hatching. Cox and  
481 Cresswell (2014) found that species whose body mass increases the most during breeding also  
482 have higher survival rates. They proposed that the lower food predictability under less  
483 pronounced seasonality triggers greater adult investment in survival than in reproduction during  
484 the breeding season. Swifts forage exclusively on flying insects, whose availability depends on  
485 weather conditions (Grüebler et al. 2008), with rainy days severely limiting adult food intake and  
486 affecting their body mass (Dumas et al. 2024). Swifts are relatively long-lived birds, and should  
487 therefore invest more in their survival (maintaining body mass and body condition) during the  
488 breeding season than in current reproduction (see e.g. Charlesworth 1980; Saether 1988),  
489 although it would also depend on certain variables, including their pre-breeding condition,  
490 which can modulate their resolution of such trade-off (Erikstad et al. 1998). Indeed, Martins and  
491 Wright (1993) showed that common swifts (*Apus apus*) rapidly restore their body mass at the  
492 end of the nestling phase, when the chicks' feeding requirements are reduced, thus limiting the  
493 cost of reproduction. In our study, we found that body mass increased from laying initiation to  
494 fledging stages, which supports the idea of prioritizing investment in adult self-maintenance for

495 breeding individuals.

**496 Age-related variation of glycated albumin**

497 Albumin glycation in female swifts increases in early life up to the age of 5, before decreasing  
498 slightly with age. This is similar to the typical bell-shape pattern (see e.g. Forslund and  
499 Pärt 1995; Saraux and Chiaradia 2022) followed by many traits in relation to age, and it is  
500 similar to what has been described before in this species for morphometric and breeding  
501 traits (Moullec et al. 2023), as well as for physiological traits such as females' erythrocyte  
502 membrane resistance to oxidative stress (Bize et al. 2008). Albumin glycation, together with  
503 other glycation markers, has been reported to increase with age in humans, although linearly  
504 instead of following a bell-shaped pattern (Selvin et al. 2018). Glucose levels, however, did not  
505 vary with age, in contrast to what is known from biomedical research in humans and other  
506 model species, where we can see for example an increase with age in primates and a bell-  
507 shaped pattern in laboratory mice (Palliyaguru et al. 2021). Our data shows also a higher  
508 survival of individuals with higher glucose levels, which together with an increased fledging  
509 success, suggests our population of swifts is being selected for higher glucose levels, being  
510 potentially quite resistant to its pervasive effects.

511 Still, this could also generate an antagonistic pleiotropy affecting ageing (Williams 1957), given  
512 the effects of glycation on survival (see below). However, our results on age-related variation of  
513 glycated albumin are derived from a cross-sectional study, where individuals of different ages  
514 are sampled once rather than from a longitudinal study where the same individuals are  
515 sampled multiple times at various ages. Findings from cross-sectional studies are therefore  
516 incorporating both effects taking place at the individual level (improvement/maturation early  
517 in life and senescence late in life) and demographic effects such as the selective appearance and  
518 disappearance of phenotypes in the population (Forslund and Pärt 1995; van de Pol and  
519 Verhulst 2006). Hence, the increase in glycation levels before the age of 5 could be explained  
520 either by an increase in glycation rates at individual level with age or by the late recruitment  
521 into the breeding population (as only individuals showing breeding attempts are sampled) of  
522 individuals with higher glycation rates (i.e. selective appearance: a later age at reproduction in  
523 females with a higher glycation level early in life). Female Alpine swifts usually start breeding  
524 for the first time between 2 and 5 years of age (Tettamanti et al. 2012). Similarly, a decrease  
525 in older age may occur either at an individual level or as a result of the selective disappearance  
526 from the population of individuals with higher glycation rates. An intra-individual decrease in  
527 glycation levels with age towards the end of life would indicate 'improved health', which  
528 contrasts sharply with the results of biomedical research in humans, which show an

529 intra-individual increase in glycation levels with age, indicating ‘senescence’ (Selvin et al.  
530 2018). There is considerable support for the hypothesis of selective disappearance in other  
531 species. Indeed, glycation of albumin and haemoglobin has been linked to mortality in  
532 humans (Wu et al. 2021; Rooney et al. 2022), whereas adult collared flycatchers with higher  
533 haemoglobin glycation levels (measured using a human blood kit) were more likely to  
534 disappear from the wild (Récapet et al. 2016). Furthermore, our data showed interesting  
535 effects of albumin glycation levels on survival to the next season, which seems to indicate  
536 that individuals with lower glycation levels have a higher probability of survival, but only when  
537 accounting for age, even when age itself does not predict survival. This can be so because the  
538 effects of age on mortality rates (potentially senescence) are mediated by factors such as  
539 glycation rates or body mass, for which our data seem to show selection in favour of higher body  
540 masses. This is in partial disagreement with what Dumas et al. (2024) found, i.e. that such  
541 an effect only occurs for non-breeders, whereas we report it for breeders. Nevertheless,  
542 they showed this effect for both sexes together, whereas we do it for females alone. This  
543 may be relevant as Dumas et al. (2024) also showed different kinds of selection pressures  
544 (although through fledging success, not through survival as in our case) between sexes (i.e.  
545 stabilizing selection for females and disruptive selection for males) for breeding body mass (i.e.  
546 measured in June).

547 As a conclusion, to finally determine whether glucose and glycated albumin can be reliably used  
548 as markers of senescence in swifts, and potentially in other birds, longer longitudinal studies are  
549 still needed to determine if the effects are sustained across different years, potentially with  
550 contrasting environmental conditions modulating the selective pressures.

551 **Pre-reproduction plasma glucose levels and albumin glycation dynamics affect female**  
552 **reproductive performance**

553 We found that plasma glucose levels before the start of reproduction were the only factor that  
554 significantly predicted fledging success. Hence, body condition of the parents (here evaluated  
555 as body mass) did not positively modulate fledging success in swifts, contrary to what has been  
556 highlighted before in this species (Dumas et al. 2024) and in other birds (with body mass  
557 residuals on size, e.g. Chastel et al. 1995; Moe et al. 2002). This discrepancy in our results may  
558 be due to our small sample size, which makes it difficult to establish significant relationships if  
559 the effect is subtle. The relationship between pre-reproduction glucose levels and fledging  
560 success was quadratic, and a further segmented analysis suggested that starting breeding with  
561 low glycaemia is not optimal for reproduction, and a glucose level over a point (estimated to be  
562 307 mg/dL in our model) does not improve reproductive success anymore. Low glycaemia

563 may indicate poor female condition, with deleterious consequences such as impaired adult  
564 foraging performance and seldom chick provisioning, two important determinants of  
565 fledging success (e.g. Jenni-Eiermann and Jenni 1994, Saraux and Chiaradia 2022). Although this  
566 is not an obvious effect according to our data, which seem to indicate a plateau for  
567 fledging success after a threshold in plasma glucose (i.e. 307 mg/dL), high glycaemia could  
568 also impair the ability of females to rear chicks successfully. This could involve the  
569 glycation process for example, although our data did not suggest that albumin glycation  
570 levels directly influence reproduction.

571 In contrast, Borger (2024) found that glycated haemoglobin in female zebra finches was  
572 positively related with clutch size and offspring production, but as they only measured it after  
573 reproduction, costs cannot be separated from constraints. It may thus be concluded that their  
574 results indicate that higher egg production and perhaps clutch care induce higher levels of  
575 protein glycation, costs we did not find. However, direct comparison between our results and  
576 those of Borger (2024) is difficult, as discrepancies may be explained by physiological differences  
577 between species studied (Bize, et al. 2014), the context (captive versus wild individuals), the  
578 nature of the targeted glycated proteins, and even the analytical method used (non-specific kits  
579 versus accurate mass-spectrometry). A more detailed study of glycaemia dynamics during the  
580 successive phases of the breeding season (mating, laying, incubation, brooding, fledging) in our  
581 species, as previously conducted in others (Remage-Healey and Romero 2000, Gayathri, et al.  
582 2004, Azeredo, et al. 2016, Bendová 2020), would help to better understand how fledging  
583 success is affected by circulating glucose levels in the parents.

584 Interestingly, the decrease in glycated albumin levels during the breeding season was positively  
585 related to clutch size in our study, without being reflected in fledging success, suggesting  
586 explanations not related to breeding performance but rather to more basic metabolic processes.  
587 For instance, our results can reflect a general increase in anabolism during the reproductive  
588 period. Reduced glycation levels may be attributed to faster protein (albumin) turnover rates,  
589 thereby reducing damage levels (Wada, et al. 2016). This is consistent with the negative  
590 relationship between variation in body mass and variation in albumin glycation levels that we  
591 recorded during the course of the breeding season: the greater the increase in body mass after  
592 reproduction, the greater the reduction in glycation levels. The negative relationship between  
593 the number of eggs produced and the increase in body mass probably reflects a trade-off  
594 between somatic and reproductive investments (see Williams 2005 for a review of the costs  
595 associated with egg laying), while the positive relationship between glycation reduction rate and  
596 clutch size might indicate a closer relationship between albumin turnover and egg production.



597 In fact, albumin production should be increased during egg-laying due to its transfer to the eggs  
598 (Patterson et al. 1962).

## 599 **Conclusions and perspectives**

600 We show here that glucose and glycated albumin levels increase and decrease respectively  
601 during reproduction in Alpine swifts, which is paralleled by an increase in body mass. We did not  
602 find any costs related to reproductive effort, evaluated using clutch or brood size. Our results  
603 on survival show that glycation levels may represent a more important constraint for other  
604 phases of the annual cycle, such as the long-distance migration swifts perform. Future studies  
605 should explore if changes in glycation levels such as those detected (around 1%) are paralleled  
606 by a significant change in other parameters better known to reflect changes in health or fitness.  
607 This would help to better understand the underlying mechanisms mediating glycation and  
608 fitness. In addition, we established that albumin glycation levels vary with age in a similar way  
609 as other age-related parameters in this species, with a peak at 5.5 years, suggesting that  
610 glycation may be part of the physiological mechanisms underlying the senescence process.  
611 Nevertheless, this is a cross-sectional study, so longitudinal data would be needed to determine  
612 if this variation is really linked to ageing per se and not to other demographical phenomena like  
613 selective appearance or disappearance, as our results on survival suggest. Finally, glucose levels  
614 before reproduction showed a positive effect on fledging success up to a certain threshold, after  
615 which a plateau is reached. A negative trend on hatching success was also found, while glycated  
616 albumin levels are negatively linked to body mass, and its dynamics to changes in body mass and  
617 clutch size, suggesting that they could influence female reproductive fitness. Although the exact  
618 mechanisms underlying our results remain unclear, we hypothesize that albumin glycation  
619 dynamics may be mainly influenced by protein turnover rate and may thus be representing the  
620 general rate of anabolism, and that glucose is likely to be an indicator of parents' nutritional  
621 state, acting positively on chicks' survival to fledging. To prove these hypotheses, more precise  
622 measures of energy metabolites and their dynamics on both parents and offspring, together  
623 with body condition and body composition changes during rearing should be determined, to see  
624 which nutrients are the most important to determine breeding success and how the costs and  
625 benefits of differential allocation between parents and chick survival are shaped.

## 626 **Statements and Declarations**

627 Authors declare having no competing interests affecting the content of this publication. Data  
628 and code are provided as Electronic Supplementary Material (ESM2 & ESM3 respectively).

629

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## 634 **Authors contributions**

635 FC and FB conceived the original collaboration, set up the project, contributed to the  
636 development of the idea and participated in the discussion of the results. AMB contributed to  
637 the development of the idea, participated in the field work and sample collection with PB, and  
638 made the statistical analyses. CS and SJO performed the glycation measurements by mass  
639 spectrometry. PB provided data from the long-term monitoring of the animals, contributed to  
640 the sample collection and participated in the discussion of the results. AMB wrote the original  
641 draft, later edited by FC, FB and PB. All the authors approved the final draft.

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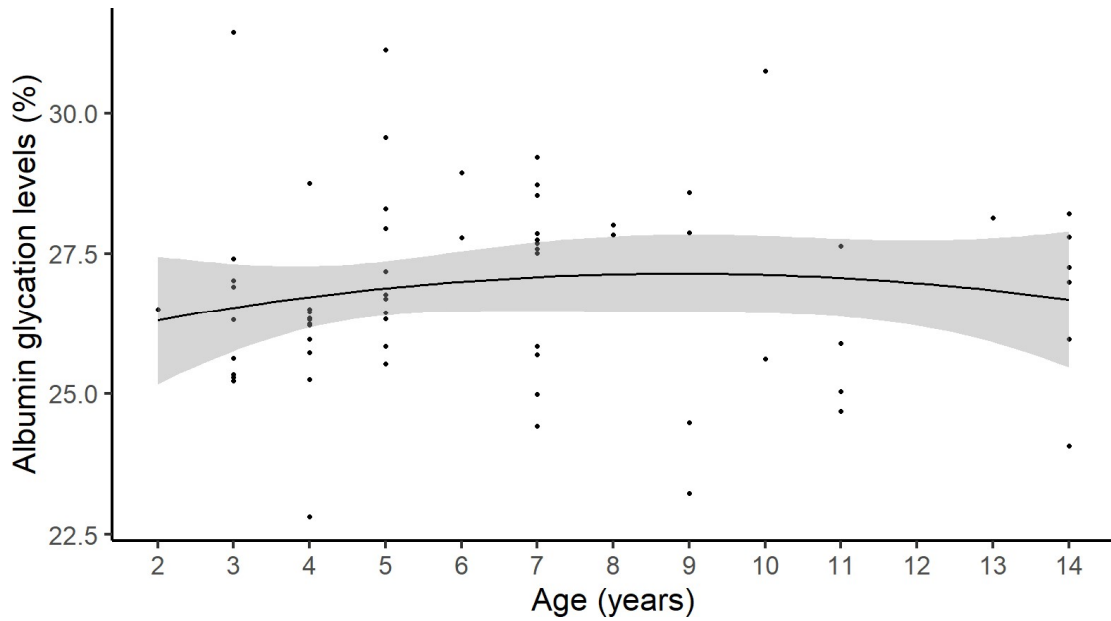
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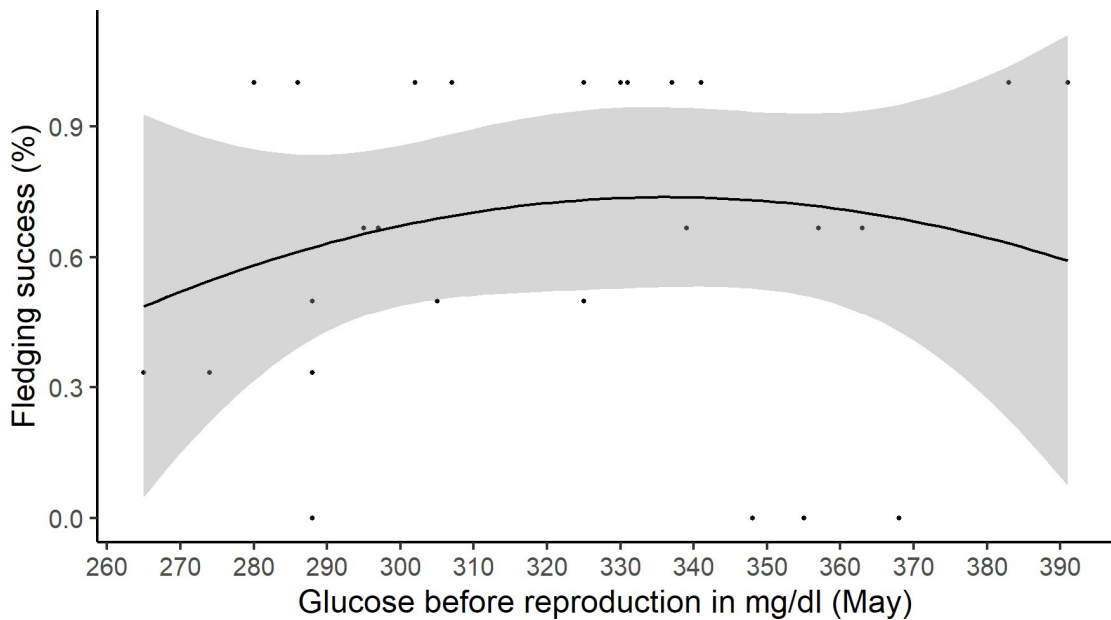
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# ESM1



**Figure ESM1.1.** Variation of albumin glycation levels (measured as a percentage of total albumin) in relation to chronological age in adult female Alpine swifts.



**Figure ESM1.2.** Variation of fledging success (measured as a percentage of chicks hatched that fledged) in relation to plasma glucose values before reproduction in adult female Alpine swifts.



11 **Table ESM1.1.** Results of general linear mixed models on the effects of reproduction investment on plasma  
 12 glucose levels (in mg/dl) difference between before and after reproduction. Two different estimates of reproductive  
 13 investment (A) clutch size, and (B) brood size at fledging, were considered in different models. The difference in  
 14 mass in grams is included as a fixed predictor.

	Estimates	Standard Errors	df	t-values	p-values
<i>(A) Clutch size</i>					
Intercept	40.83	49.45	7.26	0.826	0.435
Clutch size	-1.42	17.01	11.89	-0.084	0.935
Mass difference	1.53	1.65	7.18	0.923	0.386
<i>(B) Brood size at fledging</i>					
Intercept	40.34	18.45	3.94	2.187	0.095
Brood size at fledging	-1.96	7.95	27.72	-0.246	0.807
Mass difference	1.45	1.65	5.96	0.879	0.414

15

16 **Table ESM1.2.** Results of general linear mixed models on the effects of reproduction investment on albumin  
 17 glycation levels (in percentage of total albumin) difference between before and after reproduction. Two different  
 18 estimates of reproductive investment (A) clutch size, and (B) brood size at fledging, were considered in different  
 19 models. The difference in mass in grams and the difference in glucose in mg/dl are included as fixed predictors.

	Estimates	Standard Errors	df	t-values	p-values
<i>(A) Clutch size</i>					
Intercept	1.5	1.79	6.14	0.837	0.434
Clutch size	-0.72	0.606	10.53	-1.19	0.261
<b>Mass difference</b>	<b>-0.243</b>	<b>0.061</b>	<b>4.31</b>	<b>-3.97</b>	<b>0.014</b>
Glucose difference	0.008	0.006	26.97	1.3	0.204
<i>(B) Brood size at fledging</i>					
Intercept	-0.5	0.63	27	-0.797	0.432
Brood size at fledging	-0.028	0.28	27	-0.099	0.922
<b>Mass difference</b>	<b>-0.219</b>	<b>0.052</b>	<b>27</b>	<b>-4.3</b>	<b>0.0002</b>
Glucose difference	0.009	0.006	27	1.4	0.174

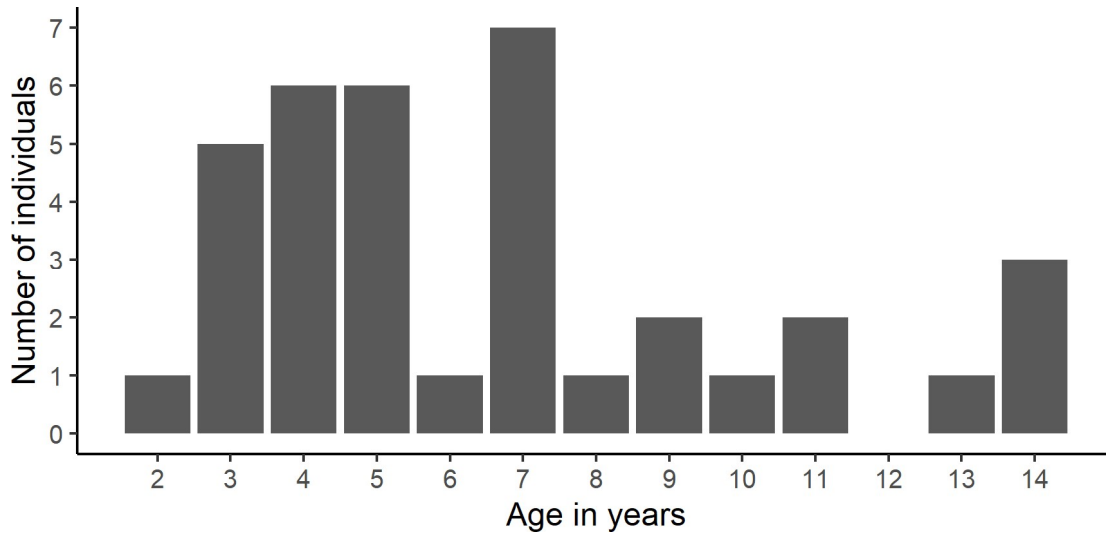
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22 **Table ESM1.3.** Results of general linear mixed model on the effects of difference between before and after  
 23 reproduction in plasma glucose (in mg/dl) and albumin glycation levels (in percentage of total albumin) on survival  
 24 to the next season (August 2023 to May 2024). The model shown is the best after model step back-wise selection  
 25 process with AIC and BIC is performed.

	Estimates	Standard Error	z value	p-values
Random effects: Vcolony = 0 (3 colonies), Vresiduals = NA (31 observations)				
Intercept	-0.099	0.597	-0.166	0.868
Glucose difference	0.001	0.008	0.119	0.905
Glycation difference	-0.368	0.232	-1.585	0.113

26



27

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**Figure ESM1.3.** Total number of individual Alpine swifts of each age included in the study.

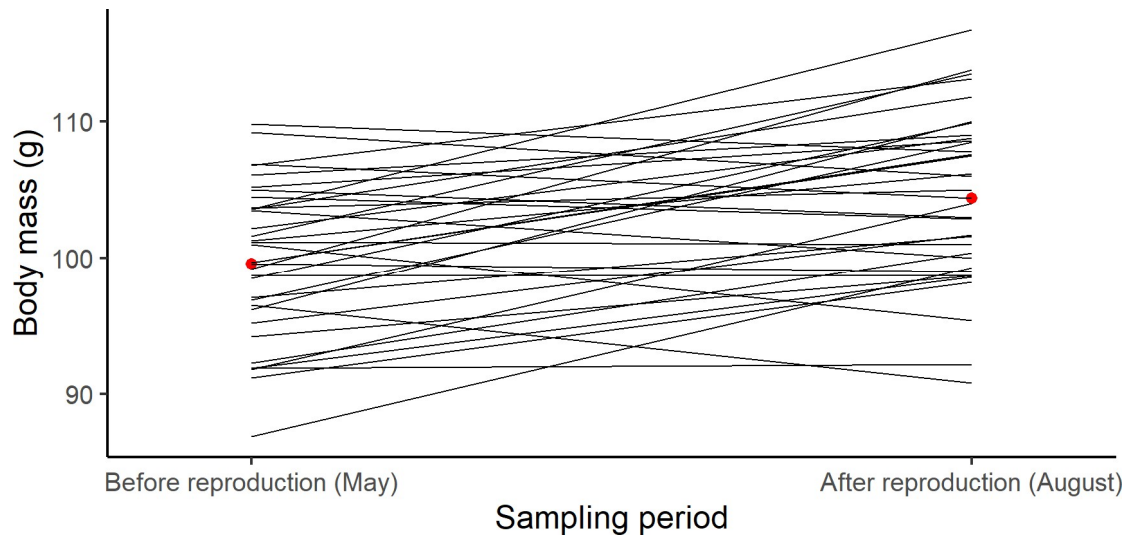
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**Table ESM1.4.** Distribution of ages in years across different colonies. Each cell shows the number of individuals for a given age and city (colony).

City	Age in years												
	2	3	4	5	6	7	8	9	10	11	12	13	14
Biel	1	0	5	0	0	3	0	1	0	2	0	0	0
Luzern	0	3	0	1	0	0	0	1	1	0	0	0	0
Solothurn	0	2	1	5	1	4	1	0	0	0	0	1	3

31

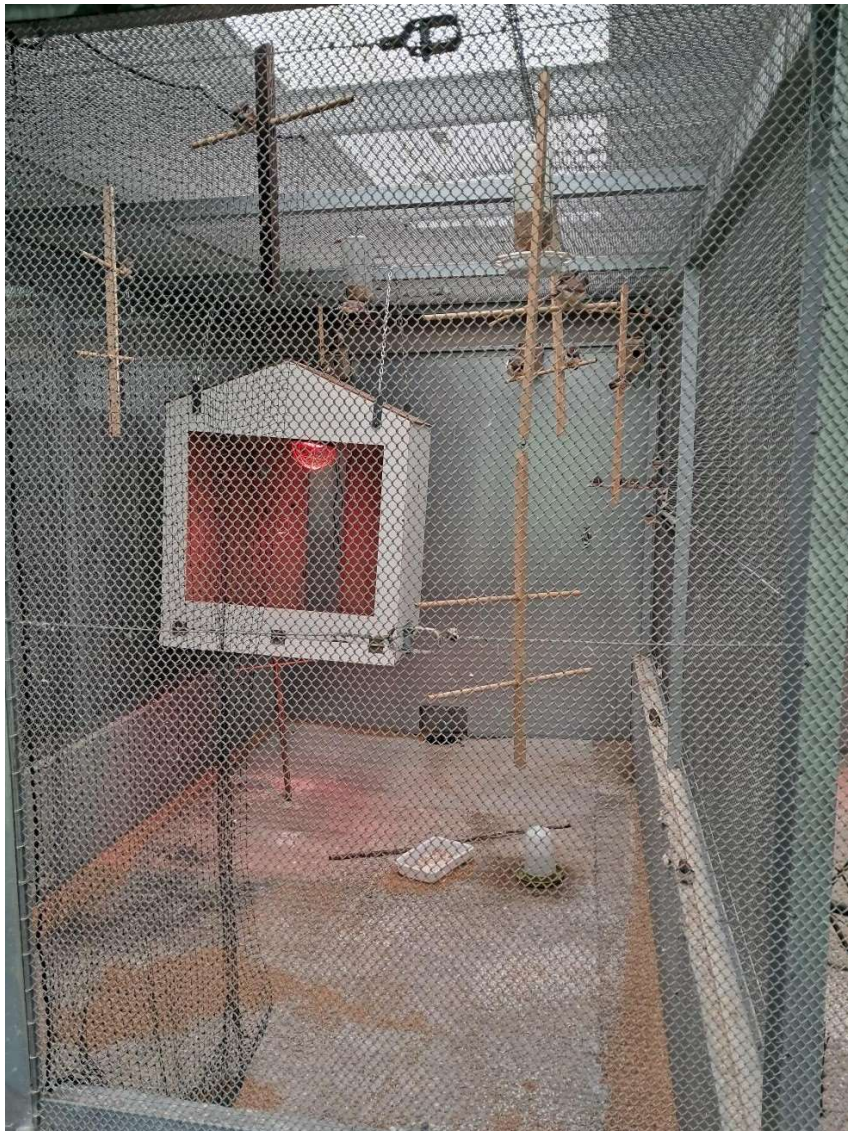


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33 **Figure ESM1.4.** Body mass variation from before (May) to after (August) reproduction in adult female Alpine swifts.  
34 Lines are describing the individual changes between May and August. Red dots represent mean values.

# Chapter 3

Experimental approach: relationship between glycaemia, glycation and AGEs with ageing traits in birds





## Expérience de supplémentation alimentaire chez les diamants mandarins

### Description générale de l'expérience et hypothèses de travail

Pour déterminer les effets du glucose sanguin, de la glycation des protéines et des produits avancés de glycation (AGE) sur le fitness des oiseaux en général, et notamment sur le vieillissement, nous avons exposé un groupe de diamants mandarins (*Taeniopygia guttata*) à un traitement par supplémentation, recevant soit du glucose, soit du méthylglyoxal (un composé dicarbonylé réactif favorisant la formation des AGE et contribuant aux pathologies semblables au diabète ; voir par ex. Turk, 2006 ; Moraru et al., 2018). Ainsi, nous devrions être en mesure d'explorer les mécanismes sous-jacents susceptibles de relier le glucose sanguin, le fitness et le vieillissement des oiseaux, comme le métabolisme et le stress oxydatif. Le traitement au glucose vise à augmenter la glycémie ou, du moins, à exposer les oiseaux à des pics plus élevés de glucose durant la phase d'absorption, tandis que le traitement au méthylglyoxal vise à accroître directement la formation d'AGE, contournant d'autres effets physiologiques du glucose sur l'oiseau, tels que des modifications du métabolisme ou du microbiote. Un groupe sans supplémentation sert non seulement de contrôle, pour déterminer si les suppléments affectent les paramètres mesurés, mais constitue également la première étude longitudinale sur le vieillissement des oiseaux en relation avec le glucose, la glycation et les AGE, comparant leurs variations à d'autres marqueurs du vieillissement déjà explorés chez les oiseaux, comme la longueur des télomères, le stress oxydatif ou le taux métabolique.

Nous nous attendons à une diminution de la survie dans les deux groupes de traitement par rapport au groupe de contrôle, avec des mécanismes potentiellement différents. Le glucose pourrait augmenter la glycation et donc la formation d'AGE, tandis que le méthylglyoxal devrait principalement augmenter les AGE directement produits par ce dernier, tels que le CEL (carboxyéthyl-lysine). De plus, il est supposé que le glucose augmenterait initialement le métabolisme, avec une élévation du quotient respiratoire (QR) due à une utilisation accrue des glucides, suivi d'un déclin plus rapide (c.-à-d. un

taux de vieillissement métabolique plus rapide) que le groupe de contrôle. Le méthylglyoxal, en revanche, ne devrait pas augmenter le métabolisme mais pourrait induire un déclin plus rapide. Les deux groupes de supplémentation devraient augmenter le stress oxydatif (et potentiellement réduire la longueur des télomères), mais le groupe au glucose devrait présenter un stress oxydatif plus élevé que le groupe au méthylglyoxal, en raison de l'activation de voies additionnelles (Brownlee, 2001).

Les traitements devraient accroître le taux de déclin des traits principaux liés au maintien homéostatique (c.-à-d. vieillissement physiologique), comme le métabolisme, la masse corporelle et la condition corporelle (donnés par les scores de muscle et de graisse), et augmenter les dommages (stress oxydatif, raccourcissement des télomères, niveaux d'apoptose). L'investissement dans la reproduction pourrait également être indiqué par un déclin de la coloration du bec, considéré comme un signal sexuel (Hill et al., 2023). Une diminution de la rougeur du bec (un facteur sélectionné par les femelles ; voir Simons et Verhulst, 2011) peut refléter une détérioration de la condition corporelle, étant liée à divers paramètres physiologiques et de fitness, chez les mâles comme chez les femelles (Simons et al., 2012 ; Romero-Haro et al., 2024). La coloration du bec diminue avec l'âge (Simons et al., 2016) et peut être affectée par divers stress (par ex. Alonso-Alvarez et al., 2004 ; Eraud et al., 2007 ; Goodchild et al., 2021). Les traitements pourraient aussi augmenter l'inflammation en raison d'une formation accrue d'AGE (comme chez Wein et al., 2020), évaluée ici par le ratio H/L (hétérophiles/lymphocytes) (voir par ex. Ewenson et al., 2001 ; Lentfer et al., 2015 ; Skwarska, 2018).

## Matériels et méthodes

### Protocole général

L'expérience a commencé le 21 septembre 2022 et s'est terminée le 25 septembre 2023. 90 diamants mandarins adultes (*Taeniopygia guttata* ; Vieillot, 1817 ; voir la section **espèce étudiée**) de différentes provenances (voir **Annexe 1, Tableau 1**), identifiés par des bagues de patte numérotées, ont été répartis de manière aléatoire en trois groupes : contrôle, supplémentation en glucose et supplémentation en méthylglyoxal. Chaque groupe comprenait 15 mâles et 15 femelles. La séparation des sexes n'était pas possible

en raison de contraintes d'espace. Ils ont été placés dans des volières extérieures (380 x 215 x 240 cm ; profondeur, largeur, hauteur) où la photopériode, la température et l'humidité variaient naturellement. Un thermomètre/hygromètre a été installé dans chaque volière pour enregistrer la température et l'humidité actuelles, minimales et maximales, avec une prise de mesures quotidienne dans l'après-midi. De la nourriture (un mélange de graines tropicales Deli Nature®), de l'eau (traitée ; voir ci-dessous), de l'os de seiche et du gravier ont été fournis en permanence.

Les traitements au glucose et au méthylglyoxal étaient administrés dans l'eau potable à raison de 50 g/L pour le glucose et d'environ 8,33 g/L pour le méthylglyoxal (7 mL de solution de méthylglyoxal Merck® à 40 % dans 993 mL d'eau du robinet), tandis que le groupe de contrôle ne recevait que de l'eau plate. Les quantités ont été déterminées par une étude pilote (voir **Annexe 2**), basée sur les taux de consommation d'eau mesurés, et les abreuvoirs étaient remplis une fois par semaine.

Des mesures trimestrielles (en août et novembre 2022, et en février, mai et août 2023) ont été effectuées pour évaluer plusieurs paramètres liés à la sénescence des oiseaux : la masse corporelle, la condition corporelle (scores de muscle et de graisse), le taux métabolique de repos (RMR) et le quotient respiratoire (QR) par respirométrie, la coloration du bec, la performance de vol, et divers autres paramètres sanguins (longueur des télomères, statut oxydatif, glycation des protéines plasmatiques, concentration plasmatique en AGE, profil immunologique, glucose, albumine, ions Ca<sup>+</sup>, Na<sup>+</sup>, K<sup>+</sup>, phosphates, acide urique, aspartate aminotransférase, protéines totales, globuline, créatine kinase et acides biliaires). Les procédures les moins intrusives (respirométrie, test de vol, pesée, évaluation de la coloration et de la condition physique) ont été réalisées à chaque session d'échantillonnage, tandis que les échantillons sanguins pour différents paramètres ont été ajustés de manière à ce que chaque paramètre soit mesuré au moins tous les six mois, et certains tous les trois mois (voir **Tableau 1**), avec deux séries différentes mais cohérentes de paramètres évaluées tous les trois mois. Toutes ces procédures ont duré 4 semaines au total lors de chaque cycle (voir **Tableau 2**), sauf dans les phases ultérieures, lorsque le nombre total d'oiseaux survivants était plus bas, auquel cas 3 semaines minimum étaient suffisantes. Les 3/4



semaines étaient consécutives sauf si des raisons d'organisation imposaient un autre aménagement.

**Tableau 1.** Calendrier des mesures effectuées. Les mesures en gris clair correspondent aux paramètres/procédures mesurés dans le sang, soit dans le plasma (jaune), dans les cellules (bleu), ou dans les deux (vert). Les zones colorées indiquent les périodes pendant lesquelles ces mesures ont été réalisées.

Mesure	Août 2022	Novembre 2022	Février 2023	Mai 2023	Août 2023
Test de vol					
Respirométrie					
Coloration					
Condition physique					
Prélèvement sanguin					
AGE et glycation					
Stress oxydatif					
Téломères					
Hématologie					
Cytométrie					

**Tableau 2.** Répartition des différentes procédures au cours des semaines d'échantillonnage. Le prélèvement sanguin a été effectué en semaines 2 et 3 lorsque le nombre d'oiseaux était réduit au point de ne plus nécessiter une deuxième semaine pour les autres procédures.

Semaine d'échantillonnage	Mardi	Mercredi	Jeudi	Vendredi
Semaine 1	Masse corporelle et condition physique, respirométrie, coloration & test de vol	Masse corporelle et condition physique, respirométrie, coloration & test de vol	Masse corporelle et condition physique, respirométrie, coloration & test de vol	Masse corporelle et condition physique, respirométrie, coloration & test de vol
Semaine 2	Masse corporelle et condition physique, respirométrie, coloration & test de vol	Masse corporelle et condition physique, respirométrie, coloration & test de vol	Masse corporelle et condition physique, respirométrie, coloration & test de vol	Masse corporelle et condition physique, respirométrie, coloration & test de vol
Semaine 3	Prélèvement sanguin : AGE + glycation ou hématologie	Prélèvement sanguin : AGE + glycation ou hématologie	Prélèvement sanguin : AGE + glycation ou hématologie	
Semaine 4	Prélèvement sanguin : Téломères, cytométrie et stress oxydatif (si nécessaire)	Prélèvement sanguin : Téломères, cytométrie et stress oxydatif (si nécessaire)	Prélèvement sanguin : Téломères, cytométrie et stress oxydatif (si nécessaire)	

Les oiseaux ont été placés en intérieur dans une pièce à 20 °C avec une photopériode semblable à celle qu'ils connaissaient à l'extérieur le jour précédant chaque mesure pour plusieurs raisons :

1. Contrôler leur état d'alimentation (ils devaient être à jeun avant les prélèvements sanguins et en état postprandial pour la respirométrie).
2. Maintenir constantes les conditions environnementales juste avant les mesures, quelle que soit la saison.
3. Réduire le stress thermique lors de leur entrée dans l'incubateur de respirométrie (à 35 °C). Il n'était pas possible d'abaisser davantage la différence de température, car les pièces de l'animalerie ne pouvaient pas être chauffées jusqu'à 35 °C.
4. Pour des raisons d'organisation, les oiseaux ne pouvaient pas être capturés le jour même des prélèvements.
5. Éloigner le processus de capture des mesures permettait de réduire le stress subi par les oiseaux juste avant celles-ci.

En général, les oiseaux subissaient les différentes mesures au cours des deux premières semaines dans l'ordre suivant : premièrement la respirométrie, immédiatement après l'évaluation de la coloration, et le test de vol le matin suivant. À cet effet, 12 oiseaux ont été sélectionnés aléatoirement avec la fonction *sample()* de R, de manière à éviter tout biais de capture dans la procédure de sélection. Ils étaient ensuite capturés la veille des mesures et placés dans de petites cages (40,5 cm x 54,5 cm x 29,5 cm) pour la nuit, avec un accès libre à l'eau et à la nourriture, qui étaient retirées à 9 h le lendemain matin. La respirométrie se déroulait l'après-midi, de 14 h à 18 h (voir section sur la **Respirométrie**). Les scores de muscle et de graisse étaient évalués avant la respirométrie, tandis que la masse corporelle et la température cloacale étaient mesurées deux fois, juste avant et après la respirométrie. La coloration était évaluée immédiatement après la respirométrie pour chaque oiseau (voir section **Coloration**). Enfin, ces oiseaux restaient dans la même pièce et dans les mêmes conditions que la nuit précédente, en vue du test de vol le matin suivant. Les vendredis, cependant, les oiseaux n'étaient pas laissés à l'intérieur, mais relâchés ce soir-là dans leurs volières. Le mardi matin, comme aucune

respirométrie n'avait eu lieu la veille, le test de vol était effectué sur des oiseaux capturés et placés à l'intérieur la veille spécialement pour cela (24 oiseaux étaient donc capturés les lundis), et ceux-ci étaient les individus destinés aux mesures de condition physique, de respirométrie et de coloration le vendredi suivant.

Pour les prélèvements sanguins, les oiseaux étaient également gardés à l'intérieur la veille, mais sans nourriture, afin qu'ils soient à jeun lors des mesures. Dans ce cas, une volière correspondant à un traitement complet était capturée chaque jour (ou plus si le nombre total d'oiseaux n'était pas trop grand) et ils étaient relâchés quelques heures après le prélèvement. Quelques plumes étaient arrachées de la poitrine des oiseaux au moment du prélèvement sanguin et conservées dans des tubes Eppendorf® pour des analyses futures de corticostérone (non montrées ici).

## **Espèce d'étude**

Le diamant mandarin (*Taeniopygia guttata*; Vieillot, 1817) (**Figure 1**) est un oiseau de l'ordre des Passeriformes, de la famille des Estrildidés, vivant en Australie et dans les îles de la Sonde en Indonésie. Il en existe deux sous-espèces : l'une de la Sonde et l'autre d'Australie, cette dernière étant celle à l'origine de toutes les lignées domestiques actuellement utilisées en laboratoire dans le monde. C'est un petit oiseau coloré, très grégaire et granivore, qui est, au moins pour la sous-espèce australienne plus étudiée, adapté aux déserts. Cette espèce a commencé à devenir un oiseau de cage courant au XIXe siècle, bien qu'il y ait encore eu un apport génétique provenant des populations sauvages, jusqu'à ce que son exportation d'Australie, dont viennent toutes les populations captives d'origine, soit interdite en 1960 (voir Zann 1995a). En raison de cette tradition d'élevage en captivité et de sa facilité à se reproduire, le diamant mandarin est depuis longtemps utilisé comme espèce modèle chez les oiseaux, notamment pour l'étude de la coloration et de la sélection sexuelle (voir section **Description générale de l'expérience et hypothèses de travail**), étant donné son dimorphisme sexuel (Morris, 1954), son apprentissage vocal avec ses bases neurodéveloppementales (Mello, 2014), la génétique quantitative (Barbara Tschirren & Postma, 2010), la génomique (e.g. Forstmeier et al., 2010) et l'écophysiologie, explorant

par exemple les fondements physiologiques du coût de la reproduction (e.g. Veasey et al., 2001; Bertrand et al., 2006) ou les conséquences sur le fitness à long terme des conditions de vie précoces (e.g. Criscuolo et al., 2008; Tschirren et al., 2009; Criscuolo et al., 2011; Crino et al., 2014; Reichert et al., 2014; Reichert et al., 2015; Briga, 2016). Le fait que son génome soit séquencé (Warren et al., 2010) facilite également son utilisation dans des études d'omiques, bien que les recherches détaillées en physiologie et développement soient encore limitées par rapport à d'autres modèles d'oiseaux, comme le poulet (*Gallus gallus*) (voir e.g. Flores-Santin & Burggren, 2021) ou la caille japonaise (*Coturnix japonica*) (Huss et al., 2014). Enfin, certaines caractéristiques facilitent son élevage en captivité, comme sa nature grégaire, qui permet de le maintenir en grands groupes dans des volières. Cependant, il n'est pas à proprement parler une espèce sociale, mais il se reproduit en couples monogames, ce qui permet aussi de l'héberger en couples dans de petites cages. Fait tout aussi important, il est un reproducteur opportuniste qui se reproduit normalement lorsque les conditions sont adéquates (normalement lorsqu'il pleut dans son environnement naturel), atteignant une maturité sexuelle assez tôt, même pour un petit passereau tropical. Les mâles peuvent produire des spermatozoïdes vers 70 jours, et les femelles pondre vers 90 jours (bien que les individus sauvages atteignent la maturité encore plus tôt que les domestiques, même lorsqu'ils sont mis en captivité), ce qui leur permet de produire plusieurs nichées par an, même dès leur première année de vie, avec une taille moyenne de ponte de 5 œufs (entre 2 et 7 œufs) (voir Zann 1995b), ce qui les rend suffisamment prolifiques pour maintenir facilement des populations captives desquelles des animaux expérimentaux peuvent être prélevés.



**Figure 1.** Diamants mandarins (*Taeniopygia guttata*; Vieillot, 1817) dans une cage de l'animalerie avant de subir les mesures décrites dans cette expérience. Gauche : femelle ; droite : mâle.

Dans notre cas, le choix de cette espèce pour l'expérience reposait sur les raisons évoquées précédemment, ainsi que sur le fait qu'une population captive est maintenue en permanence au DEPE (Département d'Écologie, Physiologie et Ethologie) à Strasbourg, où le travail a été réalisé. Diverses lignées existent, et dans notre cas, les oiseaux provenaient de plusieurs sources : à la fois des oiseaux déjà présents dans les installations animales du DEPE et d'autres apportés au cours de l'année précédant le début de l'expérimentation (2021-2022), principalement de l'Institut Max Planck d'Ornithologie, actuellement partie de l'Institut Max Planck d'Intelligence Biologique (Pöcking-Seewiesen, Bavière, Allemagne), comme indiqué en **Annexe 1 (Tableau 2)**, tous issus de lignées européennes.

# Dietary supplementation experiment with zebra finches

## General description of the experiment and working hypotheses

In order to determine the effects of blood glucose, protein glycation and AGE (Advanced Glycation End-products) effects on birds' fitness amply and on ageing particularly, we exposed a group of zebra finches (*Taeniopygia guttata*) to a supplementation experiment in which they were given either glucose or methylglyoxal (a reactive dicarbonylic compound exerting AGE formation and contributing to diabetes-like pathologies; see e.g. (Turk, 2006; Moraru et al., 2018)). This way, we should be able to explore the different underlying mechanisms potentially mediating blood glucose levels and fitness and ageing in birds, as metabolism and oxidative stress. With this in mind, while the glucose supplementation was intended to increase blood glucose levels, or at least exposing the birds to higher absorptive phase glucose peaks, the methylglyoxal treatment is intended to increase the AGE formation bypassing other effects glucose can have on the birds' physiology through alterations in, for example, metabolism or microbiota. A non-supplemented group would not only serve us as a control for the experiment, to know if the supplementations affected the measured parameters, but it also constitutes the first longitudinal study of the effects of ageing on birds' glucose, glycation and AGE, comparing the variation on those with other ageing parameters that have been explored in the past in birds, as telomere length, oxidative stress or metabolic rate.

This way, we expected both treatments to decrease survival in comparison to the control group, but potentially mediating by different mechanisms. While glucose would increase glycation and therefore potentially AGE formation (see **AGE section**), methylglyoxal would be expected to increase mainly AGE, principally those produced directly by it, as CEL (carboxyethyl lysine). Moreover, glucose is hypothesized to increase metabolism initially, with an elevation in RER (Respiratory Exchange Ratio) due to a higher carbohydrate usage, followed by a more rapid decline (i.e. faster metabolic ageing rate) than the control group. Methylglyoxal, nevertheless, would not be expected to increase metabolism and would only induce, in any case, a higher decline on it (higher ageing rate) than in the control group. Although both supplementation groups should increase oxidative stress (potentially leading to higher telomere shortening), the glucose group should show a higher oxidative stress than methylglyoxal group, as it would affect further pathways (Brownlee, 2001).

The treatments are generally hypothesized to increase the rate of decline of main traits related to homeostatic maintenance (i.e. physiological ageing), as metabolism, body mass and body condition (as given by muscle and fat scores), and increase the damage (oxidative stress, telomeres shortening, apoptosis levels). Investment in reproduction can also be indirectly shown by a decline in beak coloration, which is considered an index sexual signal (Hill et al., 2023), which means that its decrease in redness (a factor selected by females; see Simons and Verhulst, 2011 for a meta-analysis) may reflect a pattern of body condition deterioration, as it relates to several physiological (e.g. Birkhead et al., 1998) and fitness traits, in both males and females (Simons et al., 2012; Romero-Haro et al., 2024), and it has previously been reported to show age decline (Simons et al., 2016) and to be negatively impacted by multiple stressors (e.g. Alonso-Alvarez et al., 2004; Eraud et al., 2007; Goodchild et al., 2021). Treatments may also increase inflammation through an increased AGE formation (as in Wein et al. 2020), that here we assess as H/L ratio (Heterophiles/Lymphocytes) (see e.g. Ewenson et al., 2001; Lentfer et al.,

2015; Skwarska, 2018). Finally, body composition was assessed, measuring the amount of lipids in the skeletal muscles (represented by pectoral muscles), liver and carcass (the rest of the body), controlling for dry mass, to test for a higher fat deposition in the treated individuals, leading to hepatic steatosis, like in NAFLD (Non-Alcoholic Fatty Liver Disease; Chao et al., 2019; Powell et al., 2021) or to an excess of ectopic lipid accumulation (here assessed in muscles), which could lead to an impaired metabolic state, similar to which is observed in diabetes and metabolic syndrome (Grundy, 2016). This could be mediated by an increase in sugar intake in the glucose group and by an increase in the activity of Fatty Acid Synthase (FAS) by methylglyoxal (Garrido et al., 2015; Moraru et al., 2018). Water content was equally assessed to control for potential dehydration effects caused by the treatments in relation to the differential drinking rates (see **pilot study section**).

## Materials and methods

### General protocol

The experiment began on the 21<sup>st</sup> of September 2022 and finished the 25<sup>th</sup> of September 2023. 90 adult zebra finches (*Taeniopygia guttata*; Vieillot, 1817; see **study species section**) from different provenances (see **Appendix 1, Table 1**), and identified with numbered leg rings, were randomly assigned to 3 groups: control, glucose and methylglyoxal supplementation treatments. Each group consisted of 15 males and 15 females (sex differentiated given their sexual dimorphism in colouration; see **Study species section**) from which the age groups (young and old individuals, see **Appendix 1**) were the most equally possible represented. Separation of sexes was not possible due to space constraints. They were placed into outdoors aviaries (380 x 215 x 240 cm; depth, width, height), so that the photoperiod, temperature and humidity varied naturally. A thermometer/hygrometer was placed in each aviary to register current, minimum and maximum temperature and humidity, taking measurements once a day in the afternoon. Food, consisting into a commercial seed mix (Deli Nature<sup>®</sup> for tropical finches), water (tap water with the adequate treatment, see below) cuttlebone and grate were provided *ad libitum*. Both food and water were provided in one feeder each, placed on the floor, so that all the birds could have access to them easily, regardless of their health status. A shelter with an infrared light working in permanence most of the year, except late spring and summer, was set up in each aviary for protecting birds against cold. The lateral fences that delimit each aviary were coated with cardboard in winter and bedsheets during summer, so that birds from one group could not see or interact with the contiguous group or be altered by the researchers or technicians when going inside other cages, and to protect them from cold in winter. The aviaries included several roosting perches and branches and, except from late summer, the floor was covered by twigs and tree bark, contributing to environmental enrichment. Whenever an individual died during the duration of the experiment, the body was collected, weighed and stored at -20 °C until dissection could be performed for body composition analyses. The date of death was noted for survival analyses to be performed, along comments on the possible causes of it or related circumstances.

The glucose and methylglyoxal treatments were provided in the drinking water at 50 g/L for the case of glucose and about 8.33 g/L for methylglyoxal (7 mL of Merck<sup>®</sup> methylglyoxal solution of 40% in water diluted into 993 mL of tap water), while the control group received just still water. The quantities were determined by a pilot study (see **Pilot study section** and **Appendix 2**) based on measured drinking rates, and the water feeders were refilled once a week.

Every three months (i.e. August and November 2022 and February, May and August 2023; see **Table 1**), several parameters related to birds' senescence were measured: body mass and body condition (fat and muscle scores), resting metabolic rate (RMR hereon) and respiratory exchange ratio (RER hereon) by the means of respirometry, beak coloration, flying performance, and numerous others assessed in the blood (i.e. telomere length, oxidative status, plasma proteins glycation, plasma Advanced Glycation End-products (AGE) concentration, immunological profile, glucose, albumin, Ca<sup>+</sup>, Na<sup>+</sup>, K<sup>+</sup>, phosphates, uric acid, aspartate aminotransferase, total proteins, globulin, creatine kinase and bile acids). The less intrusive procedures (respirometry, flying test, weighing, coloration and body condition assessment) were performed every sampling session, while blood samples for different parameters were adjusted so that each parameter is measured at least every six months and some every three (see **Table 1**), with two different but consistent set of them assessed every three months. All these procedures lasted 4 weeks in total during each round (see **Table 2**), unless it could be shortened, as it was the case in later stages when the total number of surviving birds was lower, so a minimum of 3 weeks were enough. The 3/4 weeks were consecutive unless organizational reasons obliged to do it otherwise.

**Table 1.** Schedule of the measures performed. The ones in light grey correspond to procedure/parameters measured in blood, either plasma (yellow), cells (blue) or both (green). The filled areas indicate that such procedure/measurement was done during such sampling period.

Measurement	August 2022	November 2022	February 2023	May 2023	August 2023
Flying test					
Respirometry					
Coloration					
Body condition					
Blood sampling					
AGE and glycation					
Oxidative stress					
Telomeres					
Haematology					
Cytometry					

**Table 2.** Scheme indicating the way different procedures were distributed across the sampling weeks. Blood sampling was performed on weeks 2 and 3 when the number of birds became reduced enough to not need a second week for the other procedures.

Sampling week	Tuesday	Wednesday	Thursday	Friday
Week 1	Body mass and body condition, respirometry, coloration & flying test	Body mass and body condition, respirometry, coloration & flying test	Body mass and body condition, respirometry, coloration & flying test	Body mass and body condition, respirometry, coloration & flying test
Week 2	Body mass and body condition, respirometry, coloration & flying test	Body mass and body condition, respirometry, coloration & flying test	Body mass and body condition, respirometry, coloration & flying test	Body mass and body condition, respirometry, coloration & flying test
Week 3	Blood sampling: AGE + glycation or haematology	Blood sampling AGE + glycation or haematology	Blood sampling AGE + glycation or haematology	
Week 4	Blood sampling: Telomeres, cytometry and oxidative stress (when corresponding)	Blood sampling: Telomeres, cytometry and oxidative stress (when corresponding)	Blood sampling: Telomeres, cytometry and oxidative stress (when corresponding)	



Birds were put indoors in a room at 20°C and with a photoperiod similar to what they were experiencing outside the day before each measurement for several reasons:

1. Control the feeding state (they should be fasting before blood sampling and in postprandial state for respirometry).
2. Keep constant the across seasons environmental conditions right before the measurements.
3. Decrease possible thermal stress when entering into the incubator for respirometry (at 35 °C). Lower difference of temperature was not achievable as the animal facility rooms could not be heated up to 35 °C.
4. For organizational reasons, the birds could not be captured the same day of the sampling.
5. Distancing the capturing process from the measurements would reduce the stress suffered by the birds right before those took place.

In general, birds underwent through the different measurements of the two first weeks in the following order: 1<sup>st</sup> respirometry, right after coloration and the following morning flying test.

For that, 12 birds were randomly selected with R *sample()* function, so that no capture bias existed in the selection procedure, then captured the day before the measures and let indoors in small cages (40.5 cm x 54.5 cm x 29.5 cm) overnight, with ad libitum access to water and food, to be removed at 9:00 a.m. Respirometry took place in the afternoon, from 14:00 to 18:00 (see **Respirometry** section). Muscle and fat scores were assessed before respirometry, while body mass and cloacal temperature were assessed twice, right before and after respirometry. Coloration was assessed right after respirometry for each bird (see **Coloration** section). Finally, these birds were left in the same room and conditions they spent the previous night, for flying test to be performed on them the following morning. On Fridays, however, birds were not let inside, but released that evening into their aviaries. Also, on Tuesday morning, as no respirometry would have been performed the day before, flying test was done on birds captured and left inside the previous evening specifically for this purpose (so that 24 birds were captured on Mondays), and these ones were the individuals to be allocated for body condition, respirometry and coloration on the following Friday.

For blood sampling birds were also kept inside the day before but without food so that they were fasting when being measured. In this case, at least a whole treatment cage was captured every day (more when the total number of birds was not too big) and they would be released a few hours after the sampling. Some feathers were plucked from the chest of the birds when doing blood sampling and stored in Eppendorf® tubes for future corticosterone analyses (not shown here).

This study followed all the legal considerations, with the ethic authorisations from the French Ministry of Secondary Education and Research, APAFIS #32475-2021071910541808 v5. The research was funded by an ANR grant (AGEs – ANR21-CE02-0009).

## **Study species**

Zebra finches (*Taeniopygia guttata*; Vieillot, 1817) (**Figure 1**) are Passeriformes' birds from the Estrildidae family, inhabiting Australia and the Lesser Sunda Islands in Indonesia. There exists two subspecies of it, one from the Sunda and the other from Australia, being the latter the ones that gave rise to all the domestic breeds that are currently used in laboratories around the world. It is a small, colourful, highly gregarious granivorous bird, that is also, at least in the case of the much more studied Australian subspecies, a desert dweller. This species started becoming an

usual cage bird during the XIX<sup>th</sup> century, although still with some genetic input from the wild, until its export from Australia, from whose subspecies these captive populations come all originally, was banned in 1960 (see Zann 1995a). Partly because of this tradition as a pet animal and its ease for captive breeding, zebra finches have long been used as a model species within birds, in particular for the study of coloration and sexual selection (see **General description of the experiment and working hypotheses** section), given their sexual dimorphism (Morris, 1954), vocal learning and its underlying neurodevelopmental pathways (Mello, 2014), quantitative genetics (Barbara Tschirren & Postma, 2010), genomics (e.g. Forstmeier et al., 2010) and ecophysiology, exploring e.g. the physiological underpinnings of the cost of reproduction (e.g. Veasey et al., 2001; Bertrand et al., 2006), or the long-term fitness consequences of early life conditions (e.g. Criscuolo et al., 2008; Tschirren et al., 2009; Criscuolo et al., 2011; Crino et al., 2014; Reichert et al., 2014; Reichert et al., 2015; Briga, 2016). Also, the fact that its genome is sequenced (Warren et al., 2010) facilitates its usage in omics studies, although detailed physiological and developmental research is still reduced in comparison with other bird models, like chicken (*Gallus gallus*) (see e.g. Flores-Santin & Burggren, 2021) or Japanese quail (*Coturnix japonica*) (Huss et al., 2014). Finally, some of their characteristics facilitate their captive rearing, as their gregarious nature, which allows their stocking in big flocks in aviaries. However, they are not strictly speaking a social species, but breed in monogamous pairs, what also affords housing them in couples in small cages. Not less importantly, they are opportunistic breeders that would normally reproduce whenever the conditions are adequate (normally when it rains in their natural environment), reaching sexual maturity quite early even for small tropical passerines, males being able to produce sperm at about 70 days, and in females laying at around 90 (although wild individuals mature even earlier than domestic ones, still when put in captivity), which makes them able to produce several broods per year, even in their first year of life, with a modal clutch of 5 (2-7 eggs) (see Zann 1995b), making them prolific enough to easily sustain captive populations from whose stock experimental animals can be taken.



**Figure 1.** Zebra finches (*Taeniopygia guttata*; Vieillot, 1817) in a cage inside of the animal facilities before undergoing measurements within the presently described experiment. Left: female; right: male.

In our case, the selection of this species for the experiment was based on the previously mentioned reasons, together with the fact that a captive population is permanently kept at the DEPE (Department of Ecology Physiology and Ethology) in Strasbourg, where the work was performed. Various breeds exist, and in our case, birds were obtained from several origins, both from birds already present in the DEPE's animal facilities and brought from other sources during

the year previous to the start of the experimentation (2021-2022), mainly from the Max Plank Institute for Ornithology, currently part of the Max Planck Institute for Biological Intelligence (Pöcking-Seewiesen, Bavaria, Germany) as indicated in the **Appendix 1 (Table 2)**, and all from European pedigrees.

## **Flight performance measurements**

What is described in this section constitutes an adaptation from Reichert et al., 2015.

Flight performance, which is directly linked to an individual's capacity to forage and escape predators, is used as a proxy for individual maintenance (Lindhe Norberg, 2002). It was measured using vertical take-off when alarmed, with a similar set up to that used in previous studies (Criscuolo et al., 2011; Kullberg et al., 2002; Reichert et al., 2015). Each bird is released on a perch situated at 20 cm from the ground, at the base of a transparent vertical plastic tube covered by a metallic grid to make it possible for the bird to see its borders and not to try to escape through it (**Figure 2**). At the top of the flying tube (120 cm from the first perch), there is a perch where the bird can be collected after each flight. The birds were released five times in the base of the tube and allowed to rest for 30 seconds between each flight. If the bird did not complete any flight after 5 min, or spent more than 5 min in total without flying during all the trials, it was recorded as "no flight" and the trial was terminated to avoid unnecessary stress for the animal. During the experimental trial, all flights were recorded with a camera (Canon EOS 7D, see **colouration section**) or, on its defect, with a mobile phone. To determine flight speed, the videos were observed and the time the birds take to cover a distance of 40 cm is calculated. This measure is made by counting the number of video frames (each frame of the Canon EOS 7D covering 0.017 s) between two marks on the tube at 40 and 80 cm height. Flight speed is calculated with the formula:  $V=d/[F*0.017]$  where  $d$  is the distance between the two marks (40 cm),  $F$  is the number of frames the bird has spent between both marks. The fastest of the five flights is taken as a measurement of the bird's maximum escape flight ability (Kullberg et al., 2002). The time to start flying is also measured. 12 randomly sampled (by the means of *R sample()* function) birds that had been kept indoors in small cages (40.5 cm x54.5 cm x29.5 cm) during the previous night were measured each day (see **general protocol**) on the morning (Tuesday-Friday), from 9:00 to 12:00 approximately. They were released afterwards in their corresponding outdoors aviaries.



**Figure 2.** Tube used for flying test. The bird is put in the bottom through the curtain and startled by clapping, making it fly up to the top perch. The speed is then calculated by the number of frames spent between the marks.

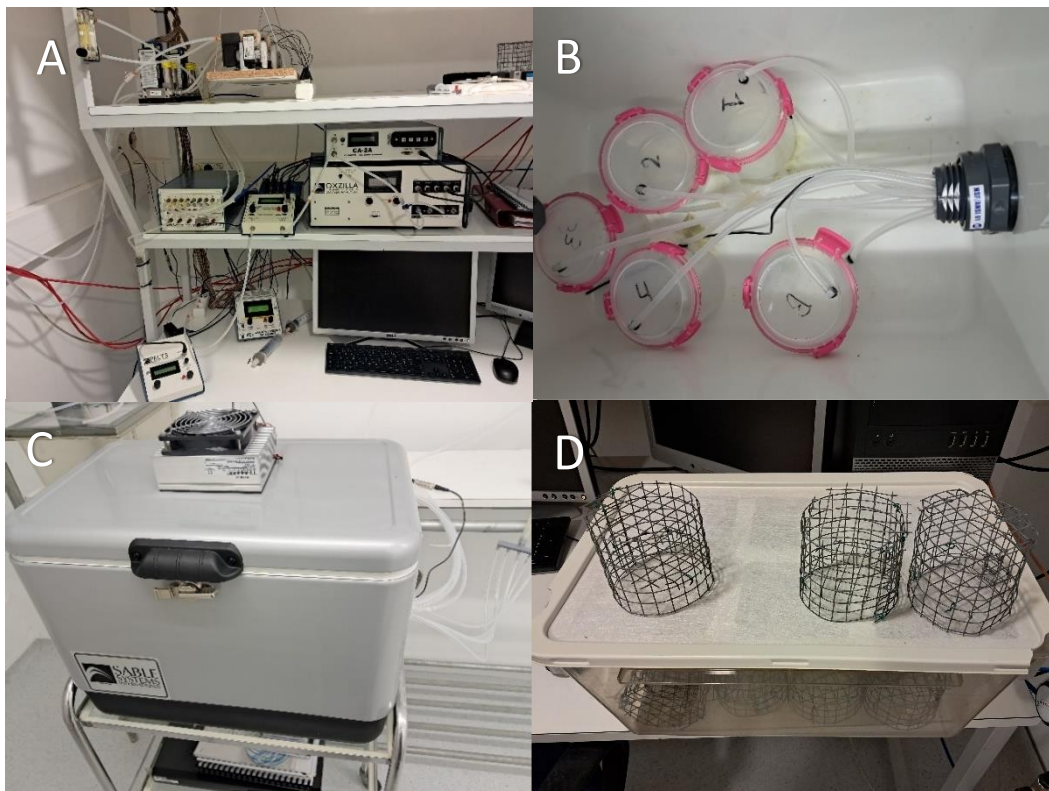
## **Respirometry**

Respirometry is a methodology that allows metabolism to be assessed indirectly by the means of changes on gases concentrations in the air around the organism (**Lighton 2008**). In our case, H<sub>2</sub>O, CO<sub>2</sub>, and O<sub>2</sub> (in that order) are measured by pull mode flow-through respirometry with a Sable System<sup>®</sup> respirometry device (**Figure 3.A**). A mathematical correction was performed on O<sub>2</sub> and CO<sub>2</sub> values, to control for the water vapour dilution effect (Equation 8.6 from Lighton 2008). That allows us to obtain not only a value of oxygen consumption equivalent to Basal Metabolic Rate (BMR), but also a Respiratory Exchange Ratio (RER hereon), defined as whole body CO<sub>2</sub> production/O<sub>2</sub> consumption, and that, to the extent that it can be used as a proxy of RQ (Respiratory Quotient, the same but for the portion of gases corresponding to catabolism) constitutes an index of the substrate being consumed (i.e. carbohydrates, proteins or lipids), and therefore the metabolic state of the animal (Péronnet and Massicotte, 1991).

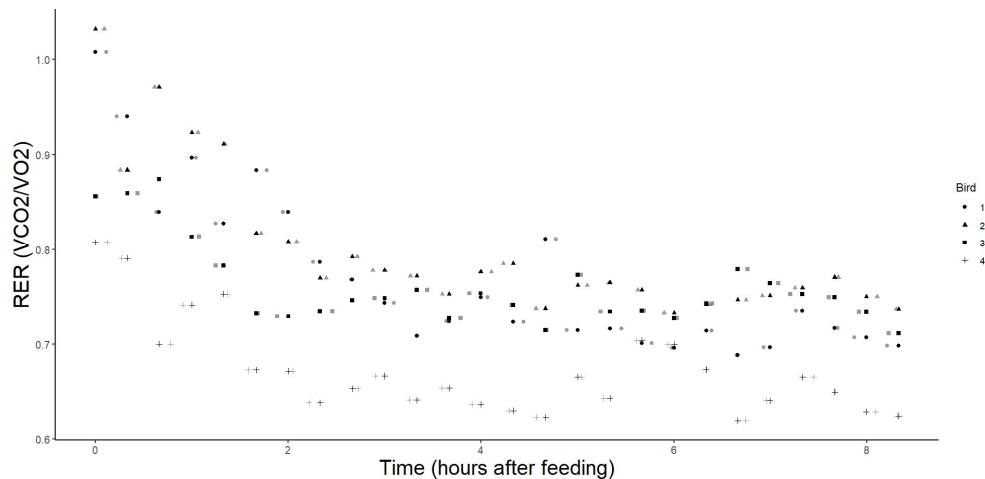
In order to measure BMR properly, it must be done at the thermoneutral zone, which is between 33 °C and 38°C in zebra finches (Calder 1964; Briga and Verhulst 2017; Wojciechowski et al., 2021) in post-absorptive state (determined for zebra finches as described below), in resting but awake individuals (McNab 1997). Even if we tried to meet these criteria, it is usually heavily argued if BMR is achievable, as some authors inquire that an actual metabolic nadir has to be reached, often after the animal has spent some time inside the metabolic chamber, and assuming it is not growing or investing any energy into reproduction (the latter not being totally controllable in our case, particularly for females, that may be producing eggs at some parts of the experiment), in order to call the measurement BMR, so we will call it Resting Metabolic Rate (RMR) here on. This RMR was calculated using equation (3) from Lighton et al. (1987), modified by dividing the VO<sub>2</sub> by 60 to convert it to mL/s and then report the RMR in Watts.

For doing these measures, the air is taken at a flow rate of 650 ml/min from a respirometry room that is at around 20° C, and it goes through the respirometry chambers (consisting of 530 mL Sistema<sup>®</sup> ToGo<sup>™</sup> plastic boxes with hermetic closing; **Figure 3.B**) placed inside of a Sable System<sup>®</sup> temperature controlling cabinet set at 35°C (see **Figure 3.C**). The air is pulled by five pumps (one per chamber) placed after those with their corresponding flow-meter before the pump to determine the excurrent flow. The respirometry system was turned on several hours before starting the measurement in order to reach the adequate stationary state needed for the sensors to work properly, and the temperature cabinet was turned on about an hour before the start of the measurements to reach the adequate temperature. Humidity was controlled by drying the air up to a level always lower than 10% of water vapour saturation entering into the chambers with a single filter used for all of them placed upwards the chambers and containing drierite or silica gel, and soda lime to deplete CO<sub>2</sub>, as these dryer agents can react with CO<sub>2</sub> in the air, capturing it and later on releasing it. These were placed in the following order (in the sense of the flow): drier, soda lime and again the drying agent, as soda lime produces water when reacting with CO<sub>2</sub>. Cotton wool or fabric filters were placed between these layers to avoid the mixing of the products. Furthermore, the bottom of the chambers was covered with mineral oil, to avoid the water released by the faeces to affect differently the humidity levels inside the chambers. All of this allowed to keep similar humidity conditions across seasons for the measurements to be more comparable. The birds were placed into hand-made wire cages (**Figure 3.D**) inside the chambers so that they were not directly in contact with the oil, and also to facilitate their handling and rapid exchange from the system. The filter was changed when the colour indicators of the drier and the soda lime had turned pink/violet, showing they were saturated and drierite/silica gel was regenerated every day putting it in a Plexiglass tray for 2 hours inside an oven at 210° C.

12 birds randomly sampled by R (see general protocol) were placed indoors in small cages (40.5 cm x54.5 cm x29.5 cm) at 20°C and the same photoperiod they were experiencing outside the day before to avoid thermal stress and maintain always the same conditions right before the measurement, regardless of outdoor temperature, which would vary with the season (see **general protocol**). They were provided unrestricted food and still water in the cages, but the food was removed at 9:00 in the morning, in order for them to be in postprandial state when measured in the afternoon. This was determined by a preliminary test in which the  $O_2$  consumption and RER were measured in a group of 4 birds from 9:00 to 18:00, showing a reduction from a RER of about 1 (representing generally carbohydrates catabolism and associated with absorptive state) at the start up to around 0.7 (representing mainly fat catabolism and associated with fasting) (see e.g. Walsberg and Wolf, 1995; Mccue et al., 2011) after about 3 hours of measurement (**Figure 4**). As occasional birds were detected storing seeds in their crops up to about 3 hours after feeding, we finally set a period of at least 4 hours of fasting before metabolic assessments. The room in which respirometry is performed is separated by a wall from the respirometry system to decrease the perturbation to the animals and it was kept in the dark during the measurements.



**Figure 3.A.** Sable System<sup>®</sup> respirometry system with **B** chambers within the **C** temperature controlling cabinet. **D.** Wire cages to restrain the birds during the measurement.



**Figure 4.** Respiratory Exchange Ratio variation over time from a preliminary test on a group of 4 zebra finches. Birds were placed in a cage in the indoors animal facilities and given free access to food and water, and then put into a respirometry chamber to measure RMR and RER.

In total, 3 groups of 4 birds each were measured every day, starting at around 14:00 for the first group. Each round lasted one hour, comprising two bouts of 30 min each, i.e. 6 min for each individual and for a baseline (divided into two spans of 3 min at the beginning and end of each round), with a multiplexer programmed to automatically change the sampling between different boxes. The baselining was done by measuring air going through a cage put inside the cabinet without any bird in it. Birds were given 15 minutes of habituation inside of the chambers before starting the measure, which also served for the temperature and humidity inside the chambers to return to their adequate values.

The data obtained from these measures was processed by an Expedata (Sable System® software for respirometry data processing) macro correcting the lag for each channel, the water vapour dilution and performing the baseline correction. The average O<sub>2</sub> and CO<sub>2</sub> of the most stable 180 seconds within each 6 min measurement spans of each cycle were calculated (determined automatically by the software) and averaged to be used as the consumption values for those birds. An average RER was then calculated from those values.

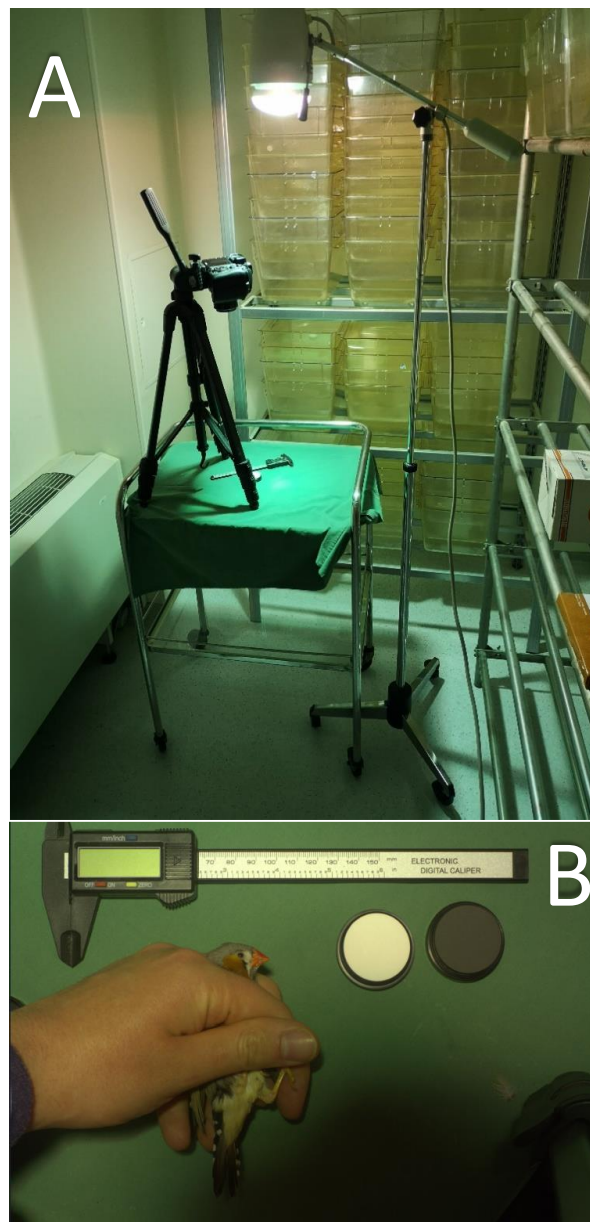
Right before and after introducing the birds inside the chambers we measured body mass to the nearest 0.1 g by weighing the birds in a Sartorius® scale and cloacal temperature with a testo® 925 thermometer coupled with a Type K probe, introducing it approx. 1 cm into the cloaca, to be able to adjust oxygen consumption to mass (as average mass from both measures) and control for possible thermal stress. Body condition parameters were also measured before (see **body condition protocol**) and coloration afterwards (see **coloration protocol**).

We left the birds indoors in the same cages they spent the previous night after all of this in order to be used for flying tests the next day, except in the case of Fridays, in which they would be released to their aviaries. The total procedure lasted a maximum of 8 days, distributed into two weeks (Tuesday-Friday) for each round of measurement performed every 3 months (see **general protocol**)

## Coloration

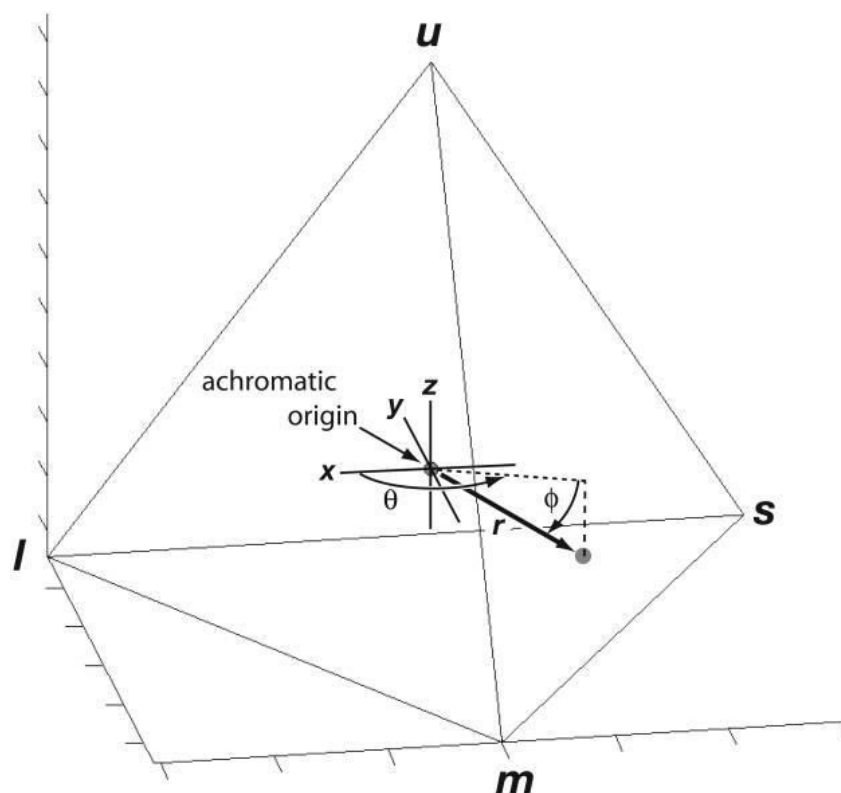
Beak coloration was measured by the means of photography with a Canon EOS 7D modified for full spectrum sensibility, to be able to detect UV. A Novoflex Novoflexar objective, with a considerable transmittance for UV, was used, together with a CMOS-optimized UV/IR-filter

(Baader UV/IR Cut) or visible-IR spectrum filter (Baader U-filter), for each of the two photos to be taken. 12 birds were photographed each day, in groups of 4, corresponding to those of respirometry bouts, and being performed after each round of it (see **general protocol**). The photographs were taken in a room without windows, with a lightbulb resembling daylight with an adequate UV production (Osram Ultravitalux UV-A) hanging at 80 cm over the table, with the abovementioned camera set up in a tripod over a table with a green cloth covering its surface to avoid reflections. Two photos were taken consecutively to each bird, one with each filter. The objective focus was set at the 1.5 feet mark of the objective (between 40 and 50 cm marks). The camera was parallel to the surface and its settings adjusted manually with an ISO of 400, a white balance of 4100 K and the metering being performed over the surface of a Spectralon (by Labsphere®) diffuse white standard (95% grey reflectance), placed over the table, used to standardize the luminance. Another grey Spectralon standard with 10 % reflectance was photographed under the same conditions to control for possible over exposition of the white standard. A calliper was included into the photos in order to provide a size reference, and the bird to be photographed was held with the left hand and therefore with the right side of the beak upwards and parallel to the surface (**Figure 5**).



**Figure 5.A.** Set up used for the photography. **B.** Example of bird picture.

Photos were stored as .CR2 files (RAW files from Canon®) in the camera SD card, and later on transferred to a computer and processed with micaToolbox ImageJ plugin, developed by Troscianko & Stevens (2015). This toolbox extracts 4 colour channels (R, G, B and UV), with both photographs of each bird being superposed in a multispectral stack, manually aligned, and signals automatically linearized and normalized, considering the sensitivities of the camera (available on the program) and the characteristics of the light source (D65 used). The toolbox can also model the animal perception of the image with a colour space specifically developed for each species. Here, blue tit (*Cyanistes caeruleus*) was used, as is the closest species available within the program, used as a model species for passerine birds. This way, cone catch quanta values (for long, medium, short wavelength and double cones) were obtained from the photos taken. The values were posteriorly expressed as coordinates in a tetrahedral avian vision space (with equation 20 from Endler & Mielke, 2005), in which the Euclidean distances between individual values are approximately equivalent to Just Noticeable Differences (JND), the minimum distances within a colour space that render colours that can be distinguished by a specific organism visual system. These coordinates are X, Y, Z (as it is a three-dimensional space) plus a “luminosity” value given by the double cone absorption values. X represent the axis going from long (red) to short (blue) wavelength, being higher the bluer the hue, Y the axis from the middle point of the previous axis to medium wavelength (green), being higher the greener the hue, and Z represents the position between the middle of all the previous three (similar to a human grey point) and UV, being higher the higher the more UV is the hue (**Figure 6**).



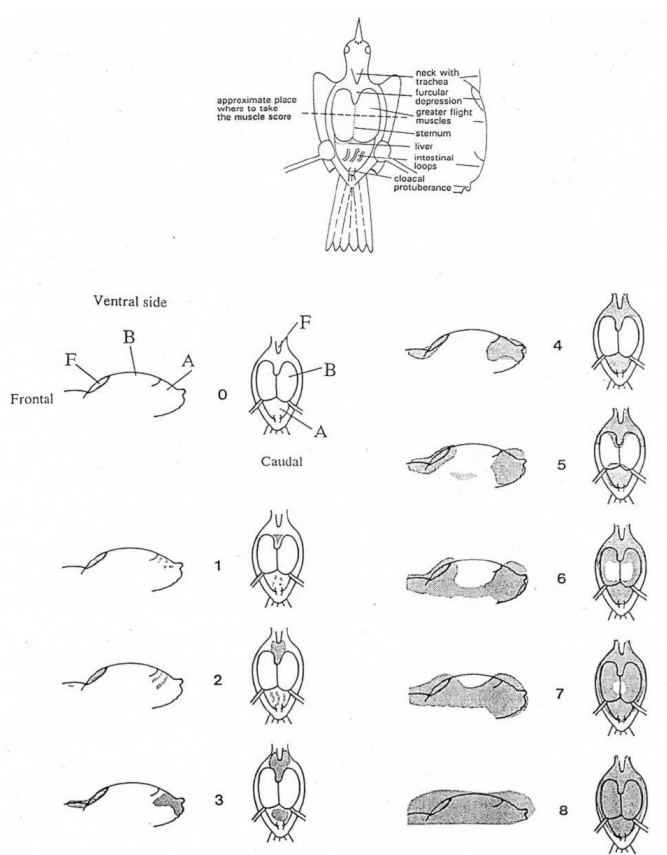
**Figure 6.** Tetrahedral space with its x, y and z axes and the vertices representing the pure “primary” colours in the tetra-chromatic visual system modelled (i.e. s=short wavelength, m=medium wavelength, l=long wavelength, u=UV). Image from Stoddard and Prum (2008).

After the photos had been taken, birds were left in the same small cages (40.5 cm x54.5 cm x29.5 cm) in which they were placed before respirometry, and in which they will stay overnight, for flying tests to be performed with them during the following day (see **general protocol**).



## Body condition

12 birds randomly sampled (see **general protocol**) were assessed each day just before respirometry. They would be grabbed from a cage (40.5 cm x54.5 cm x29.5 cm) in which they had remained indoors for the previous night and morning (see **general** and **respirometry protocols**) and weighed by the means of a Sartorius scale with a maximum resolution of 0.1 g. For that the bird was placed upside down into a plastic tube with which the scale would have been previously tared. Afterwards, a measure of both muscle and fat conditions were performed, following a score from 0 to 8 in the case of fat and from 0 to 3 in the case of muscle, such that the higher the score the more fat or muscle they had. These scores were assessed by blowing in ventral and lateral sides of the birds to remove the feathers and visually check the degree of development of each of them, and giving them a number depending on the following charts (adapted from Aranzadi Sciences Society):



**0.** Without distinguishable fat.

**1.** Traces. Clues of fat (dark zone).

**2.** Concave interclavicular region with visible fat. Abdominal fat in traces.

**3.** Interclavicular depression flat, fully covered. Belly still not fully covered.

**4.** Fully covered abdominal region.

**5.** Convex interclavicular fat. Abdominal fat covering part of the breast muscle.

**6.** Fat, visible in the sides of the breast muscle, that joins the interclavicular and abdominal fat. Partially covered musculature.

**7.** Only a small part of the breast muscle visible.

**8.** Body fully covered of fat. Musculature not visible.

If the bird's fat or muscle did not fit a specific level of the table, because of excess or defect, it was adjusted with fractional values of 0.5 over or under the given integers.



0. Quill very patent. Concave musculature.



1. Quill distinguishable. Flat musculature.



2. Quill still distinguishable. Convex muscles.



3. Quill undistinguishable. Very convex muscles.

Translation and adaptation from Aranzadi Sciences Society.

## **Blood sampling**

Birds were bled twice every season (each three months, see **general protocol**), within two consecutive weeks. The birds were left indoors in small cages (40.5 cm x54.5 cm x29.5 cm) the previous evening without food, so that they were fasting.

Blood samples were taken by brachial vein puncture with a lancet and a heparinised Microvette®. After the extraction of around 100 µL, the birds were returned to a cage with free access to food and water, until being released in their outdoor aviaries later that day. Sampling always started in the morning, beginning at as early as around 7 a.m. (although usually at around 9 a.m.) and ending at maximum at around 18:30, although normally much earlier (80 % of the cases within a range of less than 4 hours, and 60 % under 2 hours; see **statistics section** for discussion on possible stress effects). Blood was kept in ice after collected, to be centrifuged at 4° C and 3500 g for 10 min right after the end of the sampling in order to separate plasma and cells.

The plasma was separated into aliquots for future analyses and all samples were stored at -80 °C until measures were performed, except in the case of cytometry and haematology analyses with the ElementRC® machine, which were performed with fresh plasma (see **oxidative stress, AGEs, glycation and haematology protocols**). Aliquots for telomere measurements were also taken from fresh whole blood, added to culture media (Dulbecco Modified Eagle Medium (DMEM) high Glucose, L-Glutamine, Sodium Pyruvate, Dominique Dutcher®, Brumath, France)

and shipped to Paris the same day to be treated within that week, as described in the **telomeres section**.

## **Oxidative stress**

We selected five parameters to be assayed for oxidative status: total plasmatic non-enzymatic antioxidants (OXY, Diacron®), total organic hydroperoxides (d-ROMs, Diacron®), protein carbonylation, mitochondrial superoxide production (see **cytometry**) and DNA damage (as 8-d-Oxo Guanosine, Epigentek®). The three first parameters were measured in plasma from week 2/3 (see **general protocol**) and the two latter in cells from either the same blood sampling as the plasma measurements in the case of DNA damage or week 3/4 for the superoxide production (see **cytometry**), following the protocols indicated by the commercial kits and a Tecan Infinite M200® microplate reader for absorbance measurements. 10 µL of plasma were needed for protein carbonyl, 8 µL (4 µL per duplicate) for OXY and 2 µL for d-ROMs, including duplicates, so a total of 20 µL for all the measurements. When no such volume was available, no duplicates for OXY were used for that individual, or if the volume was lower than 16 µL, OXY and d-ROMs were prioritized over protein carbonyl, being considered more general measures of oxidative status. For protein carbonyl estimations, as it was pondered by the protein concentration present in each sample, 5 µL of the final samples measured were pipetted into a separate plate and diluted with another 5 µL of distilled water to perform a BCA (bicinchoninic acid). This test allowed us to determine the protein content by a colourimetry technique that does not interfere with the reagents in the protein carbonyl test, as it is the case, for example, with the Bradford test. A commercial batch BSA (Bovine Serum Albumin) was used to perform the reference curve, as we did not have access to purified zebra finches albumin or any other protein at a known concentration.

DNA for oxidative damage measurements was extracted from blood cells using 5 µL aliquots of unfrozen cell samples by the means of an EpiQuick® kit and proteinase K and RNase from Macherey-Nagel®, following their indicated protocols, and the quality of the extraction product was checked using a Nanodrop®, with the extraction being repeated if the DNA concentration was lower than 100 ng/µL or the amount of salts or proteins considered too elevated. This is determined by absorbance ratios of the sample at 260 nm/230 nm and 260 nm/280 nm above 2. This is so as 260 nm is the peak of absorbance for nucleic acids, while 230 nm and 280 nm are the peaks for salts/sugars and proteins, respectively. The resulting solutions of EDTA with DNA were frozen at -80 °C until DNA damage measurements were performed. The majority of these measurements were performed in March/April 2023 and August 2023, except from a DNA damage measurement done in April 2024.

## **Telomeres**

### **Preparation of microscope slides**

Two drops of blood from each sample were added to RPMI1640 medium and subsequently centrifuged for 7 min at 1400 rpm at room temperature. Then, the supernatant was removed and the cell pellet re-suspended in a solution of 0.075 M potassium chloride (KCl) (Merck, New Jersey, US) at 37°C and incubated for 20 min in a 37 °C water bath (hypotonic shock). The cells were pre-fixed by adding approximately five drops of fixative (3:1 ethanol/acetic acid) to each tube under agitation, and the tubes centrifuged for 7 min at 1400 rpm at room temperature. The supernatant was then removed and cells were re-suspended in the fixative solution followed by another centrifugation under the same conditions. After two additional rounds of these fixation steps, the cells were stored in the fixative solution at 4 °C overnight and then spread on

cold wet slides the next day. The slides were dried overnight at room temperature and stored at  $-20^{\circ}\text{C}$  until further use.

### **Telomere staining**

Telomeres were stained with a Cy-3-labelled PNA probe specific for telomeres (TTAGGG sequence; Cell Environment, Evy, France), as previously described in e.g. M'kacher et al. (2020). Briefly, slides were washed with 1X PBS and fixed with 4% formaldehyde at room temperature. After rinsing three times with PBS, they were treated with pepsin (0.5 mg/ml) at  $37^{\circ}\text{C}$  for 5 min. After an additional rinsing three times with PBS, the slides were sequentially dehydrated with 50%, 70%, and 100% ethanol and air-dried. The telomere and centromere probes were added to the slides and subsequently denatured on a hot plate at  $80^{\circ}\text{C}$  for 3 min and then incubated in the dark for 1 h at room temperature. The slides were subsequently rinsed with 70% formamide/10 mM Tris pH 7.2 three times during 15 min and then in 50 mM Tris pH 7.2/150 mM NaCl pH 7.5/0.05% Tween-20 (3 x 5min). After a final rinse with PBS, the slides were counterstained with DAPI and mounted in PPD at the appropriate pH.

### **Telomere quantification**

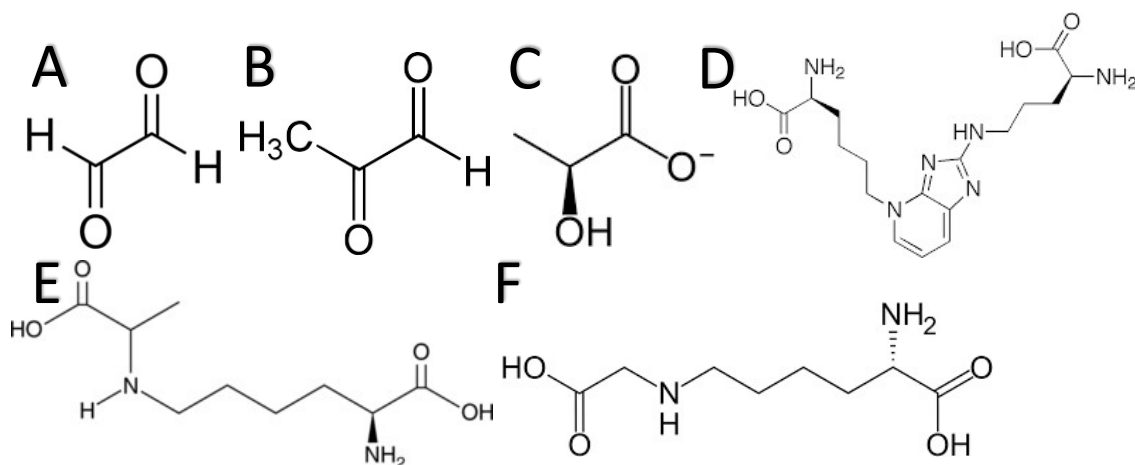
Quantitative image acquisition from slides with stained cells was performed using MetaCyte software (MetaSystem, version 3.9.1, Altlußheim, Germany). The exposure and gain settings remained constant between captures. The analysis of telomere signals was performed using TeloScore Software (Cell Environment, Paris France). The mean fluorescence intensity (FI) of telomeres was automatically quantified and analyzed in 10000 nuclei on each slide. The measurements were performed on triplicate slides.

## **AGE**

A subset of plasma sample aliquots from every treatment groups at the periods previously indicated (see **general protocol**) were processed in order to obtain the extracts to be subsequently analysed by targeted metabolomics. Methylglyoxal, glyoxal, lactate, pentosidine, CML (Carboxymethyl-lysine) and CEL (Carboxyethyl-lysine) were targeted in the analysis (**Figure 8**). For this, methylglyoxal, glyoxal and lactate had to be derived (i.e. transformed into different compounds) in order to be detected. This derivation was performed in aliquots of  $15\mu\text{L}$  from each sample, to which  $70\mu\text{L}$  of methanol with  $100\text{ ng/mL}$  of internal standard (D-Abscisic Acid: D-ABA) was added. Then, it was incubated for 2 h at  $-80^{\circ}\text{C}$  and centrifuged 10 minutes at  $4^{\circ}\text{C}$  and 13200 rpm, from where  $50\mu\text{L}$  of supernatant were recovered. Finally,  $1\mu\text{L}$  of reagent (o-phenylenediamine)  $500\mu\text{g/mL}$  was added, and the resulting samples were incubated overnight at  $4^{\circ}\text{C}$ , after which they were analysed by ultrahigh-performance liquid chromatography (UHPLC) on the UltiMate 3,000 UHPLC system (Thermo) coupled to an EvoQ Elite (Bruker) mass spectrometer equipped with an electrospray ionization (ESI) source in MS/MS mode. For this,  $5\mu\text{L}$  from each extract were injected, on an Acquity UPLC<sup>®</sup> HSS T3 C18 column ( $2.1 \times 100\text{ mm}$ ,  $1.8\mu\text{m}$ , Waters) coupled to an Acquity UPLC HSS T3 C18 precolumn ( $2.1 \times 5\text{ mm}$ ,  $1.8\mu\text{m}$ , Waters) for chromatographic separation. Samples were carried through the column following a gradient of solvent A ( $\text{H}_2\text{O}$ ; 0.1% formic acid) and B (methanol; 0.1% formic acid) at a flux of  $300\mu\text{L/min}$ , starting with 2% B for 0.5 minutes, reaching 100% B at 8.5 minutes, holding 100% B for 3 minutes and returning to 2% B in 1 minutes, for a total run time of 13.5 minutes. The column was operated at  $35^{\circ}\text{C}$ . Nitrogen was generated from pressurized air by a Nitro 35 nitrogen generator (GenGaz) and used as cone gas ( $20\text{ L/h}$ ), heated probe gas ( $25\text{ L/h}$ ) and nebulizing gas ( $25\text{ L/h}$ ). The cone and heated probe temperatures were  $300^{\circ}\text{C}$  and  $350^{\circ}\text{C}$ , respectively, and the capillary voltage was set at 3.5 kV. Metabolites were analysed by multiple reaction monitoring (MRM) after determining the retention time and mode (positive or negative) by scan and the cone voltage, daughter ion and collision energy using the MRM builder function on standards.

Washing solvent (80% H<sub>2</sub>O, 20% MeOH) was used to wash the syringe. A mix of the different standards served as a positive control.

Unfortunately, pentosidine measurements did not work, and lactate values showed a big number of zeros (under the limit of detection), so they were not considered for further analyses during this thesis (see **statistics section**). Standard curves made with standards of known concentration of each compound were used to determine the concentration of each of the sample. As glyoxal and methylglyoxal were derived, the only compounds for which the real concentration (in ng/mL) of the original molecules were estimated were CML and CEL.

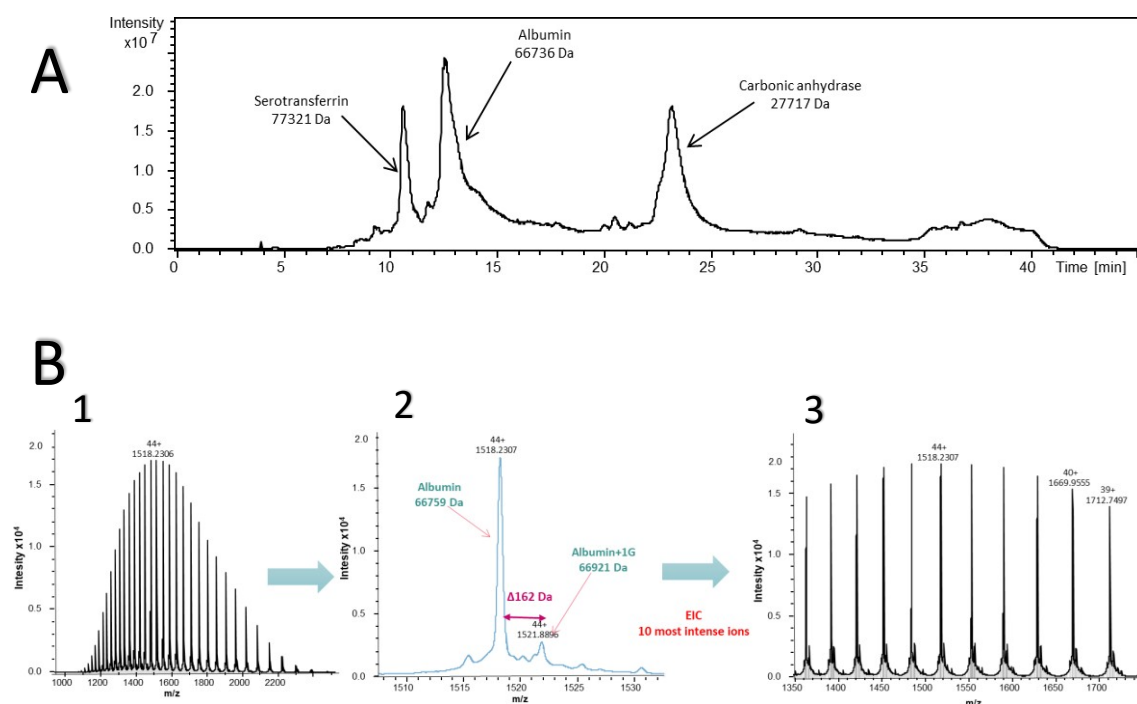


**Figure 8.** The products measured in the targeted metabolomics procedure. **A.** Glyoxal. **B.** Methylglyoxal. **C.** Lactate. **D.** Pentosidine. **E.** CEL (Carboxyethyl-lysine). **F.** CML (Carboxymethyl-lysine).

## Albumin glycation

Glycation levels for a subsample of individuals were determined using liquid chromatography coupled to mass spectrometry (LC-MS), which is considered the gold standard for the assessment of protein glycation levels (see e.g. Priego-Capote et al. 2014) and has previously been used for birds (Ingram et al. 2017; Zuck et al. 2017; Brun et al. 2022). The samples were all analysed after the end of the experiment, during late 2023. Only one sample from each individual and time slot (from the subsample selected) was measured, given logistic limitations on the total number of samples that could be processed. For this, 3  $\mu$ L of plasma were diluted with 22  $\mu$ L of distilled water containing 0.1% of formic acid, followed by injection of 5  $\mu$ L into the system. The glycation values used in the analyses represent the total percentage of glycated albumin, obtained by adding the percentages of singly and doubly glycated albumin. These percentages were calculated by dividing the areas of the peaks corresponding to each glycated molecule form (albumin plus one or two glucose) by the total albumin peak area (sum of the areas of glycated plus non-glycated molecules) observed in the mass spectre outcomes obtained (see **Figure 9** for an example). These mass spectres represent the different intensities of signal for the components differentiated in the sample by their TOF (Time Of Flight), which depends on the mass to charge ratio ( $m/z$ ) of the ionized molecules. More detailed information regarding functioning of the method and data processing can be found in (Brun et al. 2022). In cases where the spectrum could not be deconvoluted (i.e. glycation fractions differentiated from each other), resulting in missing data, or albumin glycation values were not properly detected (i.e. zero glycation levels reported), these individuals were excluded from the statistical analyses. This decision is made based on the fact that it is not possible to know why the glycated albumin signal

is under the limit of detection (i.e. if this is a real “value” or a technical issue). This is so as it is not even possible to determine reliably a limit of detection per se, as these are proportional values calculated by dividing areas of glycated versus non-glycated albumin from the mass spectrum, and this limit may also be different for each species or even for each sample, depending on relative albumin abundance (which is nevertheless irrelevant for the calculation of the index). Moreover, albumin glycation levels are normally well above 0 in all the species we measured, which makes us think that this is unnatural, and therefore discard these data, at least until a strong reason to discriminate between these options is found. These still constituted a minority of the cases.\*



**Figure 9.** Example of **A.** a chromatogram of a zebra finch plasma sample, with the main recognizable proteins and **B.** scheme of the data process to obtain the glycation values from a mass spectrometry outcome showing the intensity of the peaks for native and glycated albumin (with one glucose molecule) in zebra finches: first, the most intense of the ionized forms detected (**1**) is selected and zoomed in (**2**), recognizing the albumin and its glycated form by their masses; then, the ten most intense (abundant) ion forms from **1** are selected (**3**) to sum their areas of each fraction (glycated and native albumin) and divide the glycated by the total and multiply it by 100 to obtain a percentage.

\*The methods described here are the same as in previous chapters, that given their nature as submitted articles, needed to include their own description independently from the whole thesis manuscript. Therefore, I acknowledge the high textual similarity between this section and those included in previous chapters. Still, further details are given here.

## Cytometry

Blood samples were collected as indicated in the **Blood sampling** section. The Accuri™ C6 flow cytometer (BD, Oxford, UK) (**Figure 10**) was used to obtain (i) white cell counting (ii) mitochondrial superoxide production in red blood cells (RBC) and (iii) rate of living versus early apoptotic cells.



Figure 10. Cytometer.

### **Cell preparation.**

50  $\mu\text{L}$  of heparinized whole blood was separated in two parts: 10  $\mu\text{L}$  was taken and diluted in 390  $\mu\text{L}$  of cell culture media (Dulbecco Modified Eagle Medium (DMEM) high Glucose, L-Glutamine, Sodium Pyruvate, Dominique Dutcher®, Brumath, France) previously kept at room temperature. Diluted whole blood was further used for RBC mitochondrial superoxide production characterization. The remaining non-diluted volume of whole blood (40  $\mu\text{L}$ ) was centrifugated at 1000 g for 2 min at 4°C, and 10  $\mu\text{L}$  of the plasma containing the RBC phase was gently taken and diluted in 90  $\mu\text{L}$  of cell culture media. These 100  $\mu\text{L}$  of plasma containing white cells and cleaned of RBC were used to characterize white cells' populations.

### **RBC labelling.**

Each individual sample was deposited in duplicate in a 96-wells plate, 75  $\mu\text{L}$  each. A volume of 2.5  $\mu\text{L}$  of the stock solution of MitoSOX™ Red reagent (for ROS production measurement) was then added in each duplicate. Such solution was previously prepared as followed: a volume of 130  $\mu\text{L}$  of DMSO (dimethylsulfoxide) was added to an original tube of MitoSOX™ Red (50 $\mu\text{g}$ ) to get a stock solution of 0.5 mM of reagent. The obtained solution was distributed in 4 aliquots of 30  $\mu\text{L}$  to be stored at -20°C for further use. The plate with the samples was sealed and incubated 30 min at 40°C in the dark. After this first incubation step, the plate was centrifugated at 2000 g at room temperature, the medium was discarded and cells were washed using 75  $\mu\text{L}$  PBS. This wash step was repeated once again.

The washed cells were then resuspended with *Annexin V* binding buffer (previously diluted 10x, 0.1 M HEPES pH 7.4, 1.4 M NaCl, 25mM CaCl<sub>2</sub>) containing annexin-V-FITC (5 $\mu\text{L}$  of the stock solution of Annexin V and 1  $\mu\text{g}/\text{mL}$  of propidium iodide, BD Biosciences), for rate of apoptosis determination. The plate was then sealed and incubated at room temperature in the dark for 15 min. Cells were harvested following a centrifugation step at 2000 g at room temperature, washed using 75  $\mu\text{L}$  PBS, re-centrifuged at 2000 g, and finally resuspended with cell culture medium (75  $\mu\text{L}$ ). Reading of fluorescence signal was performed thereafter by the cytometer, and cells were subsequently stored at 6 °C for the measurement to be repeated after 24h to get an estimate of the dynamic of apoptosis and mitochondrial superoxide production. *Annexin V* binds to phosphatidylserine that becomes exposed on the outer layer of the plasma membrane in apoptotic cells. *Propidium iodide* (PI) enters necrotic cells but is excluded from apoptotic cells.

As such, using flow cytometry, *Annexin V* binding and *Propidium iodide* uptake and associated fluorescence signals allow to distinguish living (not stained), pre-apoptotic (*Annexin V* stained), apoptotic (both *Annexin V* and *propidium iodide*) and dead cells (*propidium iodide*).

### **White cells counting.**

The diluted white cell samples were distributed in a 96-wells plate and cell population characterization (heterophiles, lymphocytes and monocytes) was done using appropriate plots of cells' sizes (FSC channel) and shapes (SCC channel) as described in Seliger et al. 2012 in chicken and Jenkins et al. 2021 in American kestrel (see Fig. 5 in this last reference). We could not perform white cell characterization based on specific markers (antibodies) as they lacked specificity for zebra finch cells. Repeatability estimates were established on 10 repeated samples, leading to an ICC of 0.504 for H/L (heterophile:lymphocyte) ratio (Bootstrap repeatability estimation,  $p = 0.060$ ) and of 0.704 for H:WBC (heterophile:lymphocyte) ratio (Bootstrap repeatability estimation,  $p = 0.0037$ ).

### **Fluorescence reading.**

Samples were run in the Accuri™ C6 flow cytometer (Becton Dickinson, BD, Franklin Lakes, NJ, USA). For both white and red blood cells, up to 20000 cells were counted for each measurement, and a rinse cycle was added after each sample reading to avoid inter-samples' contamination. For RBC measurements, we used 488 nm excitation for Annexin V, propidium iodide and oxidized MitoSox Red. In each plate, control samples including negative (no staining), or cells stained only with MitoSox, Annexin V and PI were run to perform signals' compensation. Signal detection was done using: FL1-FITC 530 nm for Annexin, FL2-PE-A (580 nm) for MitoSox Red, and FL4-APC-A (625 nm) for propidium iodide. To optimize cytometry results, performance validation and fluidic maintenance (including peristaltic pumps) were done regularly prior to experimental sessions. Samples were analysed at low flow rate, using a threshold of 25000 to exclude debris and electronic noise. Cell doublets were excluded using a first gating on the FSC-A/FSC-H profiles. Analysis of the data was done using plot cell counts for Annexin V and propidium iodide to get living (no staining) and early apoptotic (Annexin V stained) cells proportions. For both categories, we determined the mean MitoSox intensity (mean values of superoxide production).

## **Haematology**

100  $\mu$ L of plasma diluted in distilled water was placed in an Avian/reptilian rotor from a veterinary ElementRC® machine, that makes automatic colourimetry measurements of albumin, globulin, total protein, uric acid, glucose, aspartate aminotransferase, creatine kinase, total biliary acids, phosphate,  $Ca^{2+}$ ,  $K^+$  and  $Na^+$ .

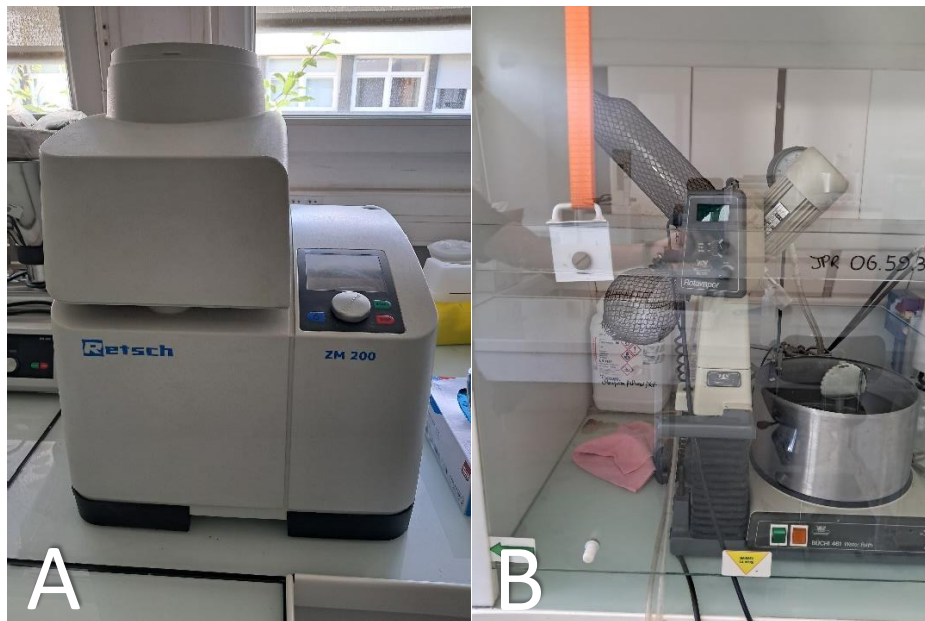
The dilution was needed for two reasons: 1) most bird' plasma samples were inferior to 100  $\mu$ L and 2) some parameters would normally be over the range of the machine measurements if no dilution was performed. Such dilutions went from a minimum of 2x to a maximum of 8x.

## **Body composition**

Individuals that died during the experiment were collected and kept frozen at  $-20\text{ }^{\circ}\text{C}$  until dissection, taking place in two sessions during 2023. Liver and breast muscle (*pectoralis major*) were separated and weighed to the nearest 0.0001 g in a precision scale along the remaining rest of the body, referred hereon as "carcase". After that, they were lyophilized by freezing at  $-20\text{ }^{\circ}\text{C}$  and subsequent sublimation by applying near void conditions (pressure 0.006 bar) during approximately two weeks and then weighed again, so the water proportion of each of the parts



(liver, *pectoralis* and carcass) could be estimated, allowing us to test for possible dehydration or water retention effects of the treatments. This weighing was repeated once a week until the mass was stable. In 2024, these portions were independently grinded (**Figure 11.A**) and the resulting powder stored at room temperature for later assessment of lipid content by the Folch method. For this, 1g of the aforementioned powder from each sample in duplicate, was introduced into labelled round vessels and 30 ml of a 2:1 mix of chloroform and methanol (CM mix hereon) was added, in order to separate the lipids from the samples. After that, these samples were exposed 30 s to ultrasounds and stirred with a glass bar (these were always rinsed with 1ml of the CM mix to recuperate the remaining on it) in order to break the cells. Subsequently, the samples were agitated during 12 hours and then filtered to remove the lumps while transferred to tubes. All the possible remaining present in the previous recipients and filters/funnels used were carefully recuperated by rinsing them with 2 ml of the CM mix. 8 ml of KCl at 0.9 % (polar compound) was added to the tubes with the samples and they were stirred as before to separate the methanol, followed by 10 min of centrifugation at 2000 rpm. After this, the superior phase (methanol with KCl) is aspirated by a void pump and the remaining transferred to a previously weighed round vessel, rinsing the tube with 2 ml of chloroform to recuperate any remainder. The samples were then left in a Rotavapor (bath at 44 °C and later cooling at 8 °C) rotating at 100 rpm at -760 mm Hg, until the chloroform evaporates (**Figure 11.B**). Finally, the vessels with the isolated lipids were left overnight in a stove at 50 °C and then weighed to calculate the percentage of lipids in the original sample.



**Figure 11. A.** Grinder and **B.** Rotavapor® used for body composition measurements.

A second extraction with a subsample equally distributed across the different experimental groups was performed on the remainders left in the paper filters previously used, in order to confirm that all lipids were adequately extracted in the first session. For this, the filters were shredded into small pieces and placed into vessels with 40 ml of CM mix, repeating the previous protocol, but with 10 ml of KCl 0.9 % this time.

The lipid percentage was calculated by subtracting the weight of the empty vessel from that of it with the extracted material, dividing this by the initial weight of the sample and multiplying by 100. The values obtained during the second extraction were averaged within groups and added to the individual values from the first extraction.

## **Pilot study**

20 birds (10 males and 10 females) were used in a pilot study to determine the dosages of both glucose and methylglyoxal that were going to be used for the main experiment. These individuals were housed indoors at 12:12 L:D cycles and approx. 22 °C in battery cages (8 equal cages per battery; battery dimensions: 100 x 40 x 183.5 cm) in groups of two per cage (1 male and 1 female), and distributed in 5 experimental groups of 4 birds each: a control group with still water and 4 different groups with water including either glucose or methylglyoxal at different concentrations: 50, 100, 150 and 200 g/l for glucose, and 500 mg/Kg per day, 1000 mg/Kg per day, 1500 mg/Kg per day, 2000 mg/Kg per day). All of them had access to *ad libitum* food consisting into a commercial seed mix (Deli Nature® for tropical finches).

The pilot study was performed in two parts: first, a 2-week session with glucose supplementation, and later a 2-week session with methylglyoxal supplementation. The birds were randomly assigned to the abovementioned groups independently for each of these sessions, with always 2 males and 2 females per group. An additional group of 2 water feeders without birds was added to control for water lost by evaporation and feeder manipulation. Water and food were weighed every day during these treatments, and final values were averaged within groups and for water also corrected for average loss by evaporation and manipulation spilling.

Body mass and body condition (see body condition) were measured and blood samples from brachial vein taken at the beginning of the experiment (i.e. before any supplementation) and after the first and the second week of supplementation, to compare either whole blood glucose or plasma methylglyoxal values. Glucose was measured by a glucometer (Ascensia Countour® plus) on a small drop of whole blood and plasma methylglyoxal by the same means as on the main experiment (see **AGE section**). The samples were performed in the morning after placing all the birds together in a single cage covered by a cloth and without access to food nor water for an hour and then randomly grabbing them to avoid correlation between sampling time and treatment group.

Results (see **Appendix 2**) showed no significant effects of glucose supplementation on whole blood glucose levels, accompanied by a moderate reduction in food intake and drinking, while methylglyoxal supplementation increased plasma methylglyoxal levels, but with a high reduction in drinking rates. Based on this, the lowest concentrations (50 g/L of glucose and 500 mg/Kg\*day of methylglyoxal) were selected for the final experiment, to reduce to the maximum the effects of dehydration that could be confounded with the effects of the supplementation (e.g. increased mortality), especially in the methylglyoxal group, and the severity of the treatment in the glucose group, given the lack of significant effects on glucose levels.

## **Statistics**

R version  $\geq 4.3.3$  with RStudio was used for all the analyses (R: A language and environment for statistical computing. 2023. R Foundation for Statistical Computing, Vienna, Austria, <https://www.R-project.org/>). For all the models mentioned in the **statistics section**,  $\alpha \leq 0.05$  were considered as significant, while  $0.05 < \alpha \leq 0.1$  were considered trends/marginal effects. LMM (linear mixed models) were used for determining the effects of the treatment (Control, Glucose or Methylglyoxal), month of measurement (Baseline, November, February, May, August) and sex (male and female), including all their interactions, on a series of variables obtained via the methodology presented here, using the *lmer()* function from the *lme4* R package (Bates et al.

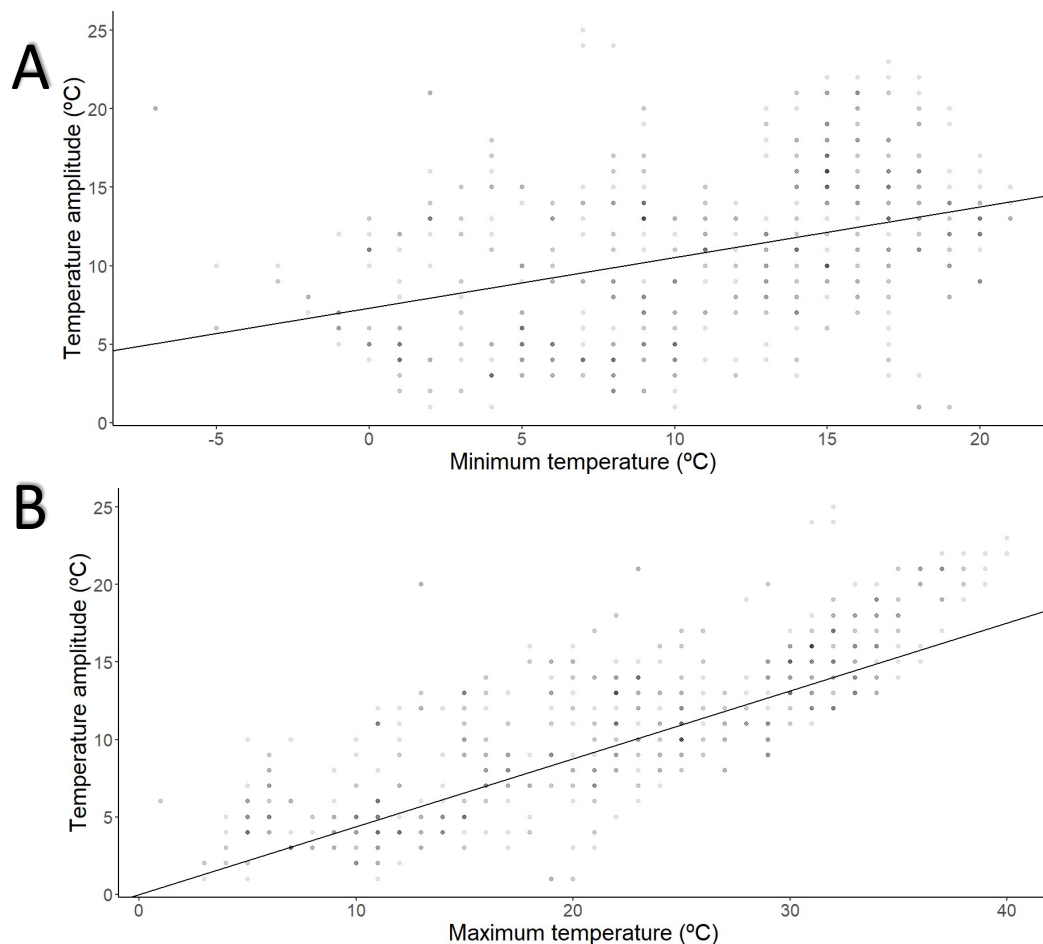
2015). Model selection was performed using the *dredge()* function from MuMIn R package (Burnham and Anderson 2002), with sex and the interactions (apart from treatment\*month) being eliminated when they did not increase the likelihood of the models as indicated by the different information criteria used by the package for comparing models. Sex was kept by principle in the coloration models, as it was deemed biologically relevant in regard of this secondary sexual trait. Transformation, mostly by decimal logarithm, was performed on the response variable when needed to attain normality of the distribution of the models' residuals, and for some dependent variables when allometric relationships are expected. The variables modelled in this way were thus the following: whole blood and plasma glucose in mg/dL, albumin glycation as a percentage of total plasma albumin, plasmatic levels of CML (carboxymethyl lysine) and CEL (carboxyethyl lysine) in ng/mL and of methylglyoxal and glyoxal [in ng/dL of derived compound, see **AGE section**], RMR, RER, body mass in grams, body condition scores (fat and muscle, see **body condition section**), lipid and water mass in grams of the liver, pectoral muscle and carcass (the rest of the body), OXY in  $\mu$ moles of HClO equivalents per ml, d-ROMs in Units of Carratelli (UCARR), protein carbonyl in ng/mg protein, 8-oxo-d-guanosine as a percentage of total DNA, H:L ratio, early apoptotic cells to living cells ratio, superoxide production by either living or early apoptotic cells (divided by the number of cells of the corresponding type) and coloration parameters (see **coloration section** and later descriptions within this section for these variables).

Plasma glucose values were considered as a covariable when predicting albumin glycation levels, OXY when predicting d-ROMs and protein carbonyl values, glyoxal and methylglyoxal levels ( $\log_{10}$  transformed) when modelling CML and CEL, average body mass (of before and after the respirometry measurement) when predicting RMR, dry mass when predicting fat mass and fresh mass when predicting water content (all these latter four variables  $\log_{10}$  transformed) and structural size (tarsus and head-beak length in cm) for the body mass model. Time (0 vs 24 hours) was also considered as an explanatory variable for the cytometer measures, including its interactions, always kept by principle in the models. Individual bird identity was always included as a random factor to control for repeated measures. All the quantitative independent variables were centred (except for age, when included), for better interpret the estimated intercepts (Schielzeth, 2010).

The effects of the experiment on beak coloration were analysed by an LMM with the *lmer()* function, having a variable formed by the combination of all the values of the coordinates in the abovementioned tetrahedral space (see **coloration section**) as a dependent variable, and the interaction of a variable representing the coordinate ion question (X, Y, Z, double cone absorption value) with the treatment, month and sex and all their interactions as a predictive variable and the birds ID as a random factor. This, together with the exclusion of the intercept, both for the general and the random part of the model, and including some additional corrections (see <https://mac-theobio.github.io/QMEE/lectures/MultivariateMixed.notes.html#a-trick-to-do-multivariate-mixed-models-using-lme4>) allows to perform an analysis equivalent to a MANOVA (Multivariate Analysis of Variance) with random factors. This is useful to deal with a trait as coloration in the way described here, with several variables representing a position in a space, in which all of these variables (coordinates) together are needed to describe any single coloration value (i.e. position in the tetrahedral space).

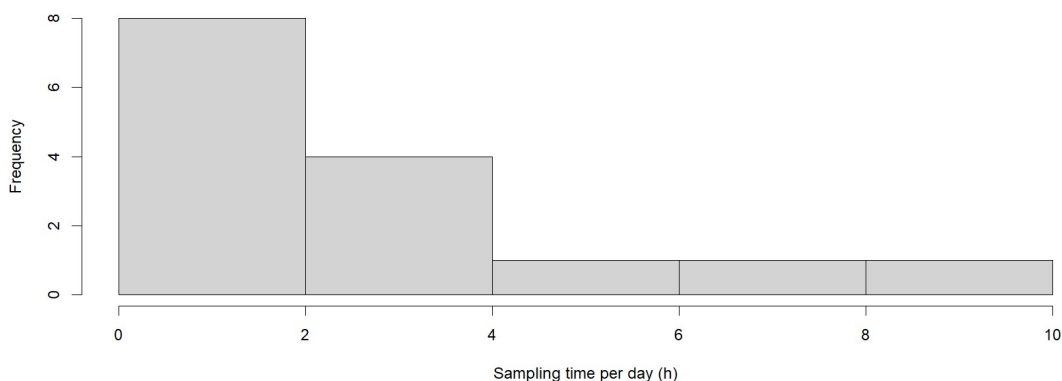
Additional models were performed to determine the effect of either RMR or RMR by gram of mass on whole blood glucose (WBG) and plasma glucose, oxygen consumption ( $VO_2$ ) on

mitochondrial superoxide production in either living or early apoptotic cells, at time 0 or 24 hours, and on oxidative damage variables (d-ROMs, protein carbonyls and 8-oxo-d-guanosine), WBG or plasma glucose on RER and oxidative damage variables and oxidative status variables on glycation and AGEs. The effects of RMR and body mass on whole blood glucose (all  $\log_{10}$  transformed) were also tested. Finally, models were performed to estimate the effects of the ambient temperature in the outdoor aviaries on blood glucose levels and RMR (both response variables  $\log_{10}$  transformed), including both daily minimum temperature and daily temperature difference (maximum temperature minus minimum temperature) recorded on the day before the blood sampling (given the birds were put indoors the day before). These variables were chosen as maximum temperature correlates (positively) with temperature difference more strongly (Multiple  $R^2=0.6377$ , Adjusted  $R^2=0.6372$ ; slope  $\pm$  s.e. =  $0.4385 \pm 0.0125$ ; P-value $<0.0001$ ; **Figure 12.A**) than minimum temperature (Multiple  $R^2=0.1466$ , Adjusted  $R^2=0.1454$ ; slope  $\pm$  s.e. =  $0.32267 \pm 0.02945$ ; P-value $<0.0001$ ; **Figure 12.B**), so minimum and difference can be enough (and would be better) to describe the temperature variation. For two days for which there was no record for the previous day, the temperature from either 2 days or 3 days before was used, considering there had not been severe temperature changes in the surrounding days and therefore the used values could be similar to the observed right the day before. The quadratic components of these variables were also included in the model, and removed if not significant. Bird ID and measurement date were included as random factors, as measurements were not evenly distributed across the year, but clumped around specific dates.



**Figure 12.** Correlation between **A** minimum daily temperature and **B** maximum daily temperature and daily temperature amplitude in the outdoor aviaries where the birds were located during the experiment. The number of values for a given temperature is represented through the intensity of the dots.

To test for possible effects of stress response on blood glucose levels, a model with  $\log_{10}(\text{Glucose})$  as the dependent variable and sampling time in hours (counting as 0 the time of the first bird sampled each day) as an explanatory variable, including bird ID and sampling day as random factors, was performed on the whole dataset. The total sampling time for a day was normally under 4 hours (within a morning), except for a few birds in the baseline, that were measured in another session in the evening, at around 6 and 8 hours after the first sampled bird (Figure 13).



**Figure 13.** Frequency distribution of daily blood sampling times expressed in hours. 80 % of the samplings were done in less than 4 hours, and 60 % in less than 2 hours (i.e. within a morning).

For time limitation and other organizational reasons, results on flying test, haematological parameters measured with the Elementary RC machine, telomeres and coloration are not shown within this thesis manuscript.

Kaplan-Meier analysis was performed over the whole population in order to show the effect of treatment on survival curves. Also, survival data was analysed with Cox proportional hazard models, using *coxph()* R function from the survival package (Therneau and Grambsch 2000; Therneau 2024). A model with all the individuals, considering only the treatment (control, glucose or methylglyoxal) and the sex was first performed on the whole experiment timespan. Additionally, models including time-varying variables (i.e. with values measured or known at different times; see Zhang et al., 2018) were performed. For this, the values of the variables as measured before each time interval in which the survival was considered were included (i.e. August and November 2023, February, May and August 2024). For the age, the initial age was taken and then the age at later stages calculated by adding to it the number of days between the start of the experiment and the day of the following measure of the other variable considered in the model (e.g. body mass) divided by 365 (to convert it into years). A first model with treatment, sex, body mass, age and all the interactions was considered, and the *dredge()* function used on it to determine the best model. Body mass was centred to allow for a proper interpretation of the estimates (Schielzeth, 2010), given the confounding effect that significant interaction terms can have on the whole model if this is not done.

The same kind of model (including treatment, sex, body mass and age) was repeated including data on either whole blood glucose, plasma glucose, albumin glycation, plasma glyoxal, methylglyoxal, CML, CEL, d-ROMs or protein carbonylation levels, or DNA damage levels (controlling for OXY in the d-ROMs model) as a covariable predicting hazard, always centred. All survival analyses were checked for the coefficients' proportionality assumption using

Schoenfeld residuals and with the *cox.zph()* function. If a variable predicted hazard showed signals of non-proportionality (i.e. significant time variation in the hazard value), a term consisting on the interaction of such variable with time was included in order to correct for it.

Shapiro-Wilks test and qq-plots were used to explore the normality distribution of the residuals of the models, and the within individual repeatability was estimated with the *rptGaussian()* function from the R package *rptR* (Nakagawa and Schielzeth 2010).

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# Results

## Effects of the treatments on the measured variables

The results of the selected final models exploring the effects of the treatments over the months of the experiment on the studied ageing related variables are shown in the following tables, as indicated in Box 1. **Figures 1-19** show the main findings from these models.

### Box 1. Tables showing each result and their corresponding final model equations.

**Table 1.A.** Whole blood glucose.  $\log_{10}(\text{Glucose}) \sim \text{Group} * \text{Month} + (1 | \text{Bird\_ID})$

**Table 1.B.** Plasma glucose.  $\text{Glucose} \sim \text{Group} * \text{Month} + (1 | \text{Bird\_ID})$

**Table 2.** Albumin glycation.  $\text{Glycation} \sim \text{Group} * \text{Month} + \text{C\_Glucose} * \text{Month} + (1 | \text{Bird\_ID})$

**Table 3.** Methylglyoxal.  $\log_{10}(\text{Methylglyoxal\_derived}) \sim \text{Group} * \text{Month} + (1 | \text{Bird\_ID})$

**Table 4.** Glyoxal.  $\log_{10}(\text{Glyoxal\_derived}) \sim \text{Group} * \text{Month} + \text{Group} : \text{Sex} + \text{Age\_years} * \text{Sex} + (1 | \text{Bird\_ID})$

**Table 5.** CML.  $\log_{10}(\text{CML\_ng\_ml}) \sim \text{Group} * \text{Month} + \text{Age\_years} * \text{Group} + (1 | \text{Bird\_ID})$

**Table 6.** CEL.  $\log_{10}(\text{CEL\_ng\_ml}) \sim \text{Group} * \text{Month} + \text{C\_logGlyoxal} + (1 | \text{Bird\_ID})$

**Table 7.** Body mass.  $\log_{10}(\text{AVG\_BM}) \sim \text{Group} * \text{Month} + \text{C\_Tarsus} + \text{C\_Head\_beak} + (1 | \text{Bird\_ID})$

**Table 8.A.** Body condition: fat score.  $\text{Fat} \sim \text{Group} * \text{Month} + (1 | \text{Bird\_ID})$

**Table 8.B.** Body condition: muscle score.  $\text{Muscle} \sim \text{Group} * \text{Month} + (1 | \text{Bird\_ID})$

**Table 9.A.1.** Body composition: carcass fat.  $\log_{10}(\text{Lipid\_mass}) \sim \text{C\_logDry\_mass} + \text{Group} + \text{Sex}$

**Table 9.A.2.** Body composition: carcass water.  $\log_{10}(\text{Water}) \sim \text{C\_logFresh\_mass} * \text{Sex} + \text{Group}$

**Table 9.B.1.** Body composition: liver fat.  $\log_{10}(\text{Lipid\_mass}) \sim \text{C\_logDry\_mass} + \text{Group} + \text{Sex}$

**Table 9.B.2.** Body composition: liver water.  $\log_{10}(\text{Water}) \sim \text{C\_logFresh\_mass} + \text{Group}$

**Table 9.C.1.** Body composition: muscle (pectoralis) fat.  $\log_{10}(\text{Lipid\_mass}) \sim \text{C\_logDry\_mass} + \text{Group} + \text{Sex}$

**Table 9.C.2.** Body composition: muscle (pectoralis) water.  $\log_{10}(\text{Water}) \sim \text{C\_logFresh\_mass} + \text{Group}$

**Table 10.A.** Metabolism: RMR.  $\text{RMR} \sim \text{Group} * \text{Month} + \text{Sex} + \text{Age\_years} + \text{C\_BM} * \text{Group} + \text{C\_BM} * \text{Month} + (1 | \text{Bird\_ID})$

**Table 10.B.** Metabolism: RER.  $\text{RQ} \sim \text{Group} * \text{Month} + \text{Month} * \text{Sex} + \text{Age\_years} * \text{Month} * \text{Sex} + (1 | \text{Bird\_ID})$

**Table 11.A.** Oxidative status: OXY.  $\text{OXY} \sim \text{Group} * \text{Month} + \text{Month} * \text{Sex} + (1 | \text{Bird\_ID})$

**Table 11.B.** Oxidative status: d-ROMs.  $\log_{10}(\text{ROM}) \sim \text{Group} * \text{Month} + \text{C\_OXY} + \text{Month} * \text{Sex} + (1 | \text{Bird\_ID})$

**Table 11.C.** Oxidative status: Protein carbonyls.  $\log_{10}(\text{PC}) \sim \text{Group} * \text{Month} + \text{OXY} + \text{Sex} + (1 | \text{Bird\_ID})$

**Table 11.D.** Oxidative status: DNA damage.  $\log_{10}(\text{DNA}) \sim \text{Group} * \text{Month} + \text{Sex} + (1 | \text{Bird\_ID})$

**\*Table 11.E.** CV of the oxidative stress markers.

**Table 12.** Apoptosis.  $\log_{10}(\text{Ratio\_EA\_L}) \sim \text{Group} * \text{Month} * \text{Time} + (1 | \text{Bird\_ID})$

**Table 13.A.** Superoxide ( $\text{O}_2^-$ ) production per living cell.  $\log_{10}(\text{Mean\_mitosox\_Cell\_type}) \sim \text{Group} * \text{Month} * \text{Time} + (1 | \text{Bird\_ID})$

**Table 13.B.** Superoxide ( $\text{O}_2^-$ ) production per early apoptotic cell.  $\log_{10}(\text{Mean\_mitosox\_Cell\_type}) \sim \text{Group} * \text{Month} * \text{Time} + (1 | \text{Bird\_ID})$

**Table 14.** Heterophile by lymphocyte (H/L) ratio.  $\log_{10}(\text{H\_L}) \sim \text{Group} * \text{Month} + \text{Month} * \text{Sex} + (1 | \text{Bird\_ID})$

**Table 1.A. Whole blood glucose**

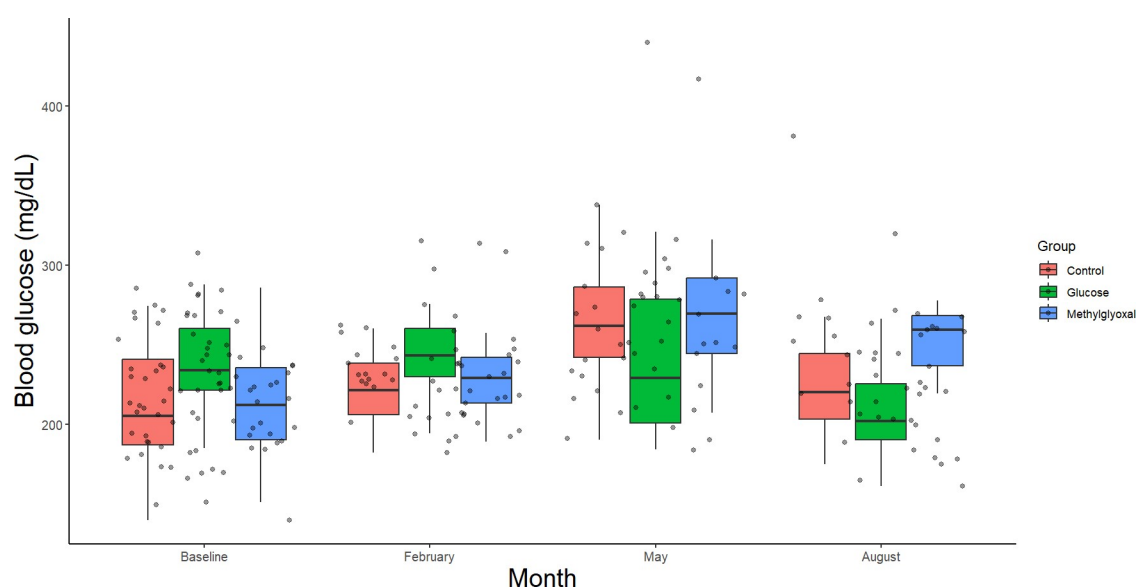
	Estimate	Std. Error	df	t value	P-value
<b>Intercept</b>	<b>2.319883</b>	<b>0.012385</b>	<b>220.115315</b>	<b>187.315</b>	<b>&lt; 2*10<sup>-16</sup></b>
<b>GroupGlucose</b>	<b>0.048261</b>	<b>0.017233</b>	<b>218.980611</b>	<b>2.801</b>	<b>0.00556</b>
GroupMethylglyoxal	0.010640	0.017233	218.980611	0.617	0.53758
MonthFebruary	0.033755	0.017243	182.213455	1.958	0.05181
<b>MonthMay</b>	<b>0.103333</b>	<b>0.018156</b>	<b>187.934923</b>	<b>5.691</b>	<b>4.77*10<sup>-8</sup></b>
MonthAugust	0.015203	0.018511	189.187884	0.821	0.41250
GroupGlucose:MonthFebruary	-0.018232	0.027656	200.621691	-0.659	0.51050
GroupMethylglyoxal:MonthFebruary	-0.003019	0.023691	178.422241	-0.127	0.89873
<b>GroupGlucose:MonthMay</b>	<b>-0.093409</b>	<b>0.028873</b>	<b>203.417747</b>	<b>-3.235</b>	<b>0.00142</b>
GroupMethylglyoxal:MonthMay	-0.007179	0.024849	183.834923	-0.289	0.77297
<b>GroupGlucose:MonthAugust</b>	<b>-0.065816</b>	<b>0.029840</b>	<b>204.178286</b>	<b>-2.206</b>	<b>0.02852</b>
<b>GroupMethylglyoxal:MonthAugust</b>	<b>0.057420</b>	<b>0.025500</b>	<b>185.541880</b>	<b>2.252</b>	<b>0.02551</b>

Shapiro-Wilk normality test

W = 0.98241, P-value = 0.004762

Repeatability for Bird\_ID

R = 0.198 ; SE = 0.076; CI<sub>95</sub>[0.054, 0.351]; P-value = 0.000793



**Figure 1.** Whole blood glucose in mg/dL in the different treatment groups over the course of the experiment. Due to logistical issues, November is missing.

**Table 1.B. Plasma glucose**

	Estimate	Std. Error	df	t value	P-value
<b>Intercept</b>	<b>366.526</b>	<b>10.487</b>	<b>124</b>	<b>34.949</b>	<b>&lt;2*10<sup>-16</sup></b>
GroupGlucose	10.003	15.261	124	0.655	0.513
GroupMethylglyoxal	-2.947	14.831	124	-0.199	0.843
MonthFebruary	2.759	16.101	124	0.171	0.864
MonthAugust	27.174	17.859	124	1.522	0.131
GroupGlucose:MonthFebruary	9.438	23.920	124	0.395	0.694
GroupMethylglyoxal:MonthFebruary	9.728	22.551	124	0.431	0.667
GroupGlucose:MonthAugust	-23.370	25.963	124	-0.900	0.370
GroupMethylglyoxal:MonthAugust	-2.053	25.257	124	-0.081	0.935

Shapiro-Wilk normality test

W = 0.98828, P-value = 0.3696

Repeatability for Bird\_ID

R = 0; SE = 0.067; CI<sub>95</sub>[0, 0.229]; P-value = 0.5

**Table 2. Glycation**

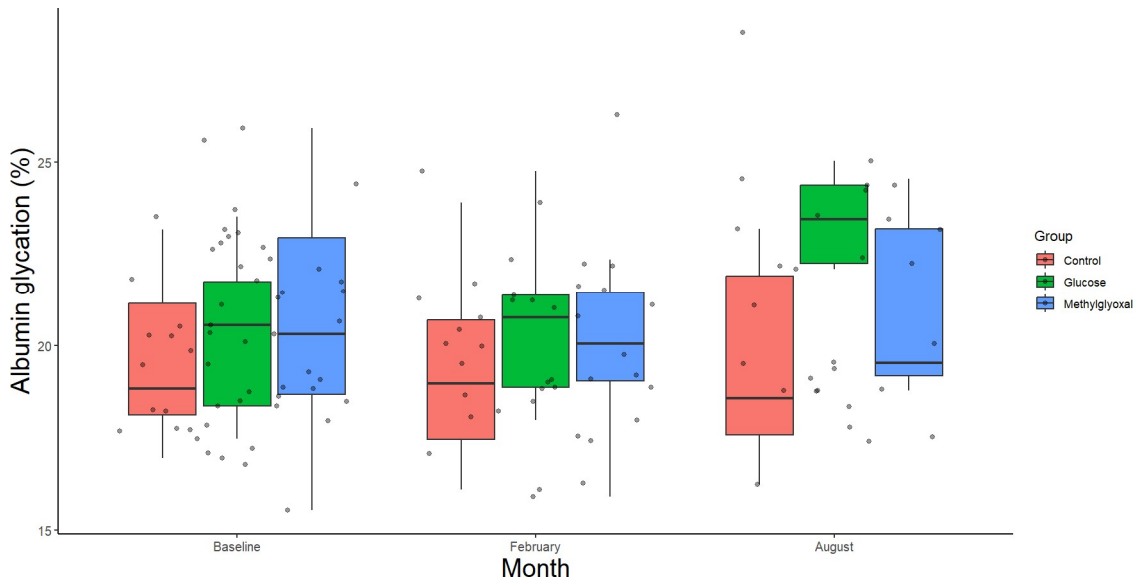
	Estimate	Std. Error	df	t value	P-value
<b>Intercept</b>	<b>19.638339</b>	<b>0.508379</b>	<b>110.798160</b>	<b>38.629</b>	<b>&lt; 2*10<sup>-16</sup></b>
GroupGlucose	0.749024	0.736044	111.946319	1.018	0.31105
GroupMethylglyoxal	1.233134	0.713369	111.293630	1.729	0.08665
MonthFebruary	-0.277062	0.669129	79.367637	-0.414	0.67994
MonthAugust	0.068806	0.765388	86.101251	0.090	0.92858
C_Glucose	0.010027	0.006398	121.526937	1.567	0.11965
GroupGlucose:MonthFebruary	0.339929	1.012227	84.607040	0.336	0.73784
GroupMethylglyoxal:MonthFebruary	-0.222936	0.933034	79.584459	-0.239	0.81177
<b>GroupGlucose:MonthAugust</b>	<b>2.818070</b>	<b>1.107210</b>	<b>89.254208</b>	<b>2.545</b>	<b>0.01264</b>
GroupMethylglyoxal:MonthAugust	0.280646	1.061155	83.702620	0.264	0.79207
MonthFebruary:C_Glucose	-0.012498	0.010204	113.400325	-1.225	0.22319
<b>MonthAugust:C_Glucose</b>	<b>-0.029791</b>	<b>0.009516</b>	<b>110.081215</b>	<b>-3.130</b>	<b>0.00223</b>

Shapiro-Wilk normality test

W = 0.98472, P-value = 0.1769

Repeatability for Bird\_ID

R = 0.302; SE = 0.117; CI<sub>95</sub>[0.063, 0.523]; P-value = 0.00283



**Figure 2.** Albumin glycation rate as a percentage of total plasma albumin in the different treatment groups over the course of the experiment.

**Table 3. Methylglyoxal**

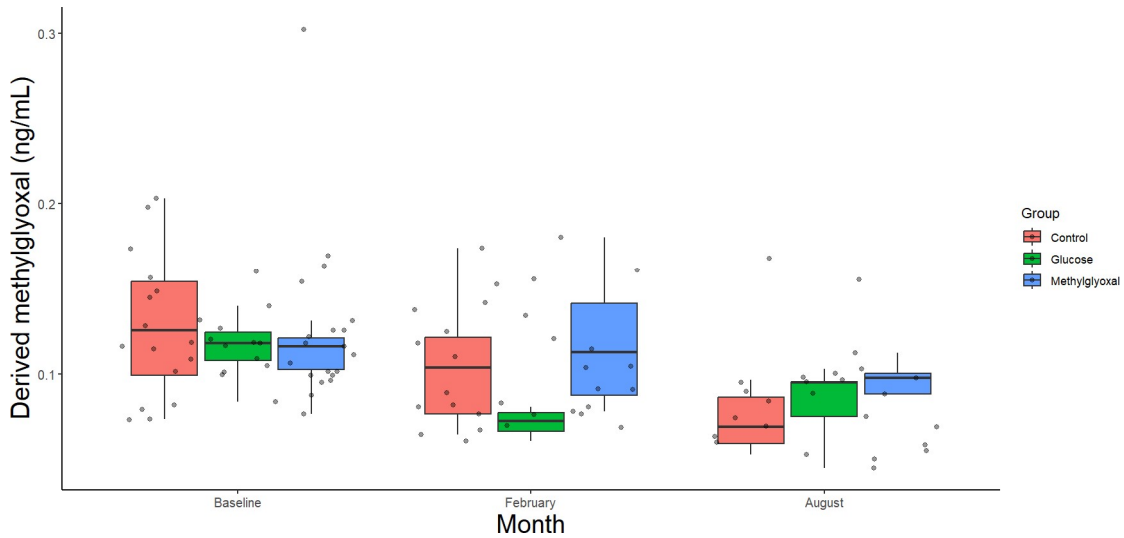
	Estimate	Std. Error	df	t value	P-value
<b>Intercept</b>	<b>-0.90481</b>	<b>0.02658</b>	<b>105.00000</b>	<b>-34.042</b>	<b>&lt; 2*10<sup>-16</sup></b>
GroupGlucose	-0.05125	0.04680	105.00000	-1.095	0.2760
GroupMethylglyoxal	-0.02821	0.03912	105.00000	-0.721	0.4725
<b>MonthFebruary</b>	<b>-0.09499</b>	<b>0.04118</b>	<b>105.00000</b>	<b>-2.307</b>	<b>0.0230</b>
<b>MonthAugust</b>	<b>-0.21999</b>	<b>0.04533</b>	<b>105.00000</b>	<b>-4.853</b>	<b>4.25*10<sup>-6</sup></b>
GroupGlucose:MonthFebruary	-0.09475	0.08299	105.00000	-1.142	0.2562
GroupMethylglyoxal:MonthFebruary	0.08232	0.06129	105.00000	1.343	0.1821
GroupGlucose:MonthAugust	0.07759	0.08066	105.00000	0.962	0.3383
GroupMethylglyoxal:MonthAugust	0.11710	0.06729	105.00000	1.740	0.0847

Shapiro-Wilk normality test

W = 0.9839, p-value = 0.2346

Repeatability for Bird\_ID

R = 0; SE = 0.078; CI<sub>95</sub>[0, 0.266]; P-value = 1



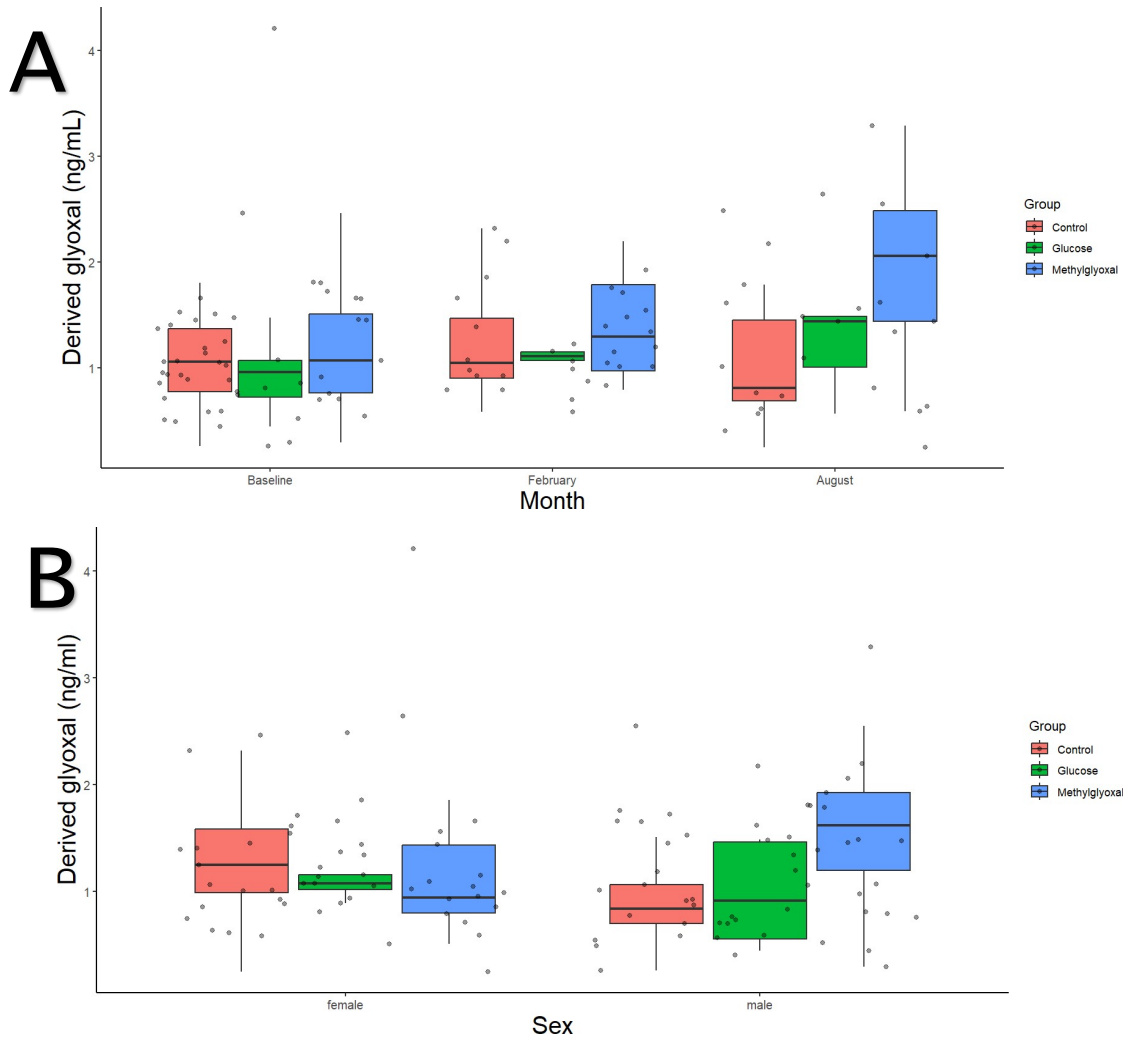
**Figure 3.** Plasma methylglyoxal levels in ng/mL of derived methylglyoxal (see **Methods**) in the different treatment groups over the course of the experiment.

**Table 4. Glyoxal**

	Estimate	Std. Error	df	t value	P-value
Intercept	0.296518	0.227077	67.096458	1.306	0.196079
GroupGlucose	-0.118644	0.108339	72.040294	-1.095	0.277116
<b>GroupMethylglyoxal</b>	<b>-0.156977</b>	<b>0.078169</b>	<b>73.343217</b>	<b>-2.008</b>	<b>0.048306</b>
MonthFebruary	0.004844	0.065552	72.038561	0.074	0.941293
MonthAugust	-0.110814	0.073215	85.088884	-1.514	0.133851
<b>Sexmale</b>	<b>-0.764580</b>	<b>0.260489</b>	<b>65.338149</b>	<b>-2.935</b>	<b>0.004597</b>
Age_years	-0.037320	0.047041	63.696047	-0.793	0.430525
GroupGlucose:MonthFebruary	0.083130	0.136236	78.319279	0.610	0.543503
GroupMethylglyoxal:MonthFebruary	0.072720	0.094657	67.729256	0.768	0.445013
<b>GroupGlucose:MonthAugust</b>	<b>0.274682</b>	<b>0.129487</b>	<b>79.318176</b>	<b>2.121</b>	<b>0.037019</b>
<b>GroupMethylglyoxal:MonthAugust</b>	<b>0.276383</b>	<b>0.102684</b>	<b>73.733622</b>	<b>2.692</b>	<b>0.008795</b>
GroupGlucose:Sexmale	0.159520	0.121557	49.373782	1.312	0.195487
<b>GroupMethylglyoxal:Sexmale</b>	<b>0.383103</b>	<b>0.092623</b>	<b>37.413195</b>	<b>4.136</b>	<b>0.000192</b>
<b>Sexmale:Age_years</b>	<b>0.110748</b>	<b>0.050684</b>	<b>64.898010</b>	<b>2.185</b>	<b>0.032502</b>

Shapiro-Wilk normality test  
 W = 0.9785, p-value = 0.0982

Repeatability for Bird\_ID  
 R = 0.114; SE = 0.115; CI<sub>95</sub>[0, 0.388]; P-value = 0.313



**Figure 4.A.** Plasma methylglyoxal levels in ng/mL of derived methylglyoxal (see **Methods**) in the different treatment groups over the course of the experiment and **B** across sexes.

**Table 5. CML**

	Estimate	Std. Error	df	t value	P-value
<b>Intercept</b>	<b>0.662569</b>	<b>0.013208</b>	<b>47.442379</b>	<b>50.165</b>	<b>&lt; 2*10<sup>-16</sup></b>
GroupGlucose	-0.023035	0.025110	68.137478	-0.917	0.36219
GroupMethylglyoxal	0.024070	0.016670	48.031493	1.444	0.15526
MonthFebruary	0.004709	0.003411	74.691280	1.381	0.17154
MonthAugust	0.007872	0.004280	96.513536	1.839	0.06896
Age_years	<b>0.006364</b>	<b>0.002560</b>	<b>45.843363</b>	<b>2.485</b>	<b>0.01665</b>
GroupGlucose:MonthFebruary	0.012281	0.007044	75.785354	1.744	0.08530
GroupMethylglyoxal:MonthFebruary	<b>0.010337</b>	<b>0.004905</b>	<b>71.044478</b>	<b>2.108</b>	<b>0.03859</b>
GroupGlucose:MonthAugust	<b>0.020589</b>	<b>0.007477</b>	<b>98.768713</b>	<b>2.754</b>	<b>0.00701</b>
GroupMethylglyoxal:MonthAugust	0.006866	0.006014	92.208066	1.142	0.25655
GroupGlucose:Age_years	0.003521	0.005393	66.640445	0.653	0.51613
GroupMethylglyoxal:Age_years	-0.005902	0.003365	46.394521	-1.754	0.08609

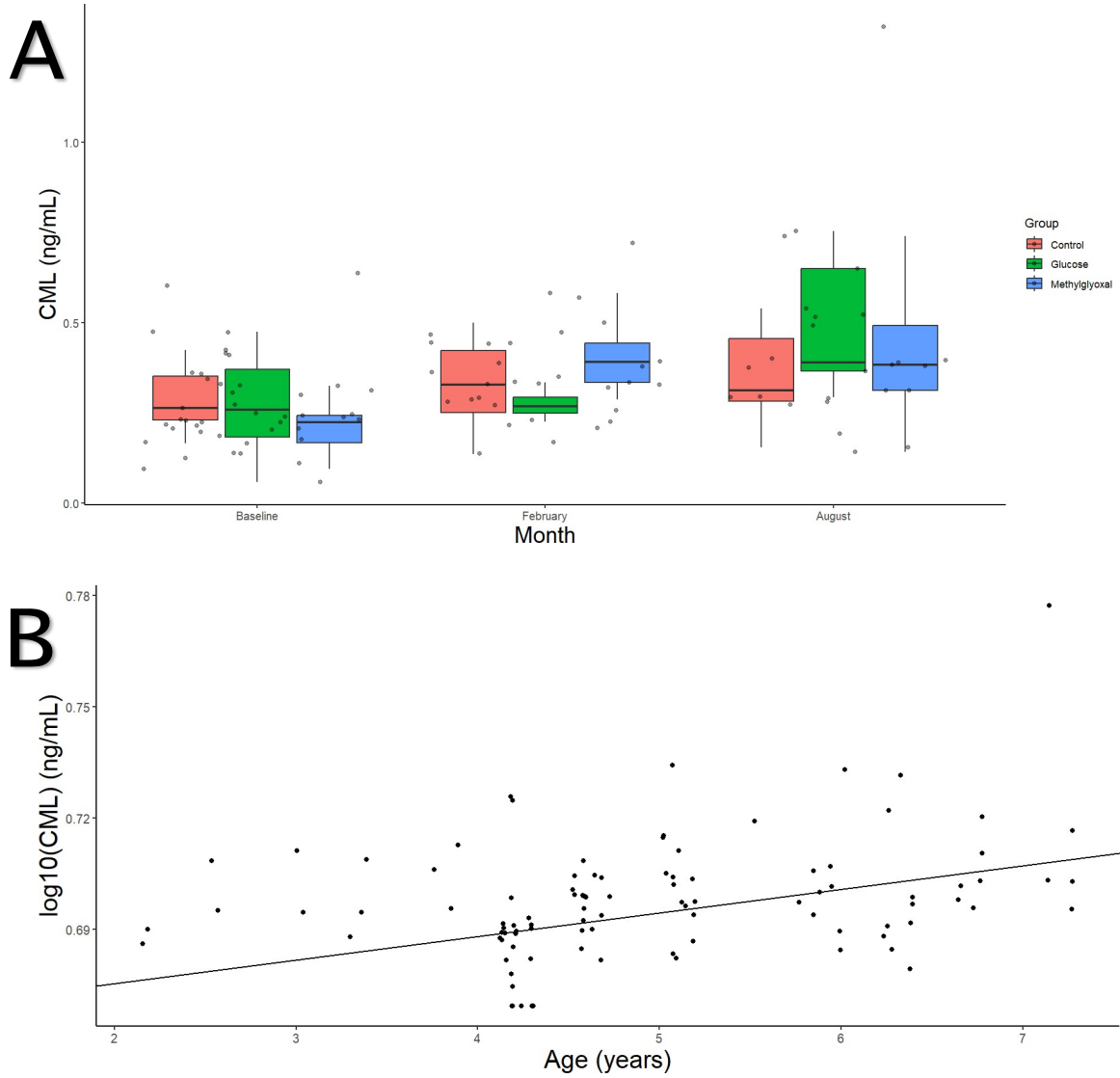


Shapiro-Wilk normality test

W = 0.92305, p-value =  $1.874 \times 10^{-5}$

Repeatability for Bird\_ID

R = 0.52; SE = 0.112; CI<sub>95</sub>[0.263, 0.714]; P-value =  $7.38 \times 10^{-6}$



**Figure 5.A.** Plasma CML levels ng/mL in the different treatment groups over the course of the experiment and **B** plasma log<sub>10</sub>(CML) levels in relation to age in years.

**Table 6. CEL**

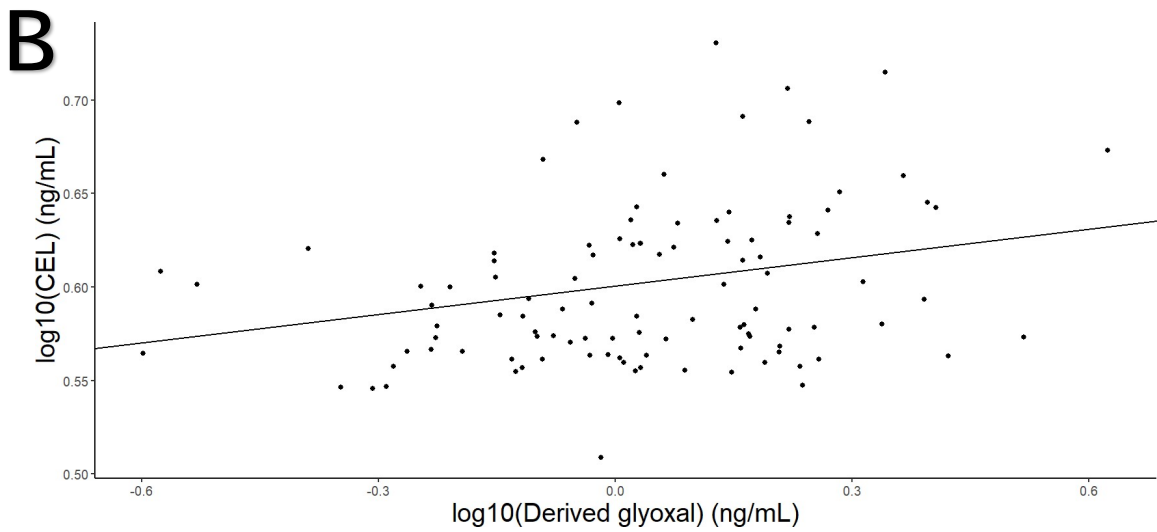
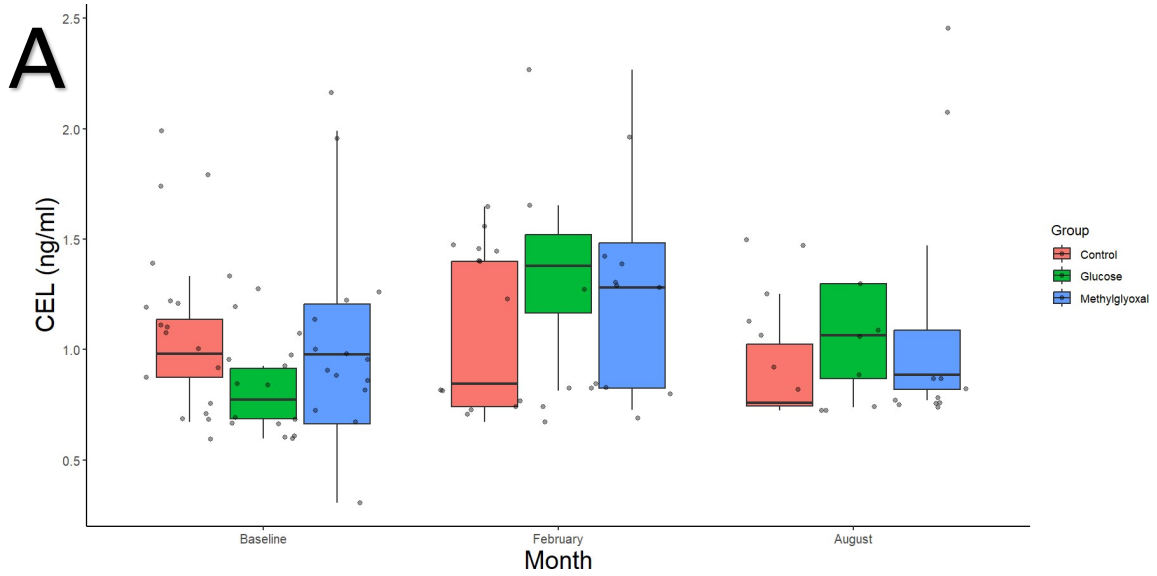
	Estimate	Std. Error	df	t value	P-value
<b>Intercept</b>	<b>0.602318</b>	<b>0.008573</b>	<b>100.917194</b>	<b>70.259</b>	<b>&lt; 2*10<sup>-16</sup></b>
GroupGlucose	-0.014645	0.015113	101.025700	-0.969	0.33484
GroupMethylglyoxal	-0.009591	0.012647	98.131345	-0.758	0.45006
MonthFebruary	-0.003810	0.012475	58.216796	-0.305	0.76117
MonthAugust	-0.006058	0.013913	67.574367	-0.435	0.66463
<b>C_logGlyoxal</b>	<b>0.050705</b>	<b>0.018480</b>	<b>104.976362</b>	<b>2.744</b>	<b>0.00714</b>
GroupGlucose:MonthFebruary	0.041347	0.025598	77.346253	1.615	0.11033
GroupMethylglyoxal:MonthFebruary	0.026054	0.018533	57.239766	1.406	0.16517
GroupGlucose:MonthAugust	0.029389	0.025173	77.369653	1.167	0.24659
GroupMethylglyoxal:MonthAugust	-0.001898	0.021035	63.911451	-0.090	0.92837

Shapiro-Wilk normality test

W = 0.96471, P-value = 0.006767

Repeatability for Bird\_ID

R = 0.14; SE = 0.113; CI<sub>95</sub>[0, 0.38]; P-value = 0.179



**Figure 6.A.** Plasma CEL levels ng/mL in the different treatment groups over the course of the experiment and **B** plasma log<sub>10</sub>(CEL) levels in relation to plasma log<sub>10</sub>(glyoxal) in ng/mL of derived glyoxal (see **Methods**).

**Table 7. Body mass**

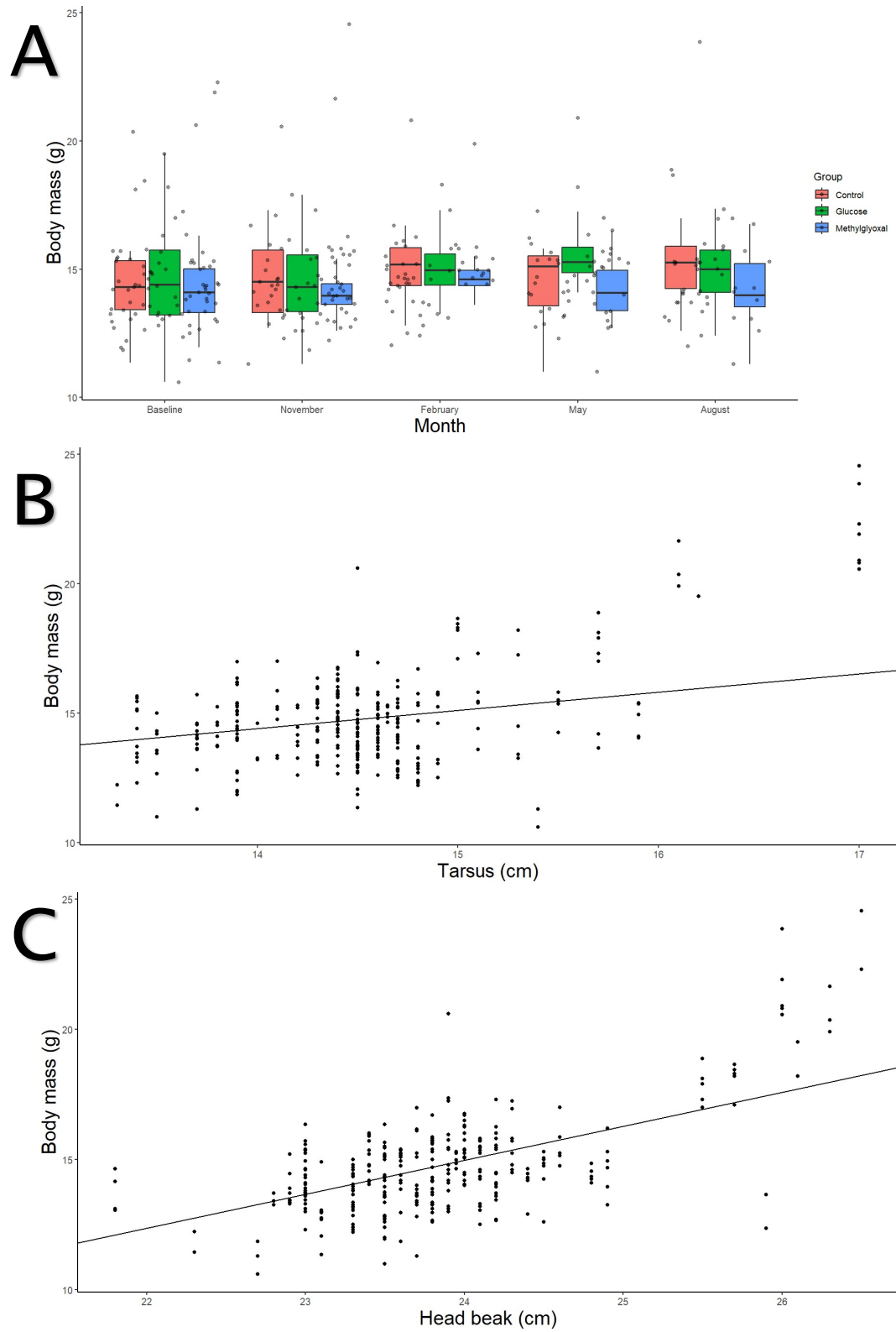
	<b>Estimate</b>	<b>Std. Error</b>	<b>df</b>	<b>t value</b>	<b>P-value</b>
<b>Intercept</b>	<b>14.77</b>	<b>0.2647</b>	<b>161.8</b>	<b>55.818</b>	<b>&lt; 2*10<sup>-16</sup></b>
GroupGlucose	0.3233	0.3384	153.3	0.955	0.34094
GroupMethylglyoxal	0.1301	0.2560	318.2	0.508	0.61156
<i>MonthNovember</i>	<i>0.3459</i>	<i>0.1912</i>	<i>237.8</i>	<i>1.810</i>	<i>0.07163</i>
MonthFebruary	0.3245	0.2126	240.4	1.527	0.12817
MonthMay	0.2284	0.2163	240.5	1.056	0.29213
<b>MonthAugust</b>	<b>0.5703</b>	<b>0.2288</b>	<b>240.6</b>	<b>2.492</b>	<b>0.01337</b>
<b>Sexmale</b>	<b>-0.7306</b>	<b>0.2776</b>	<b>8753</b>	<b>-2.632</b>	<b>0.01003</b>
<b>C_Tarsus</b>	<b>0.7040</b>	<b>0.2330</b>	<b>8811</b>	<b>3.022</b>	<b>0.00329</b>
<b>C_Head_beak</b>	<b>1.304</b>	<b>0.1879</b>	<b>9049</b>	<b>6.940</b>	<b>5.73*10<sup>-10</sup></b>
<i>GroupGlucose:MonthNovember</i>	<i>-0.5280</i>	<i>0.2776</i>	<i>239.9</i>	<i>-1.902</i>	<i>0.05836</i>
GroupMethylglyoxal:MonthNovember	-0.4231	0.2738	238.6	-1.545	0.12363
GroupGlucose:MonthFebruary	-0.4482	0.3438	244.4	-1.304	0.19355
GroupMethylglyoxal:MonthFebruary	4.541*10 <sup>-2</sup>	0.2935	241.1	0.155	0.87715
GroupGlucose:MonthMay	-0.4684	0.3542	244.2	-1.322	0.18736
GroupMethylglyoxal:MonthMay	6.427*10 <sup>-4</sup>	0.3005	241.2	0.002	0.99830
<i>GroupGlucose:MonthAugust</i>	<i>-0.6342</i>	<i>0.3710</i>	<i>243.7</i>	<i>-1.710</i>	<i>0.08862</i>
<i>GroupMethylglyoxal:MonthAugust</i>	<i>-0.5525</i>	<i>0.3146</i>	<i>241.2</i>	<i>-1.756</i>	<i>0.08034</i>

Shapiro-Wilk normality test

W = 0.96665, P-value = 9.392\*10<sup>-7</sup>

Repeatability for Bird\_ID

R = 0.751; SE = 0.039; CI<sub>95</sub>[0.661, 0.818]; P-value = 5.64\*10<sup>-47</sup>



**Figure 7.** Body mass in grams **A** in the different treatment groups over the course of the experiment and in relation to **B** tarsus length and **C** head-beak length.

**Table 8.A. Fat**

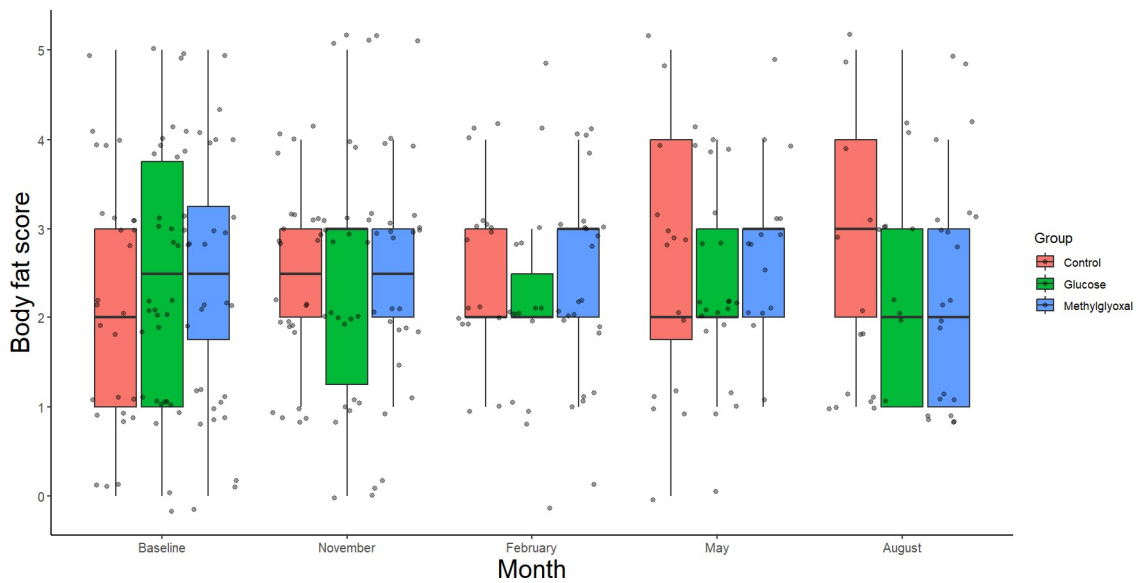
	Estimate	Std. Error	df	t value	P-value
<b>Intercept</b>	<b>2.13636</b>	<b>0.22347</b>	<b>261.55963</b>	<b>9.560</b>	<b>&lt; 2*10<sup>-16</sup></b>
GroupGlucose	0.23031	0.31899	224.34936	0.722	0.47106
GroupMethylglyoxal	0.29120	0.29065	318.04855	1.002	0.31715
<i>MonthNovember</i>	<i>0.46775</i>	<i>0.25190</i>	<i>244.35652</i>	<i>1.857</i>	<i>0.06453</i>
MonthFebruary	0.11925	0.27571	251.38531	0.433	0.66574
MonthMay	0.15803	0.28042	252.16776	0.564	0.57356
<b>MonthAugust</b>	<b>0.70917</b>	<b>0.29652</b>	<b>253.25963</b>	<b>2.392</b>	<b>0.01750</b>
GroupGlucose:MonthNovember	-0.45612	0.36258	247.21457	-1.258	0.20958
GroupMethylglyoxal:MonthNovember	-0.51241	0.35819	247.45201	-1.431	0.15382
GroupGlucose:MonthFebruary	-0.44241	0.44287	260.33205	-0.999	0.31874
GroupMethylglyoxal:MonthFebruary	-0.21420	0.37997	253.42530	-0.564	0.57343
GroupGlucose:MonthMay	-0.24771	0.45624	260.71338	-0.543	0.58764
GroupMethylglyoxal:MonthMay	-0.06852	0.38887	254.33870	-0.176	0.86028
<b>GroupGlucose:MonthAugust</b>	<b>-1.12436</b>	<b>0.47803</b>	<b>260.43499</b>	<b>-2.352</b>	<b>0.01941</b>
<b>GroupMethylglyoxal:MonthAugust</b>	<b>-1.25728</b>	<b>0.40701</b>	<b>255.31493</b>	<b>-3.089</b>	<b>0.00223</b>

Shapiro-Wilk normality test

W = 0.99711, P-value = 0.8419

Repeatability for Bird\_ID

R = 0.461; SE = 0.065; CI<sub>95</sub>[0.319, 0.57]; P-value = 2.13\*10<sup>-18</sup>



**Figure 8.** Body fat score in the different treatment groups over the course of the experiment.

**Table 8.B. Muscle**

	Estimate	Std. Error	df	t value	P-value
<b>Intercept</b>	<b>2.29179</b>	<b>0.10191</b>	<b>241.60503</b>	<b>22.488</b>	<b>&lt;2*10<sup>-16</sup></b>
GroupGlucose	0.04154	0.14714	196.85824	0.282	0.778
GroupMethylglyoxal	0.09352	0.12926	319.42598	0.723	0.470
MonthNovember	0.06892	0.10790	238.51565	0.639	0.524
MonthFebruary	0.10324	0.11831	244.31700	0.873	0.384
MonthMay	0.12117	0.12035	244.88735	1.007	0.315
MonthAugust	-0.11247	0.12729	245.51982	-0.884	0.378
GroupGlucose:MonthNovember	-0.18307	0.15543	241.55665	-1.178	0.240
GroupMethylglyoxal:MonthNovember	0.13171	0.15354	240.80224	0.858	0.392
GroupGlucose:MonthFebruary	0.12160	0.19047	251.98031	0.638	0.524
GroupMethylglyoxal:MonthFebruary	0.05899	0.16313	245.98092	0.362	0.718
GroupGlucose:MonthMay	-0.04100	0.19623	252.14609	-0.209	0.835
GroupMethylglyoxal:MonthMay	0.05089	0.16698	246.59190	0.305	0.761
GroupGlucose:MonthAugust	-0.01669	0.20558	251.68043	-0.081	0.935
GroupMethylglyoxal:MonthAugust	0.18350	0.17481	247.17496	1.050	0.295

Shapiro-Wilk normality test

W = 0.98377, P-value = 0.001095

Repeatability for Bird\_ID

R = 0.542; SE = 0.059; CI<sub>95</sub>[0.414, 0.649]; P-value = 5.86\*10<sup>-22</sup>

**Table 9.A.1. Body composition: carcass fat**

	Estimate	Std. Error	t value	P-value
<b>Intercept</b>	<b>-0.474844</b>	<b>0.041945</b>	<b>-11.321</b>	<b>9.74*10<sup>-14</sup></b>
<b>C_logDry_mass</b>	<b>2.235854</b>	<b>0.221611</b>	<b>10.089</b>	<b>2.67*10<sup>-12</sup></b>
GroupGlucose	0.034724	0.046355	0.749	0.458
GroupMethylglyoxal	0.007832	0.057608	0.136	0.893
Sexmale	-0.021303	0.044012	-0.484	0.631

Residual standard error: 0.1347 on 38 degrees of freedom

Multiple R<sup>2</sup>: 0.7594, Adjusted R<sup>2</sup>: 0.7341

F-statistic: 29.99 on 4 and 38 DF, P-value: 2.701\*10<sup>-11</sup>

Shapiro-Wilk normality test

W = 0.94737, P-value = 0.0478

**Table 9.A.2. Body composition: carcass water**

	Estimate	Std. Error	t value	P-value
<b>Intercept</b>	<b>0.734821</b>	<b>0.006877</b>	<b>106.855</b>	<b>&lt;2*10<sup>-16</sup></b>
<b>C_logFresh_mass</b>	<b>0.974862</b>	<b>0.047286</b>	<b>20.616</b>	<b>&lt;2*10<sup>-16</sup></b>
<i>Sexmale</i>	<i>0.013198</i>	<i>0.007184</i>	<i>1.837</i>	<i>0.0742</i>
GroupGlucose	0.008910	0.007805	1.141	0.2610
GroupMethylglyoxal	0.006292	0.009549	0.659	0.5141
C_logFresh_mass:Sexmale	0.147045	0.091405	1.609	0.1162

Residual standard error: 0.02247 on 37 degrees of freedom

Multiple R<sup>2</sup>: 0.9475, Adjusted R<sup>2</sup>: 0.9404

F-statistic: 133.5 on 5 and 37 DF, P-value: < 2.2\*10<sup>-16</sup>

Shapiro-Wilk normality test

W = 0.96823, P-value = 0.2744

**Table 9.B.1. Body composition: liver fat**

	Estimate	Std. Error	t value	P-value
<b>Intercept</b>	<b>-1.57189</b>	<b>0.02824</b>	<b>-55.670</b>	<b>&lt; 2*10<sup>-16</sup></b>
<b>C_logDry_mass</b>	<b>0.51579</b>	<b>0.08957</b>	<b>5.758</b>	<b>1.33*10<sup>-6</sup></b>
GroupGlucose	0.02540	0.03230	0.786	0.437
GroupMethylglyoxal	0.01651	0.03896	0.424	0.674
Sexmale	-0.03125	0.02972	-1.052	0.300

Residual standard error: 0.09211 on 37 degrees of freedom

Multiple R<sup>2</sup>: 0.5304, Adjusted R<sup>2</sup>: 0.4796

F-statistic: 10.45 on 4 and 37 DF, P-value: 9.146\*10<sup>-6</sup>

Shapiro-Wilk normality test

W = 0.96202, P-value = 0.1743

**Table 9.B.2. Body composition: liver water**

	Estimate	Std. Error	t value	P-value
<b>Intercept</b>	<b>-0.585973</b>	<b>0.003656</b>	<b>-160.264</b>	<b>&lt;2*10<sup>-16</sup></b>
<b>C_logFresh_mass</b>	<b>0.993673</b>	<b>0.014416</b>	<b>68.926</b>	<b>&lt;2*10<sup>-16</sup></b>
GroupGlucose	-0.008036	0.005097	-1.577	0.123
GroupMethylglyoxal	-0.004484	0.006092	-0.736	0.466

Residual standard error: 0.01461 on 38 degrees of freedom

Multiple R<sup>2</sup>: 0.9921, Adjusted R<sup>2</sup>: 0.9915

F-statistic: 1586 on 3 and 38 DF, P-value: < 2.2\*10<sup>-16</sup>

Shapiro-Wilk normality test

W = 0.97786, P-value = 0.5799

**Table 9.C.1. Body composition: muscle (pectorals) fat**

	Estimate	Std. Error	t value	P-value
<b>Intercept</b>	<b>-1.29958</b>	<b>0.03437</b>	<b>-37.809</b>	<b>&lt; 2*10<sup>-16</sup></b>
<b>C_logDry_mass</b>	<b>0.93117</b>	<b>0.11247</b>	<b>8.279</b>	<b>4.92*10<sup>-10</sup></b>
GroupGlucose	0.01706	0.03924	0.435	0.6663
GroupMethylglyoxal	-0.03274	0.04652	-0.704	0.4858
<b>Sexmale</b>	<b>-0.07614</b>	<b>0.03445</b>	<b>-2.210</b>	<b>0.0332</b>

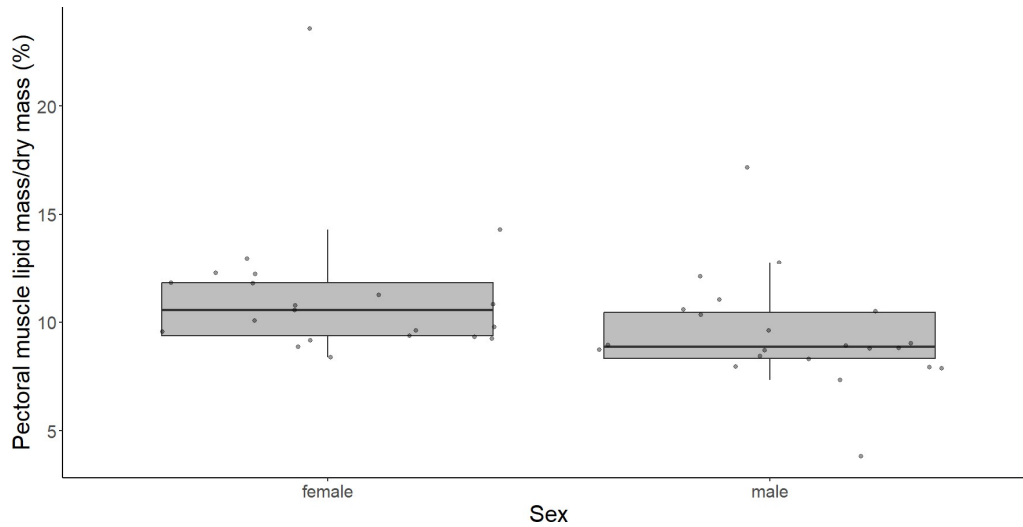
Residual standard error: 0.1112 on 38 degrees of freedom

Multiple R<sup>2</sup>: 0.6725, Adjusted R<sup>2</sup>: 0.6381

F-statistic: 19.51 on 4 and 38 DF, P-value: 8.464\*10<sup>-9</sup>

Shapiro-Wilk normality test

W = 0.87466, P-value = 0.0002322



**Figure 9.** Pectoral muscle lipid mass in birds dead during the experiment as a percentage of total dry mass across sexes.

**Table 9.C.2. Body composition: muscle (pectorals) water**

	Estimate	Std. Error	t value	P-value
<b>Intercept</b>	<b>0.122431</b>	<b>0.003093</b>	<b>39.584</b>	<b>&lt;2*10<sup>-16</sup></b>
<b>C_logFresh_mass</b>	<b>1.011731</b>	<b>0.012237</b>	<b>82.676</b>	<b>&lt;2*10<sup>-16</sup></b>
GroupGlucose	-0.003535	0.004305	-0.821	0.417
GroupMethylglyoxal	-0.002117	0.005123	-0.413	0.682

Residual standard error: 0.0123 on 39 degrees of freedom

Multiple R<sup>2</sup>: 0.9946, Adjusted R<sup>2</sup>: 0.9942

F-statistic: 2380 on 3 and 39 DF, P-value: < 2.2\*10<sup>-16</sup>

Shapiro-Wilk normality test

W = 0.92583, p-value = 0.008387

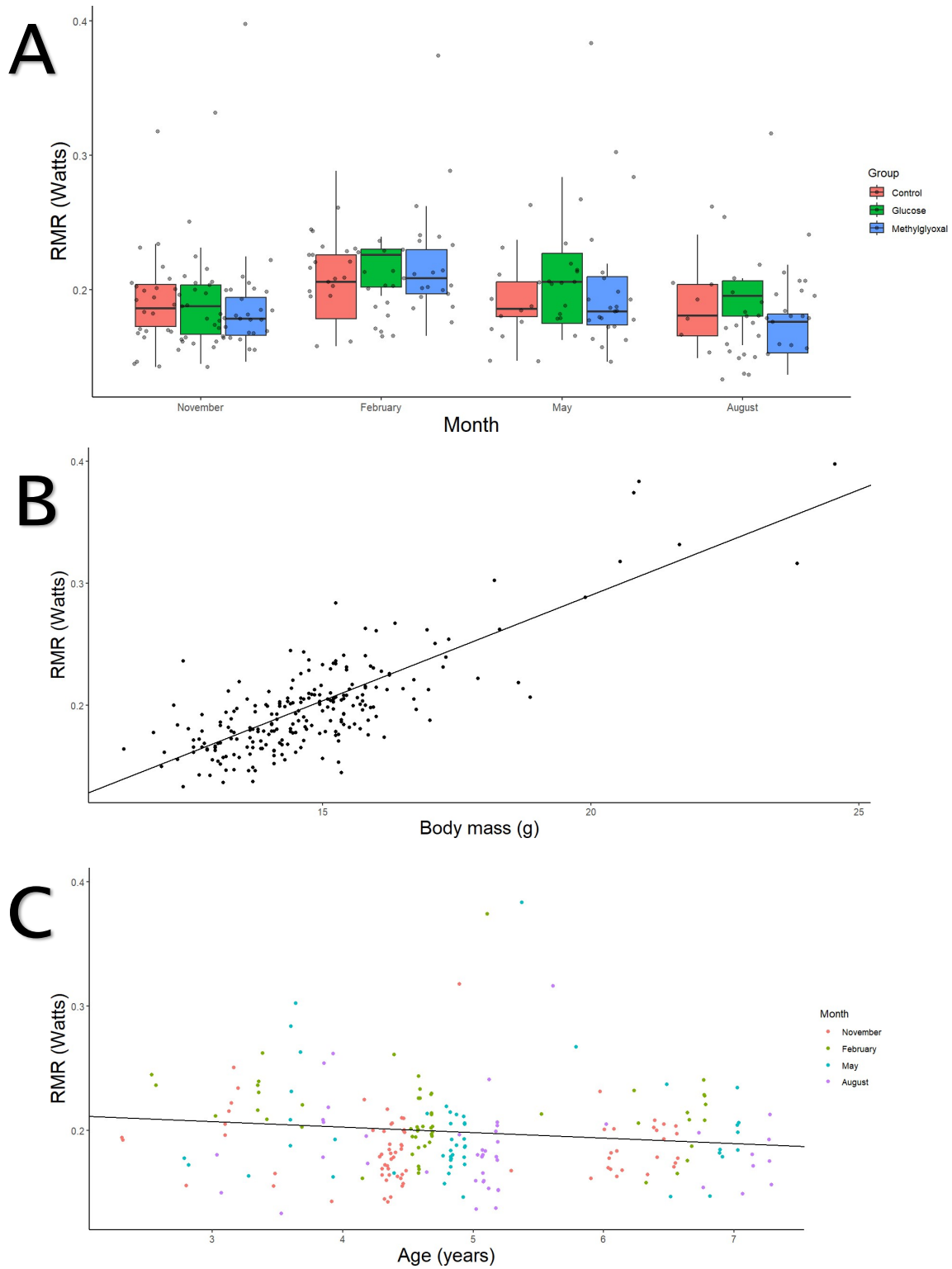
**Table 10.A. Metabolism: RMR.**

	Estimate	Std. Error	df	t value	P-value
<b>Intercept</b>	<b>0.220360</b>	<b>0.009016</b>	<b>87.110422</b>	<b>24.442</b>	<b>&lt; 2*10<sup>-16</sup> ***</b>
GroupGlucose	-0.006311	0.005752	192.221197	-1.097	0.27395
GroupMethylglyoxal	-0.005556	0.005700	200.403718	-0.975	0.33083
<i>MonthFebruary</i>	<i>0.009128</i>	<i>0.005147</i>	<i>170.811341</i>	<i>1.773</i>	<i>0.07796</i>
MonthMay	0.008151	0.005411	177.811181	1.506	0.13374
MonthAugust	-0.005683	0.005678	186.553563	-1.001	0.31818
<i>Sexmale</i>	<i>-0.005756</i>	<i>0.003345</i>	<i>66.334275</i>	<i>-1.721</i>	<i>0.08994</i>
<b>Age_years</b>	<b>-0.004418</b>	<b>0.001530</b>	<b>70.403647</b>	<b>-2.887</b>	<b>0.00516</b>
<b>C_BM</b>	<b>0.017283</b>	<b>0.001991</b>	<b>162.596394</b>	<b>8.681</b>	<b>3.94*10<sup>-15</sup></b>
<b>GroupGlucose:MonthFebruary</b>	<b>0.016600</b>	<b>0.008378</b>	<b>182.350765</b>	<b>1.981</b>	<b>0.04906</b>
<b>GroupMethylglyoxal:MonthFebruary</b>	<b>0.015917</b>	<b>0.007245</b>	<b>170.500365</b>	<b>2.197</b>	<b>0.02936</b>
GroupGlucose:MonthMay	0.001453	0.008823	185.673462	0.165	0.86940
GroupMethylglyoxal:MonthMay	0.001915	0.007498	174.712806	0.255	0.79867
GroupGlucose:MonthAugust	0.007491	0.009097	184.631355	0.823	0.41130
GroupMethylglyoxal:MonthAugust	-0.006725	0.007834	176.416616	-0.858	0.39182
<b>GroupGlucose:C_BM</b>	<b>-0.008210</b>	<b>0.002579</b>	<b>116.264557</b>	<b>-3.184</b>	<b>0.00186</b>
GroupMethylglyoxal:C_BM	-0.002943	0.002524	87.107451	-1.166	0.24675
MonthFebruary:C_BM	0.002783	0.002269	177.965437	1.227	0.22148
<b>MonthMay:C_BM</b>	<b>0.005920</b>	<b>0.002243</b>	<b>179.711212</b>	<b>2.639</b>	<b>0.00905</b>
MonthAugust:C_BM	-0.002122	0.002008	183.910981	-1.056	0.29221



Shapiro-Wilk normality test  
W = 0.9884, P-value = 0.0658

Repeatability for Bird\_ID  
R = 0.262; SE = 0.079 ; CI<sub>95</sub>[0.099, 0.412] ; P-value = 0.00015



**Figure 10.** A RMR in Watts of birds in the different treatment groups over the course of the experiment, B in relation to body mass in grams and C in relation to age in years (measurement months indicated).

**Table 10.B. Metabolism: RER.**

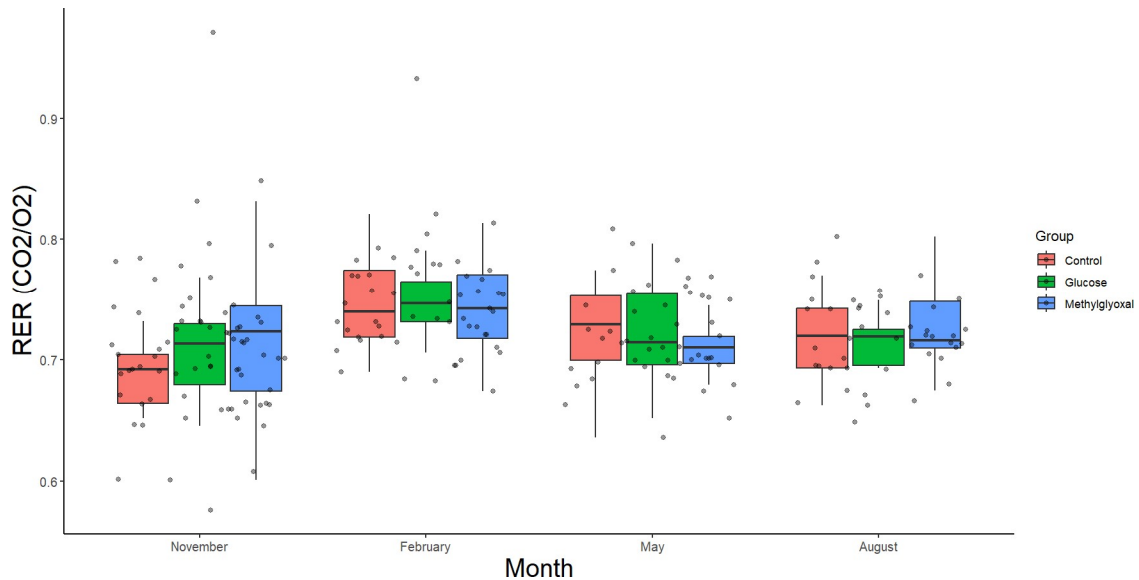
	Estimate	Std. Error	df	t value	P-value
<b>Intercept</b>	<b>0.692573</b>	<b>0.009047</b>	<b>217.354230</b>	<b>76.551</b>	<b>&lt; 2*10<sup>-16</sup></b>
GroupGlucose	0.025571	0.013217	218.914481	1.935	0.054313
GroupMethylglyoxal	0.022996	0.012664	219.282688	1.816	0.070760
<b>MonthFebruary</b>	<b>0.052800</b>	<b>0.013229</b>	<b>135.617890</b>	<b>3.991</b>	<b>0.000107</b>
<b>MonthMay</b>	<b>0.030724</b>	<b>0.014070</b>	<b>149.415687</b>	<b>2.184</b>	<b>0.030542</b>
MonthAugust	0.022409	0.014070	149.415687	1.593	0.113338
GroupGlucose:MonthFebruary	-0.021330	0.021174	146.559429	-1.007	0.315416
GroupMethylglyoxal:MonthFebruary	-0.019982	0.018386	126.350875	-1.087	0.279184
GroupGlucose:MonthMay	-0.026599	0.022132	155.342112	-1.202	0.231248
GroupMethylglyoxal:MonthMay	-0.030060	0.019124	144.862669	-1.572	0.118176
GroupGlucose:MonthAugust	-0.028463	0.022638	158.863006	-1.257	0.210481
GroupMethylglyoxal:MonthAugust	-0.011480	0.019659	151.695414	-0.584	0.560111

Shapiro-Wilk normality test

W = 0.77914, P-value < 2.2\*10<sup>-16</sup>

Repeatability for Bird\_ID

R = 0; SE = 0.039; CI<sub>95</sub>[0, 0.129]; P-value = 1



**Figure 11.** RER in the different treatment groups over the course of the experiment.

**Table 11.A. Oxidative status: OXY.**

	Estimate	Std. Error	df	t value	P-value
<b>Intercept</b>	<b>266.2003</b>	<b>11.7899</b>	<b>176.3853</b>	<b>22.579</b>	<b>&lt;2*10<sup>-16</sup></b>
GroupGlucose	3.3987	14.0150	175.2462	0.243	0.8087
GroupMethylglyoxal	0.5595	13.8029	174.8362	0.041	0.9677
MonthFebruary	5.6744	16.7976	138.9939	0.338	0.7360
MonthAugust	7.1485	17.8491	144.4146	0.400	0.6894
Sexmale	3.4090	11.3076	174.8306	0.301	0.7634
GroupGlucose:MonthFebruary	-7.5275	22.8787	149.8539	-0.329	0.7426
GroupMethylglyoxal:MonthFebruary	4.8159	19.7597	134.1410	0.244	0.8078
GroupGlucose:MonthAugust	12.4857	24.2438	152.6500	0.515	0.6073
<b>GroupMethylglyoxal:MonthAugust</b>	<b>-43.5021</b>	<b>20.5763</b>	<b>137.1904</b>	<b>-2.114</b>	<b>0.0363</b>
MonthFebruary:Sexmale	-12.7519	16.9684	139.7647	-0.752	0.4536
<b>MonthAugust:Sexmale</b>	<b>-40.2382</b>	<b>18.0965</b>	<b>144.8428</b>	<b>-2.224</b>	<b>0.0277</b>

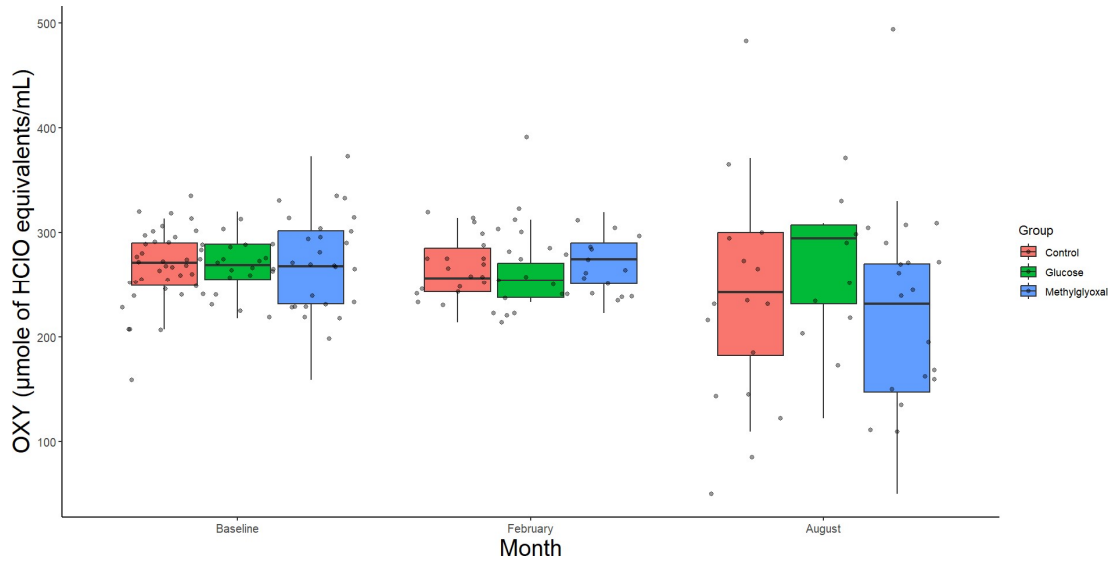
Shapiro-Wilk normality test

W = 0.93092, P-value = 1.362\*10<sup>-7</sup>

Repeatability for Bird\_ID

R = 0.201; SE = 0.097; CI<sub>95</sub>[0, 0.387]; P-value = 0.00328

**A**



**B**

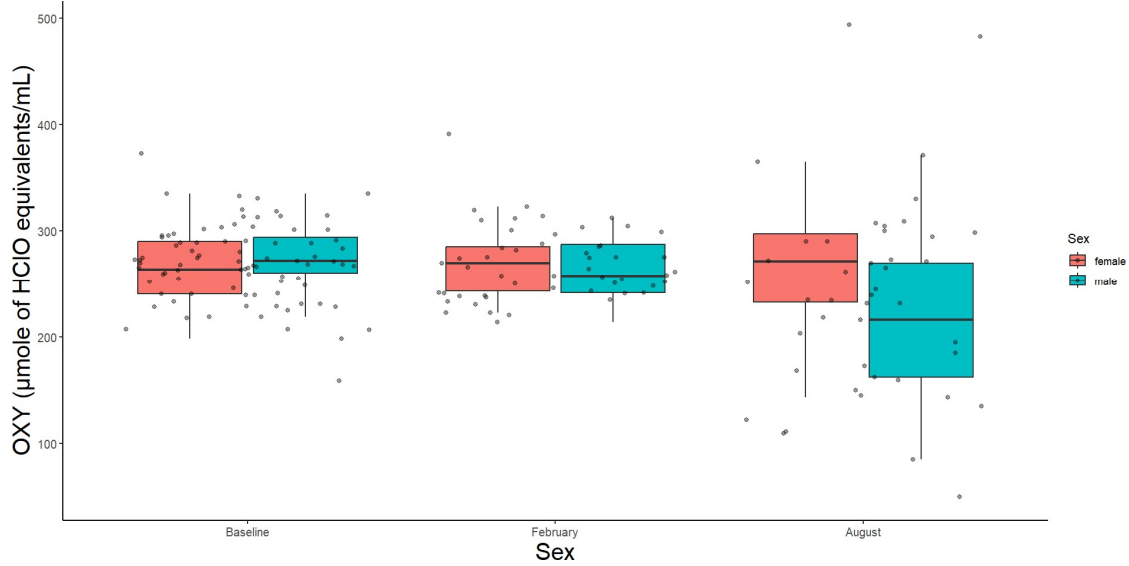


Figure 12.A. OXY values in the different treatment groups and B across sex over the course of the experiment.

**Table 11.B. Oxidative status: d-ROMs.**

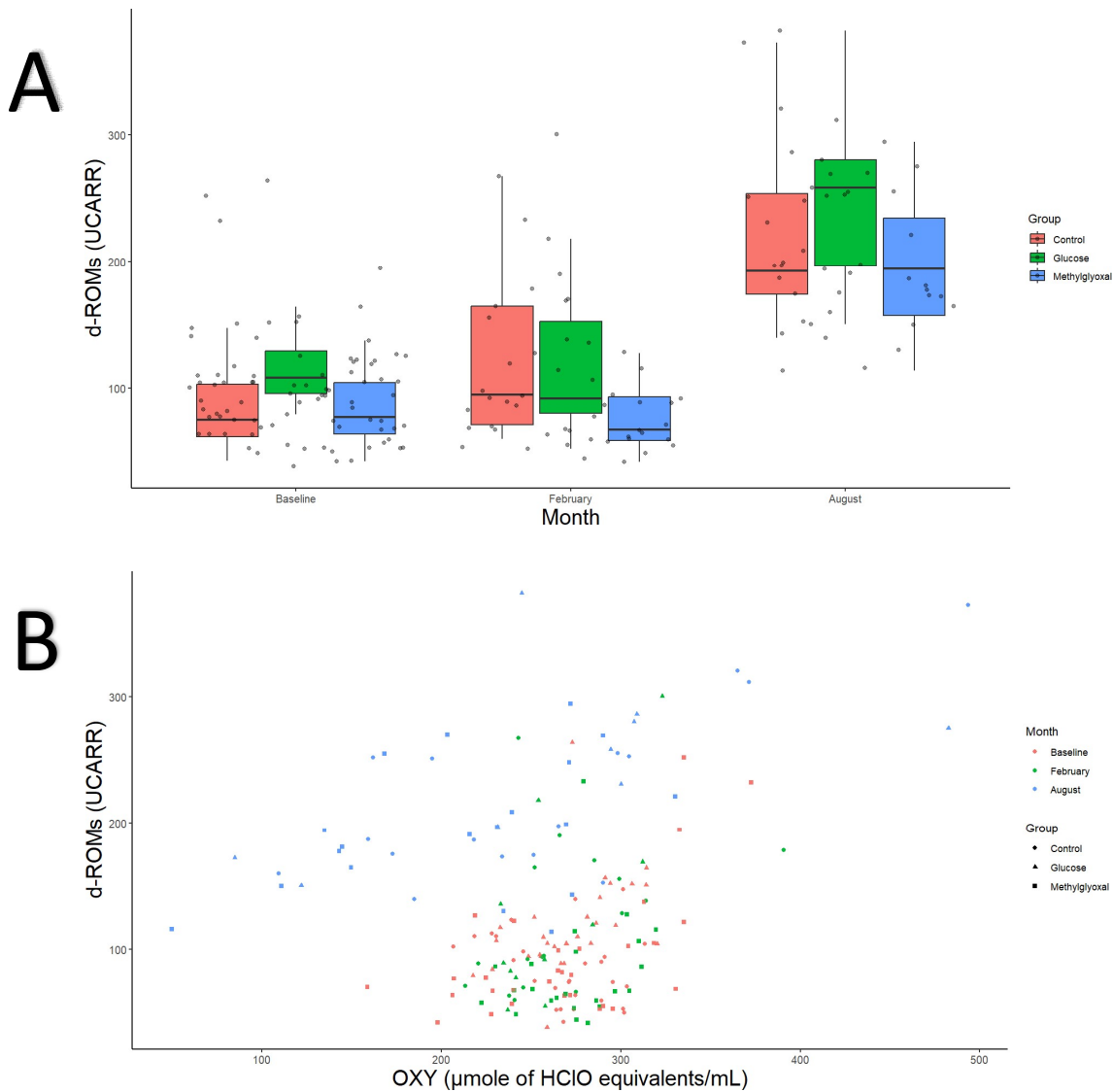
	<b>Estimate</b>	<b>Std. Error</b>	<b>df</b>	<b>t value</b>	<b>P-value</b>
<b>Intercept</b>	<b>1.927</b>	<b>3.096*10<sup>-2</sup></b>	<b>1.782*10<sup>2</sup></b>	<b>62.252</b>	<b>&lt; 2*10<sup>-16</sup></b>
<b>GroupGlucose</b>	<b>1.104*10<sup>-1</sup></b>	<b>3.743*10<sup>-2</sup></b>	<b>1.775*10<sup>2</sup></b>	<b>2.949</b>	<b>0.003617</b>
GroupMethylglyoxal	-1.310*10 <sup>-3</sup>	3.597*10 <sup>-2</sup>	1.774*10 <sup>2</sup>	-0.036	0.970985
<b>MonthFebruary</b>	<b>1.716*10<sup>-1</sup></b>	<b>4.640*10<sup>-2</sup></b>	<b>1.086*10<sup>2</sup></b>	<b>3.698</b>	<b>0.000343</b>
<b>MonthAugust</b>	<b>3.795*10<sup>-1</sup></b>	<b>4.827*10<sup>-2</sup></b>	<b>1.180*10<sup>2</sup></b>	<b>7.862</b>	<b>1.98*10<sup>-12</sup></b>
<i>C_OXY</i>	-1.540*10 <sup>-3</sup>	9.160*10 <sup>-4</sup>	1.785*10 <sup>2</sup>	-1.681	0.094567
Sexmale	-2.442*10 <sup>-2</sup>	2.891*10 <sup>-2</sup>	1.768*10 <sup>2</sup>	-0.845	0.399368
GroupGlucose:MonthFebruary	-8.685*10 <sup>-2</sup>	6.178*10 <sup>-2</sup>	1.201*10 <sup>2</sup>	-1.406	0.162357
<b>GroupMethylglyoxal:MonthFebruary</b>	<b>-1.574*10<sup>-1</sup></b>	<b>5.561*10<sup>-2</sup></b>	<b>1.054*10<sup>2</sup></b>	<b>-2.830</b>	<b>0.005571</b>
GroupGlucose:MonthAugust	-7.881*10 <sup>-2</sup>	6.530*10 <sup>-2</sup>	1.275*10 <sup>2</sup>	-1.207	0.229688
GroupMethylglyoxal:MonthAugust	-2.960*10 <sup>-2</sup>	5.986*10 <sup>-2</sup>	1.175*10 <sup>2</sup>	-0.494	0.621924
<b>GroupGlucose:C_OXY</b>	<b>4.166*10<sup>-3</sup></b>	<b>1.319*10<sup>-3</sup></b>	<b>1.800*10<sup>2</sup></b>	<b>3.159</b>	<b>0.001860</b>
<b>GroupMethylglyoxal:C_OXY</b>	<b>3.865*10<sup>-3</sup></b>	<b>1.044*10<sup>-3</sup></b>	<b>1.781*10<sup>2</sup></b>	<b>3.703</b>	<b>0.000284</b>
<b>MonthFebruary:C_OXY</b>	<b>3.013*10<sup>-3</sup></b>	<b>1.222*10<sup>-3</sup></b>	<b>1.787*10<sup>2</sup></b>	<b>2.467</b>	<b>0.014580</b>
<b>MonthAugust:C_OXY</b>	<b>2.678*10<sup>-3</sup></b>	<b>9.794*10<sup>-4</sup></b>	<b>1.728*10<sup>2</sup></b>	<b>2.734</b>	<b>0.006909</b>
<b>MonthFebruary:Sexmale</b>	<b>-1.235*10<sup>-1</sup></b>	<b>4.701*10<sup>-2</sup></b>	<b>1.134*10<sup>2</sup></b>	<b>-2.626</b>	<b>0.009819</b>
<i>MonthAugust:Sexmale</i>	8.513*10 <sup>-2</sup>	4.975*10 <sup>-2</sup>	1.228*10 <sup>2</sup>	1.711	0.089572
GroupGlucose:MonthFebruary:C_OXY	-1.041*10 <sup>-3</sup>	2.017*10 <sup>-3</sup>	1.772*10 <sup>2</sup>	-0.516	0.606353
<b>GroupMethylglyoxal:MonthFebruary:C_OXY</b>	<b>-3.437*10<sup>-3</sup></b>	<b>1.691*10<sup>-3</sup></b>	<b>1.747*10<sup>2</sup></b>	<b>-2.032</b>	<b>0.043661</b>
<b>GroupGlucose:MonthAugust:C_OXY</b>	<b>-4.573*10<sup>-3</sup></b>	<b>1.417*10<sup>-3</sup></b>	<b>1.777*10<sup>2</sup></b>	<b>-3.227</b>	<b>0.001491</b>
<b>GroupMethylglyoxal:MonthAugust:C_OXY</b>	<b>-4.319*10<sup>-3</sup></b>	<b>1.175*10<sup>-3</sup></b>	<b>1.675*10<sup>2</sup></b>	<b>-3.675</b>	<b>0.000320</b>

Shapiro-Wilk normality test

W = 0.98738, P-value = 0.1076

Repeatability for Bird\_ID

R = 0.082; SE = 0.085; CI<sub>95</sub>[0, 0.291]; P-value = 0.215



**Figure 13.A.** d-ROMs values in Carratelli Units (UCARR) in the different treatment groups and **B** in relation to OXY over the course of the experiment.

**Table 11.C. Oxidative status: Protein carbonyls.**

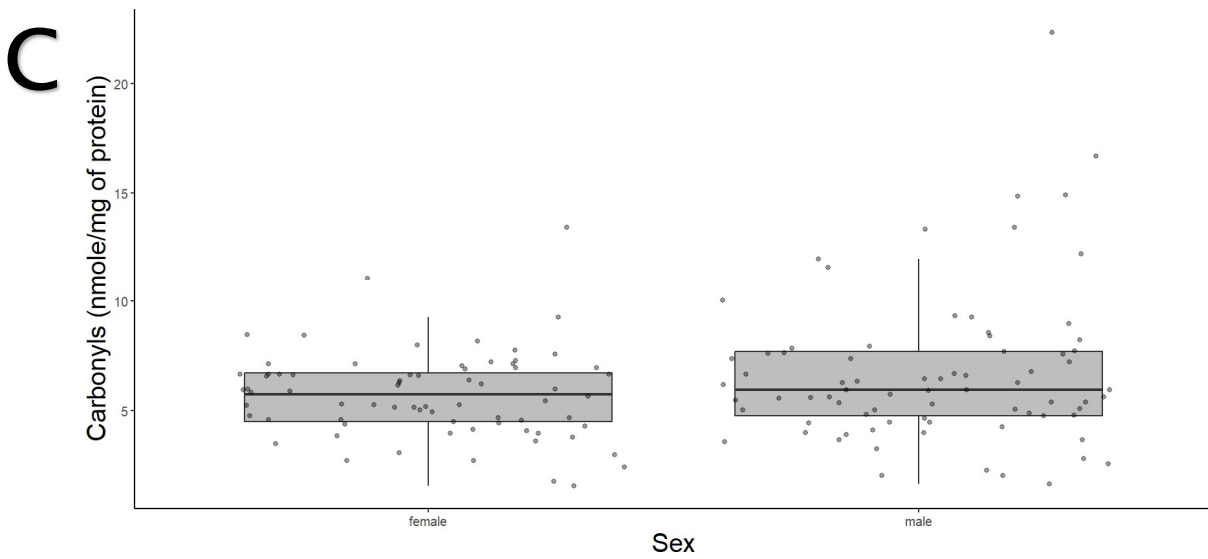
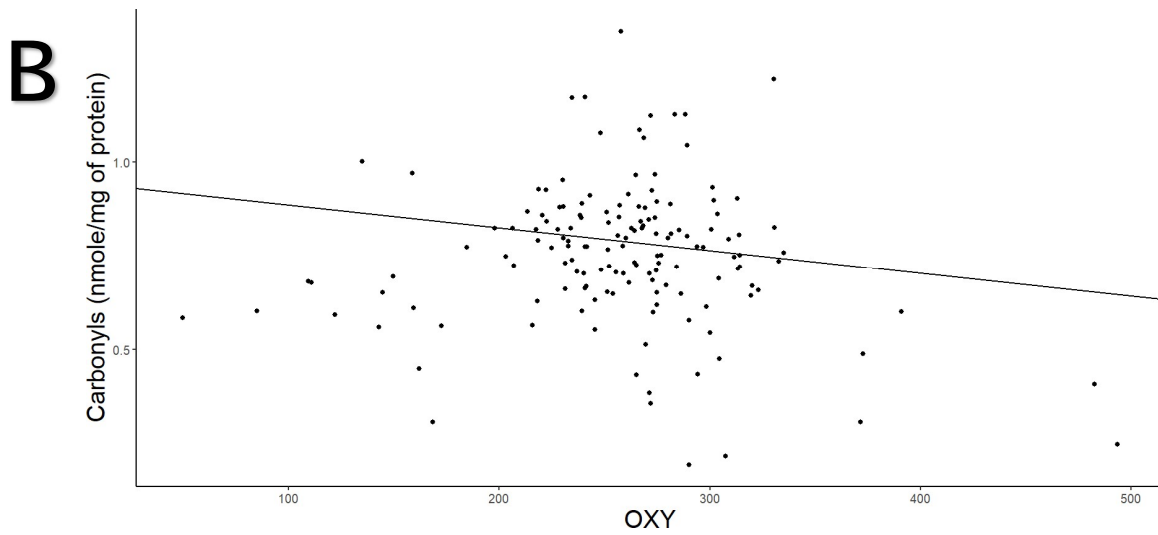
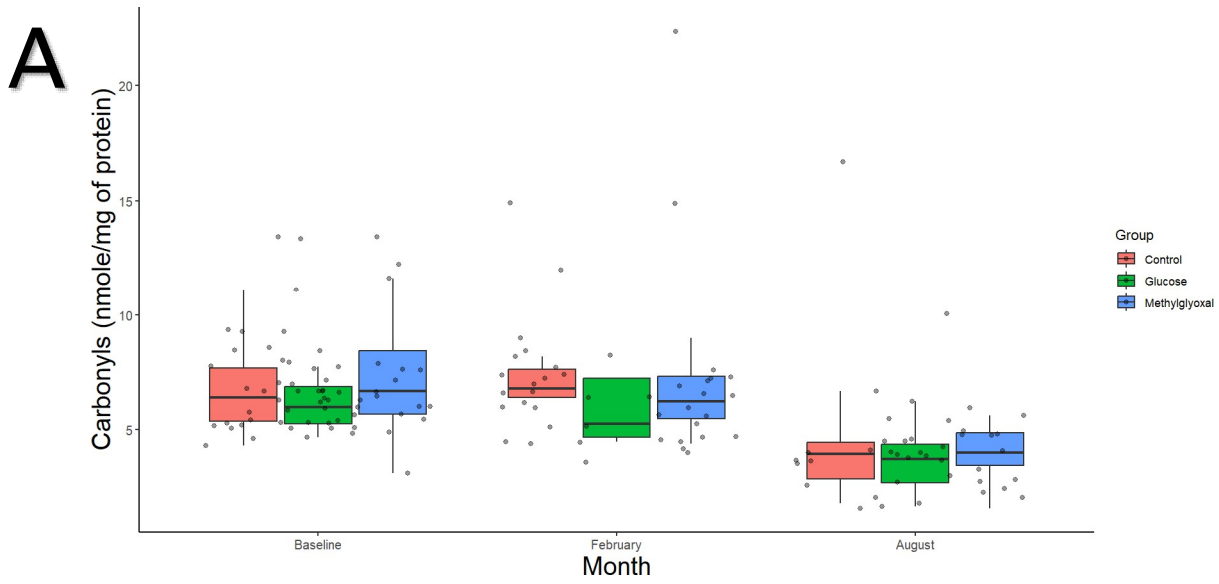
	Estimate	Std. Error	df	t value	P-value
<b>Intercept</b>	<b>9.475*10<sup>-1</sup></b>	<b>6.933*10<sup>-2</sup></b>	<b>1.489*10<sup>2</sup></b>	<b>13.665</b>	<b>&lt; 2*10<sup>-16</sup></b>
GroupGlucose	3.756*10 <sup>-3</sup>	4.648*10 <sup>-2</sup>	1.461*10 <sup>2</sup>	0.081	0.93571
GroupMethylglyoxal	2.013*10 <sup>-2</sup>	4.398*10 <sup>-2</sup>	1.466*10 <sup>2</sup>	0.458	0.64790
MonthFebruary	2.202*10 <sup>-2</sup>	4.764*10 <sup>-2</sup>	1.144*10 <sup>2</sup>	0.462	0.64490
<b>MonthAugust</b>	<b>-2.627*10<sup>-1</sup></b>	<b>4.829*10<sup>-2</sup></b>	<b>1.244*10<sup>2</sup></b>	<b>-5.439</b>	<b>2.73*10<sup>-7</sup></b>
<b>OXY</b>	<b>-6.108*10<sup>-4</sup></b>	<b>2.157*10<sup>-4</sup></b>	<b>1.481*10<sup>2</sup></b>	<b>-2.832</b>	<b>0.00526</b>
<b>Sexmale</b>	<b>5.463*10<sup>-2</sup></b>	<b>2.722*10<sup>-2</sup></b>	<b>80.16</b>	<b>2.007</b>	<b>0.04814</b>
GroupGlucose:MonthFebruary	-1.370*10 <sup>-2</sup>	7.302*10 <sup>-2</sup>	1.195*10 <sup>2</sup>	-0.188	0.85154
GroupMethylglyoxal:MonthFebruary	-5.612*10 <sup>-2</sup>	6.245*10 <sup>-2</sup>	1.103*10 <sup>2</sup>	-0.899	0.37082
GroupGlucose:MonthAugust	-3.657*10 <sup>-2</sup>	7.555*10 <sup>-2</sup>	1.243*10 <sup>2</sup>	-0.484	0.62924
GroupMethylglyoxal:MonthAugust	9.642*10 <sup>-3</sup>	6.372*10 <sup>-2</sup>	1.173*10 <sup>2</sup>	0.151	0.87998

Shapiro-Wilk normality test

W = 0.94367, P-value = 1.078\*10<sup>-5</sup>

Repeatability for Bird\_ID

R = 0.213; SE = 0.109; CI<sub>95</sub>[0, 0.417]; P-value = 0.0145



**Figure 14.A.** Plasma carbonyl levels in mmole/mg of protein in the different treatment groups over the course of the experiment, **B** in relation to OXY and **C** in relation to sex.

**Table 11.D. Oxidative status: DNA damage.**

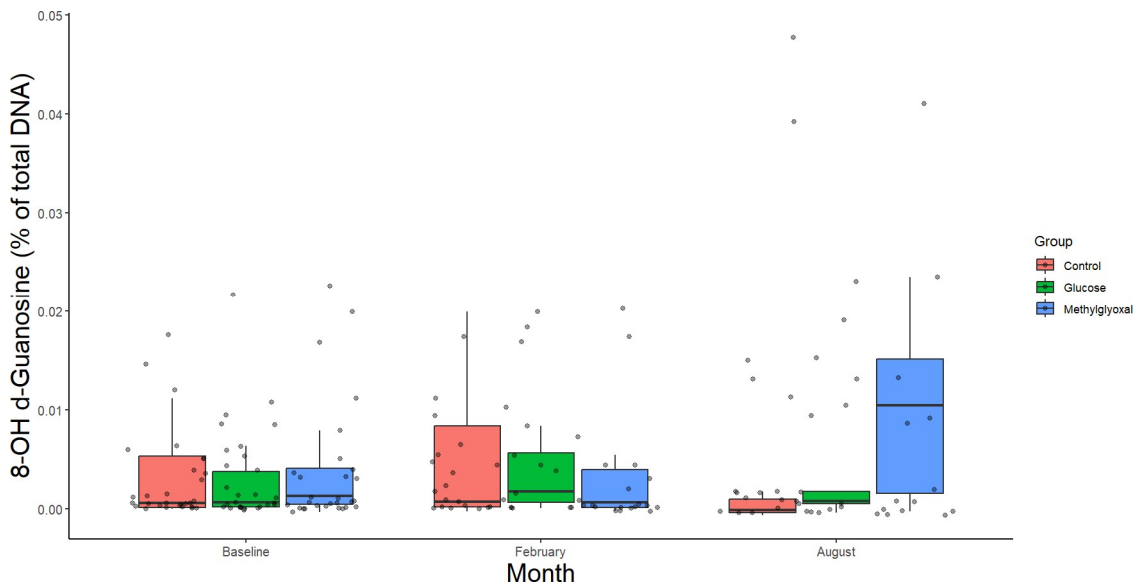
	Estimate	Std. Error	df	t value	P-value
<b>Intercept</b>	<b>-2.61857</b>	<b>0.09914</b>	<b>184</b>	<b>-26.412</b>	<b>&lt; 2*10<sup>-16</sup></b>
GroupGlucose	-0.01386	0.12848	184	-0.108	0.91420
GroupMethylglyoxal	0.06985	0.13193	184	0.529	0.59713
MonthFebruary	0.07579	0.14578	184	0.520	0.60377
<b>MonthAugust</b>	<b>-0.46337</b>	<b>0.15364</b>	<b>184</b>	<b>-3.016</b>	<b>0.00292</b>
Sexmale	-0.09638	0.07329	184	-1.315	0.19015
GroupGlucose:MonthFebruary	0.09474	0.22704	184	0.417	0.67696
GroupMethylglyoxal:MonthFebruary	-0.14090	0.20123	184	-0.700	0.48469
<b>GroupGlucose:MonthAugust</b>	<b>0.50065</b>	<b>0.24277</b>	<b>184</b>	<b>2.062</b>	<b>0.04059</b>
<b>GroupMethylglyoxal:MonthAugust</b>	<b>0.93168</b>	<b>0.21321</b>	<b>184</b>	<b>4.370</b>	<b>2.08*10<sup>-5</sup></b>

Shapiro-Wilk normality test

W = 0.96309, P-value = 9.105\*10<sup>-5</sup>

Repeatability for Bird\_ID

R = 0; SE = 0.054; CI<sub>95</sub>[0, 0.183]; P-value = 0.5



**Figure 15.** DNA damage measured by 8-OH d-Guanosine levels as percentage of total DNA in the different treatment groups over the course of the experiment.

**Table 11.E. Coefficients of variance of the oxidative stress markers.**

CV Interplate protein carbonyls	CV Intraplate protein carbonyls	CV Interplate OXY	CV Intraplate OXY	CV Interplate d-ROMs	CV Intraplate d-ROMs	CV Intraplate DNA damage	CV Interplate DNA damage
1,075 %	5,847 %	2,05 %	7,039 %	2,601 %	5,224 %	5,781 %	71,417 %

**Table 12. Apoptosis.**

	<b>Estimate</b>	<b>Std. Error</b>	<b>df</b>	<b>t value</b>	<b>P-value</b>
Intercept	0.07808	0.09855	57.55995	0.792	0.431409
<b>GroupGlucose</b>	<b>-0.47015</b>	<b>0.16156</b>	<b>65.03766</b>	<b>-2.910</b>	<b>0.004943</b>
<b>GroupMethylglyoxal</b>	<b>-0.37835</b>	<b>0.13791</b>	<b>57.55995</b>	<b>-2.744</b>	<b>0.008091</b>
<b>MonthFebruary</b>	<b>-1.31606</b>	<b>0.08223</b>	<b>295.41962</b>	<b>-16.005</b>	<b>&lt; 2*10<sup>-16</sup></b>
<b>MonthMay</b>	<b>-1.64998</b>	<b>0.08800</b>	<b>297.52678</b>	<b>-18.749</b>	<b>&lt; 2*10<sup>-16</sup></b>
<b>MonthAugust</b>	<b>-1.39731</b>	<b>0.08974</b>	<b>297.75536</b>	<b>-15.570</b>	<b>&lt; 2*10<sup>-16</sup></b>
TimeT24	0.06885	0.07961	291.11780	0.865	0.387859
<b>GroupGlucose:MonthFebruary</b>	<b>1.00785</b>	<b>0.14556</b>	<b>316.82425</b>	<b>6.924</b>	<b>2.45*10<sup>-11</sup></b>
<b>GroupMethylglyoxal:MonthFebruary</b>	<b>0.81573</b>	<b>0.11330</b>	<b>293.38191</b>	<b>7.200</b>	<b>5.08*10<sup>-12</sup></b>
<b>GroupGlucose:MonthMay</b>	<b>0.53259</b>	<b>0.15427</b>	<b>315.24790</b>	<b>3.452</b>	<b>0.000632</b>
<b>GroupMethylglyoxal:MonthMay</b>	<b>0.92920</b>	<b>0.11979</b>	<b>295.46833</b>	<b>7.757</b>	<b>1.42*10<sup>-13</sup></b>
<b>GroupGlucose:MonthAugust</b>	<b>0.42748</b>	<b>0.15653</b>	<b>318.28920</b>	<b>2.731</b>	<b>0.006668</b>
<b>GroupMethylglyoxal:MonthAugust</b>	<b>0.73527</b>	<b>0.12280</b>	<b>295.85397</b>	<b>5.987</b>	<b>6.17*10<sup>-9</sup></b>
GroupGlucose:TimeT24	0.13713	0.13932	298.83779	0.984	0.325768
GroupMethylglyoxal:TimeT24	0.01507	0.11141	291.11780	0.135	0.892493
<b>MonthFebruary:TimeT24</b>	<b>0.38674</b>	<b>0.11524</b>	<b>291.11780</b>	<b>3.356</b>	<b>0.000896</b>
<b>MonthMay:TimeT24</b>	<b>0.30820</b>	<b>0.12212</b>	<b>291.11780</b>	<b>2.524</b>	<b>0.012144</b>
MonthAugust:TimeT24	0.06761	0.12430	291.11780	0.544	0.586911
<b>GroupGlucose:MonthFebruary:TimeT24</b>	<b>-0.64493</b>	<b>0.19901</b>	<b>294.89542</b>	<b>-3.241</b>	<b>0.001329</b>
<b>GroupMethylglyoxal:MonthFebruary:TimeT24</b>	<b>-0.38367</b>	<b>0.15946</b>	<b>291.11780</b>	<b>-2.406</b>	<b>0.016752</b>
<i>GroupGlucose:MonthMay:TimeT24</i>	<i>-0.38507</i>	<i>0.21020</i>	<i>294.50339</i>	<i>-1.832</i>	<i>0.067969</i>
<b>GroupMethylglyoxal:MonthMay:TimeT24</b>	<b>-0.37302</b>	<b>0.16712</b>	<b>291.11780</b>	<b>-2.232</b>	<b>0.026377</b>
GroupGlucose:MonthAugust:TimeT24	-0.29321	0.21147	294.46276	-1.387	0.166631
GroupMethylglyoxal:MonthAugust:TimeT24	-0.21218	0.17087	291.11780	-1.242	0.215321

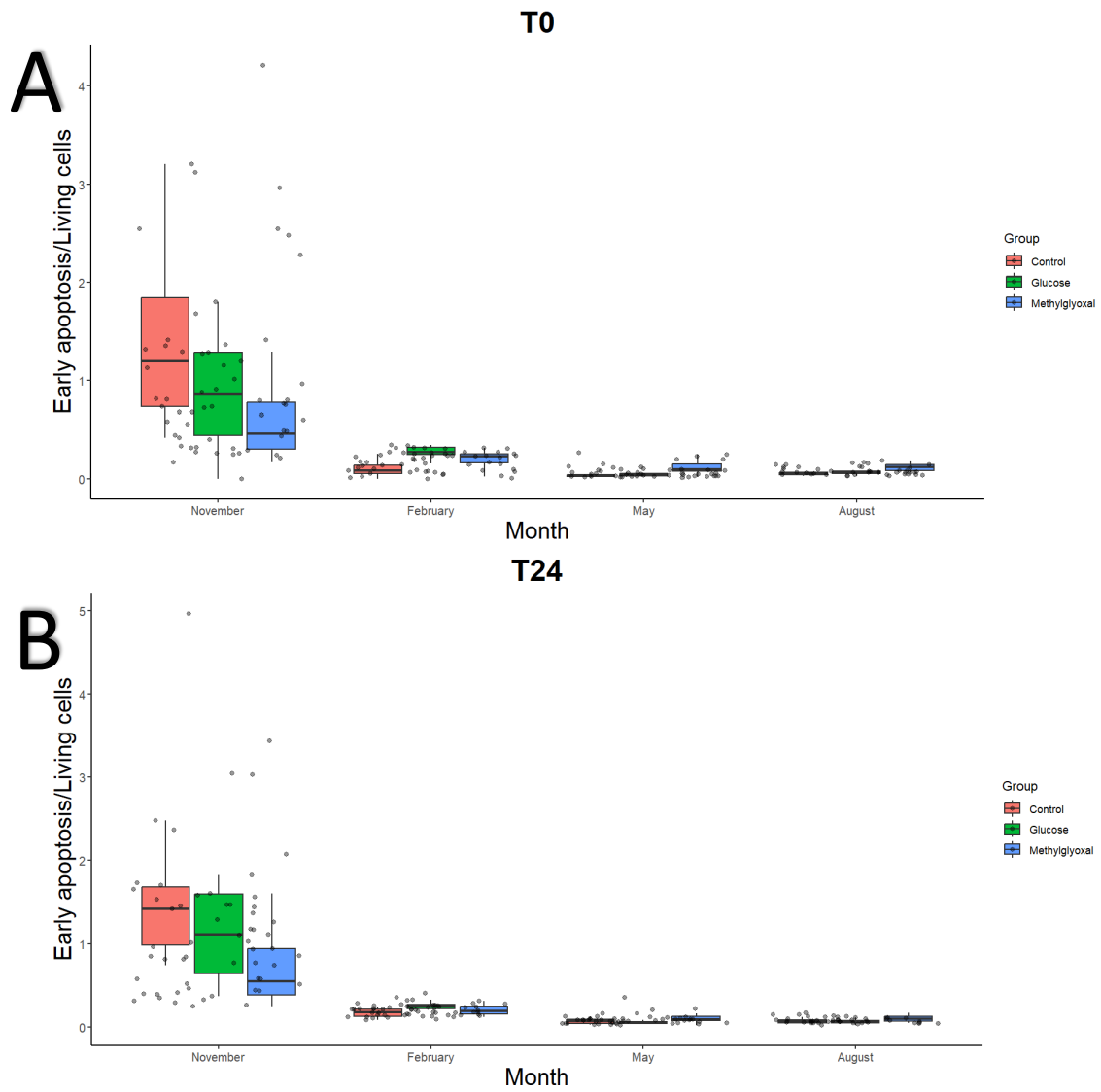
Shapiro-Wilk normality test

W = 0.81498, P-value < 2.2\*10<sup>-16</sup>

Repeatability for Bird\_ID

R = 0.637; SE = 0.052; CI<sub>95</sub>[0.516, 0.721]; P-value = 0.000285





**Figure 16.** Early apoptosis to living blood cells ratio **A** at the sampling day (T0) and **B** the next day (T24) in the different treatment groups over the course of the experiment.

**Table 13.A. Superoxide (O<sub>2</sub><sup>-</sup>) production per living cell.**

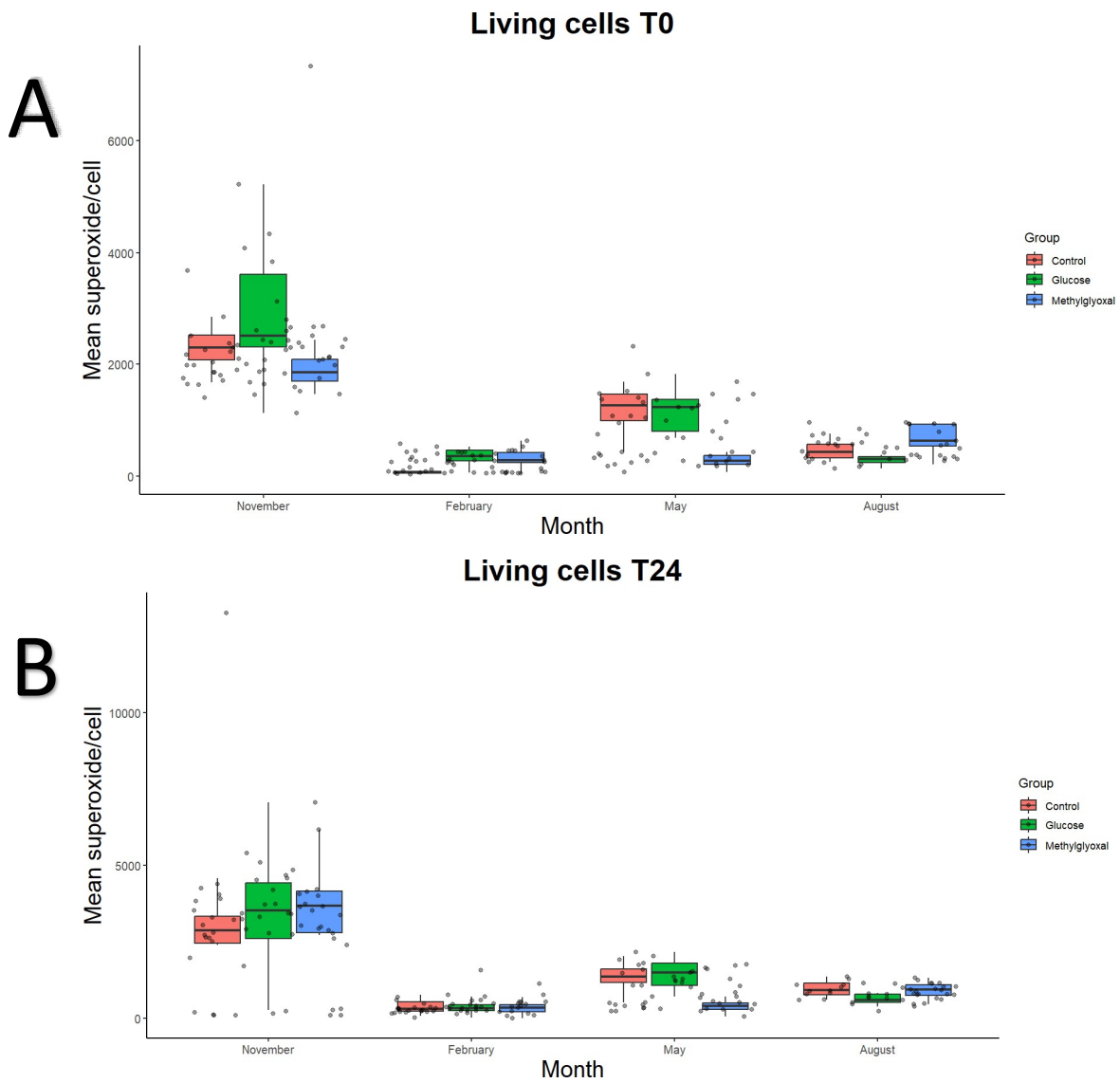
	Estimate	Std. Error	df	t value	P-value
<b>Intercept</b>	<b>3.35921</b>	<b>0.05761</b>	<b>393.38399</b>	<b>58.311</b>	<b>&lt; 2*10<sup>-16</sup></b>
GroupGlucose	0.07013	0.09169	393.38399	0.765	0.444849
GroupMethylglyoxal	-0.07571	0.08062	393.38399	-0.939	0.348226
<b>MonthFebruary</b>	<b>-1.50750</b>	<b>0.07929</b>	<b>364.48398</b>	<b>-19.013</b>	<b>&lt; 2*10<sup>-16</sup></b>
<b>MonthMay</b>	<b>-0.29216</b>	<b>0.08434</b>	<b>372.24295</b>	<b>-3.464</b>	<b>0.000594</b>
<b>MonthAugust</b>	<b>-0.71602</b>	<b>0.08592</b>	<b>373.86651</b>	<b>-8.333</b>	<b>1.52*10<sup>-15</sup></b>
<i>TimeT24</i>	<i>-0.14808</i>	<i>0.07731</i>	<i>359.03058</i>	<i>-1.915</i>	<i>0.056225</i>
<b>GroupGlucose:MonthFebruary</b>	<b>0.57089</b>	<b>0.13141</b>	<b>372.35112</b>	<b>4.345</b>	<b>1.80*10<sup>-5</sup></b>
<b>GroupMethylglyoxal:MonthFebruary</b>	<b>0.64916</b>	<b>0.10961</b>	<b>361.90187</b>	<b>5.923</b>	<b>7.37*10<sup>-9</sup></b>
GroupGlucose:MonthMay	-0.09072	0.13993	378.63664	-0.648	0.517161
<b>GroupMethylglyoxal:MonthMay</b>	<b>-0.56012</b>	<b>0.11524</b>	<b>368.80728</b>	<b>-4.860</b>	<b>1.74*10<sup>-6</sup></b>
<i>GroupGlucose:MonthAugust</i>	<i>-0.26856</i>	<i>0.14089</i>	<i>379.12883</i>	<i>-1.906</i>	<i>0.057388</i>
<i>GroupMethylglyoxal:MonthAugust</i>	<i>0.22102</i>	<i>0.11798</i>	<i>371.04020</i>	<i>1.873</i>	<i>0.061794</i>
GroupGlucose:TimeT24	0.16720	0.12304	359.03058	1.359	0.175042
<b>GroupMethylglyoxal:TimeT24</b>	<b>0.24404</b>	<b>0.10818</b>	<b>359.03058</b>	<b>2.256</b>	<b>0.024683</b>
<b>MonthFebruary:TimeT24</b>	<b>0.82172</b>	<b>0.11190</b>	<b>359.03058</b>	<b>7.343</b>	<b>1.41*10<sup>-12</sup></b>
<i>MonthMay:TimeT24</i>	<i>0.19942</i>	<i>0.11858</i>	<i>359.03058</i>	<i>1.682</i>	<i>0.093496</i>
<b>MonthAugust:TimeT24</b>	<b>0.47421</b>	<b>0.12069</b>	<b>359.03058</b>	<b>3.929</b>	<b>0.000102</b>
<b>GroupGlucose:MonthFebruary:TimeT24</b>	<b>-0.87987</b>	<b>0.18488</b>	<b>359.03058</b>	<b>-4.759</b>	<b>2.82*10<sup>-6</sup></b>
<b>GroupMethylglyoxal:MonthFebruary:TimeT24</b>	<b>-0.85571</b>	<b>0.15484</b>	<b>359.03058</b>	<b>-5.526</b>	<b>6.30*10<sup>-8</sup></b>
GroupGlucose:MonthMay:TimeT24	-0.13261	0.19621	359.03058	-0.676	0.499558
GroupMethylglyoxal:MonthMay:TimeT24	-0.15481	0.16228	359.03058	-0.954	0.340732
GroupGlucose:MonthAugust:TimeT24	-0.15845	0.19749	359.03058	-0.802	0.422914
<b>GroupMethylglyoxal:MonthAugust:TimeT24</b>	<b>-0.44331</b>	<b>0.16592</b>	<b>359.03058</b>	<b>-2.672</b>	<b>0.007887</b>

Shapiro-Wilk normality test

W = 0.83462, P-value < 2.2\*10<sup>-16</sup>

Repeatability for Bird\_ID

R = 0.099; SE = 0.041; CI<sub>95</sub>[0.024, 0.185]; P-value = 0.000772



**Figure 17.** Superoxide ( $O_2^{\cdot-}$ ) production by living cells measured as MitoSox signal intensity divided by number of living cells in a sample of 20000 blood cells (see **Methods**) **A** at the sampling day (T0) and **B** the next day (T24) in the different treatment groups over the course of the experiment.

**Table 13.B. Superoxide (O<sub>2</sub><sup>-</sup>) production per early apoptotic cell.**

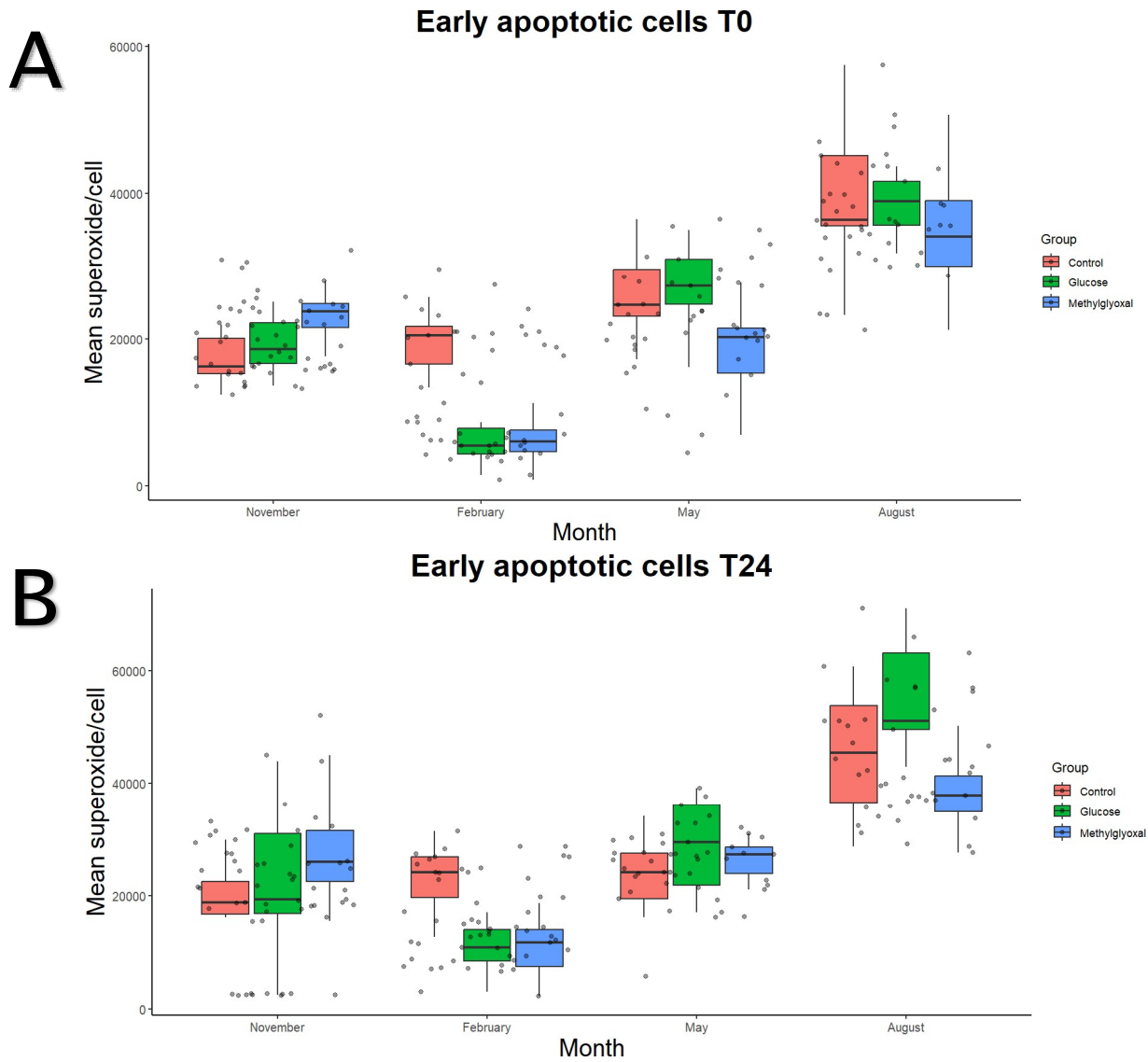
	Estimate	Std. Error	df	t value	P-value
<b>Intercept</b>	<b>4.23497</b>	<b>0.04198</b>	<b>382.09974</b>	<b>100.880</b>	<b>&lt; 2*10<sup>-16</sup></b>
GroupGlucose	0.05746	0.07154	391.01815	0.803	0.422348
<i>GroupMethylglyoxal</i>	<i>0.11294</i>	<i>0.05875</i>	<i>382.09974</i>	<i>1.922</i>	<i>0.055298</i>
MonthFebruary	0.02871	0.05778	347.60457	0.497	0.619602
<b>MonthMay</b>	<b>0.17834</b>	<b>0.06147</b>	<b>356.51942</b>	<b>2.901</b>	<b>0.003944</b>
<b>MonthAugust</b>	<b>0.34953</b>	<b>0.06262</b>	<b>358.39553</b>	<b>5.582</b>	<b>4.70*10<sup>-8</sup></b>
<b>TimeT24</b>	<b>-0.11771</b>	<b>0.05634</b>	<b>341.38898</b>	<b>-2.089</b>	<b>0.037415</b>
<b>GroupGlucose:MonthFebruary</b>	<b>-0.55100</b>	<b>0.09936</b>	<b>369.04189</b>	<b>-5.545</b>	<b>5.60*10<sup>-8</sup></b>
<b>GroupMethylglyoxal:MonthFebruary</b>	<b>-0.58929</b>	<b>0.07988</b>	<b>344.65676</b>	<b>-7.377</b>	<b>1.22*10<sup>-12</sup></b>
GroupGlucose:MonthMay	-0.08407	0.10527	372.36017	-0.799	0.424999
<b>GroupMethylglyoxal:MonthMay</b>	<b>-0.29411</b>	<b>0.08399</b>	<b>352.56312</b>	<b>-3.502</b>	<b>0.000521</b>
GroupGlucose:MonthAugust	-0.05700	0.10620	377.94691	-0.537	0.591771
<i>GroupMethylglyoxal:MonthAugust</i>	<i>-0.16856</i>	<i>0.08598</i>	<i>355.13390</i>	<i>-1.961</i>	<i>0.050712</i>
GroupGlucose:TimeT24	0.03911	0.09778	346.15887	0.400	0.689430
GroupMethylglyoxal:TimeT24	0.08413	0.07884	341.38898	1.067	0.286657
<b>MonthFebruary:TimeT24</b>	<b>0.20472</b>	<b>0.08155</b>	<b>341.38898</b>	<b>2.510</b>	<b>0.012523</b>
MonthMay:TimeT24	0.08631	0.08642	341.38898	0.999	0.318613
<b>MonthAugust:TimeT24</b>	<b>0.17478</b>	<b>0.08796</b>	<b>341.38898</b>	<b>1.987</b>	<b>0.047717</b>
GroupGlucose:MonthFebruary:TimeT24	0.12338	0.14027	343.71581	0.880	0.379687
GroupMethylglyoxal:MonthFebruary:TimeT24	0.07397	0.11284	341.38898	0.656	0.512557
GroupGlucose:MonthMay:TimeT24	0.05353	0.14821	343.47370	0.361	0.718190
GroupMethylglyoxal:MonthMay:TimeT24	0.10326	0.11826	341.38898	0.873	0.383214
GroupGlucose:MonthAugust:TimeT24	0.06011	0.14912	343.44862	0.403	0.687140
GroupMethylglyoxal:MonthAugust:TimeT24	-0.08933	0.12092	341.38898	-0.739	0.460538

Shapiro-Wilk normality test

W = 0.84665, P-value < 2.2\*10<sup>-16</sup>

Repeatability for Bird\_ID

R = 0.124; SE = 0.045; CI<sub>95</sub>[0.04, 0.215]; P-value = 0.00054



**Figure 18.A.** Superoxide ( $O_2^{\cdot-}$ ) production by early apoptotic cells measured as MitoSox signal intensity divided by number of early apoptotic cells in a sample of 20000 blood cells (see **Methods**) **A** at the sampling day (T0) and **B** the next day (T24) in the different treatment groups over the course of the experiment.

**Table 14. Heterophile by lymphocyte (H/L) ratio.**

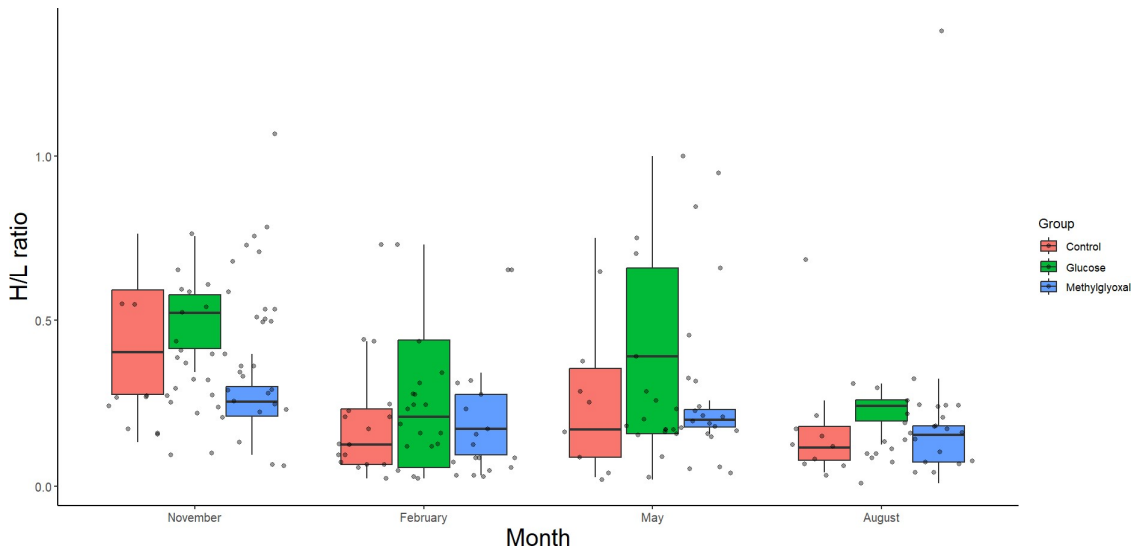
	Estimate	Std. Error	df	t value	P-value
<b>Intercept</b>	<b>-0.402182</b>	<b>0.077887</b>	<b>200</b>	<b>-5.164</b>	<b>5.83*10<sup>-7</sup></b>
GroupGlucose	0.101751	0.109406	200	0.930	0.353472
<b>GroupMethylglyoxal</b>	<b>-0.233897</b>	<b>0.094674</b>	<b>200</b>	<b>-2.471</b>	<b>0.014328</b>
<b>MonthFebruary</b>	<b>-0.600300</b>	<b>0.111366</b>	<b>200</b>	<b>-5.390</b>	<b>1.97*10<sup>-7</sup></b>
<b>MonthMay</b>	<b>-0.256330</b>	<b>0.116762</b>	<b>200</b>	<b>-2.195</b>	<b>0.029291</b>
<b>MonthAugust</b>	<b>-0.452231</b>	<b>0.117446</b>	<b>200</b>	<b>-3.851</b>	<b>0.000159</b>
Sexmale	0.004383	0.082818	200	0.053	0.957844
GroupGlucose:MonthFebruary	-0.026844	0.162012	200	-0.166	0.868569
<b>GroupMethylglyoxal:MonthFebruary</b>	<b>0.297291</b>	<b>0.137819</b>	<b>200</b>	<b>2.157</b>	<b>0.032190</b>
GroupGlucose:MonthMay	0.182377	0.171721	200	1.062	0.289493
<b>GroupMethylglyoxal:MonthMay</b>	<b>0.317689</b>	<b>0.143503</b>	<b>200</b>	<b>2.214</b>	<b>0.027972</b>
GroupGlucose:MonthAugust	0.306736	0.177212	200	1.731	0.085012
GroupMethylglyoxal:MonthAugust	0.283611	0.145543	200	1.949	0.052738
MonthFebruary:Sexmale	0.208507	0.121802	200	1.712	0.088476
MonthMay:Sexmale	-0.236226	0.128056	200	-1.845	0.066559
MonthAugust:Sexmale	-0.208601	0.131185	200	-1.590	0.113386

Shapiro-Wilk normality test

W = 0.98219, p-value = 0.01228

Repeatability for Bird\_ID

R = 0; SE = 0.038; CI<sub>95</sub>[0, 0.13]; P-value = 1

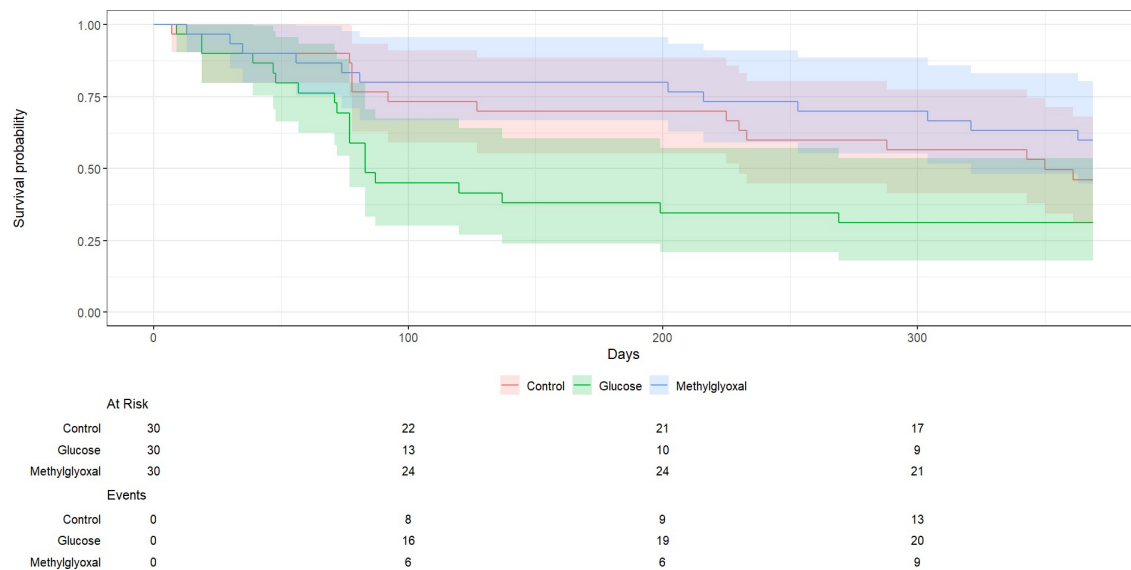


**Figure 19.** Heterophile to lymphocyte ratio in the different treatment groups over the course of the experiment.

## Survival models

Kaplan-Meier survival curves per treatment group are shown in **Figure 20**. For Cox models, the one performed over the complete population, only with treatment, sex and their interaction, reported a trend to significance for the hazard ratio of the glucose group, in comparison to the control (coefficient  $\pm$  s. e.:  $\beta=0.876 \pm 0.468$ , P-value=0.061), showing a 2.4 times greater death hazard for this group CI<sub>95</sub>[0.96, 6]. After including the age and body mass in the model (which implies the elimination of 4 birds of unknown age, two from control and two from glucose group), and the interaction of the latter with treatment (as it was identified as the best model

by the *dredge()* function), glucose treatment hazard became significantly higher than the control group (coefficient  $\pm$  s. e.:  $\beta=1.51 \pm 0.473$ , P-value=0.001), with a 4.5 times higher hazard  $CI_{95}[1.79, 11.4]$ . Moreover, both body mass and age have an effect on hazard, with the former being a protective factor (i.e. higher body mass predicts lower death hazard; coefficient  $\pm$  s. e.:  $\beta=-1.08 \pm 0.27$ , P-value<0.001; 34 % less hazard per gram of body mass  $CI_{95}[0.2, 0.58]$ ) and the latter increasing hazard (i.e. higher age predicts higher death hazard; coefficient  $\pm$  s. e.:  $\beta=0.6143 \pm 0.1432$ , P-value<0.001; 1.85 times higher hazard per year of age ( $CI_{95}[1.4, 2.45]$ ). Finally, the interaction between body mass and glucose group was significant, thus reporting an increased hazard with body mass within the glucose group (coefficient  $\pm$  s. e.:  $\beta=0.8 \pm 0.32$ , P-value=0.013) of 2.21 per gram.



**Figure 20.** Survival probability across time for each experimental treatment group.

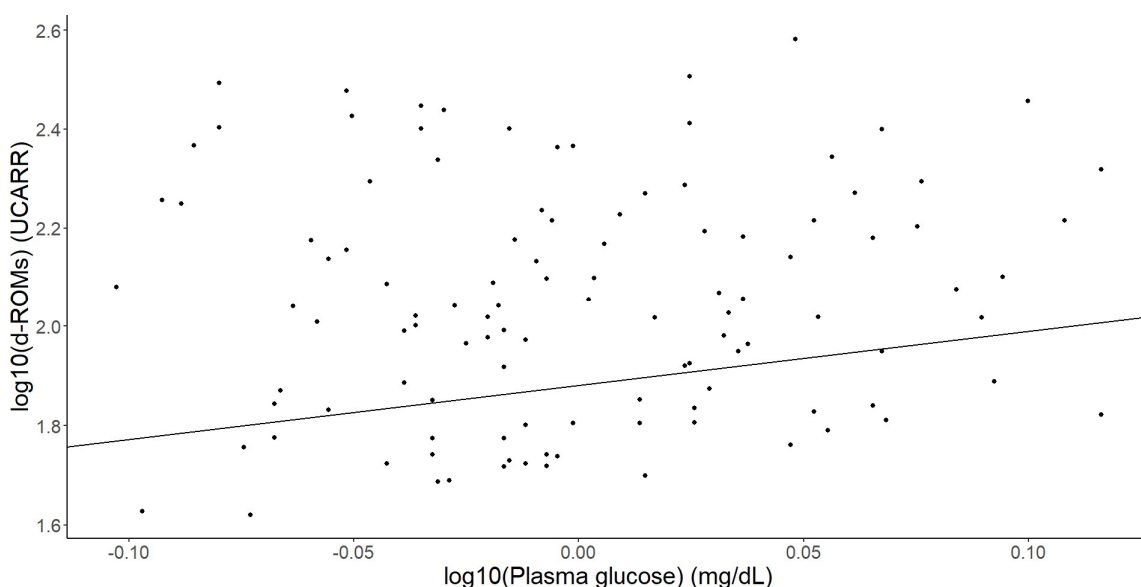
Of the remaining variables whose effect on survival was tested, the only one that showed a significant effect on hazard was CEL, which had a positive effect on hazard ratio (decreased survival; estimate  $\pm$  s.e.:  $\beta=2.798 \pm 1.164$ , P-value=0.0162), with a risk of death 16.41 times higher per ng/mL of plasmatic CEL increase ( $CI_{95}[1.676, 160.7]$ ). For plasma glucose levels, a trend was found (estimate  $\pm$  s.e.:  $\beta=-0.006 \pm 0.004$ , P-value=0.0899), with a death risk  $\sim$ 0.6% lower per mg/dL of plasma glucose increase ( $CI_{95}[-1.3, 0.1]$ ). Finally, a negative significant effect of the interaction of age with protein carbonyl levels was found (estimate  $\pm$  s.e.:  $\beta= -0.154 \pm 0.076$ , P-value=0.043).

## Other models

Models testing the effects of oxygen consumption rates ( $VO_2$  in mL/min) on mitochondrial superoxide ( $O_2^{\cdot-}$ ) production (either by living or early apoptotic cells, at T0 or T24), and on oxidative stress (d-ROMs, protein carbonyls or DNA damage), always controlling for month and treatment and their interaction (see **methods**) did not provide any significant effects. Whole blood glucose levels did not influence any of the oxidative stress variables, but plasma glucose positively correlated with d-ROMs levels (both variables  $\log_{10}$  transformed:  $\alpha=1.881$ ,  $\beta=1.087$ , P-value=0.049; **Figure 21**; final model:  $\log ROM \sim Month * Group + C\_logPGlu * Month * Sex + C\_OXY + (1 | Bird\_ID)$ ). No significant effects of oxidative stress on albumin glycation levels were found in any of the three performed models (d-ROMs, protein carbonyls and DNA damage on glycation), although positive relationship between OXY and glycation levels were found in the d-

ROMs ( $\alpha=19.4$ ,  $\beta=0.008$ ,  $P\text{-value}=0.043$ ) and DNA models ( $\alpha=19.62$ ,  $\beta=0.008$ ,  $P\text{-value}=0.046$ ), while a trend (also positive in sign) was reported in the protein carbonyl model for both OXY ( $\alpha=19.12$ ,  $\beta=0.008$ ,  $P\text{-value}=0.063$ ) and protein carbonyl values ( $\beta=2.71$ ,  $P\text{-value}=0.077$ ). Finally, CML and CEL levels were affected by DNA damage, with a significant and a marginal positive relationship, respectively ( $\alpha=0.695$ ,  $\beta=0.01$ ,  $P\text{-value}=0.013$ ;  $\alpha=0.599$ ,  $\beta=0.02$ ,  $P\text{-value}=0.073$ ).

Regarding the relationship between glucose and metabolism, RER was not affected by whole blood glucose, and blood glucose was not affected either by RMR per gram of mass. For plasma glucose levels, no significant effects of neither body mass nor RMR consumption rate were found. The results of the model of blood glucose in function of temperature variables are shown in **Table 15** and **Figure 22**. No significant effects of sampling time on whole blood glucose were found ( $\beta \pm \text{s.e.} = -0.004 \pm 0.003$ ,  $P\text{-value}=0.166$ ). For RMR variation with aviary temperature, only the minimum temperature had a negative significant effect ( $\beta \pm \text{s.e.} = -0.003 \pm 0.001$ ;  $P\text{-value}=0.0137$ ; **Figure 23**).



**Figure 21.** Positive effect of plasma glucose on plasma d-ROMs levels (both  $\log_{10}$  transformed).

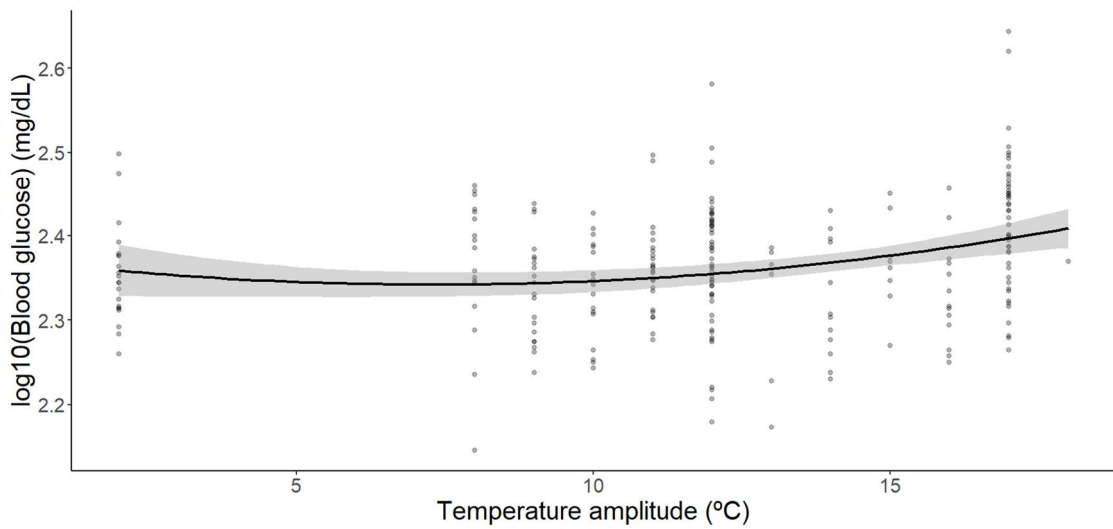
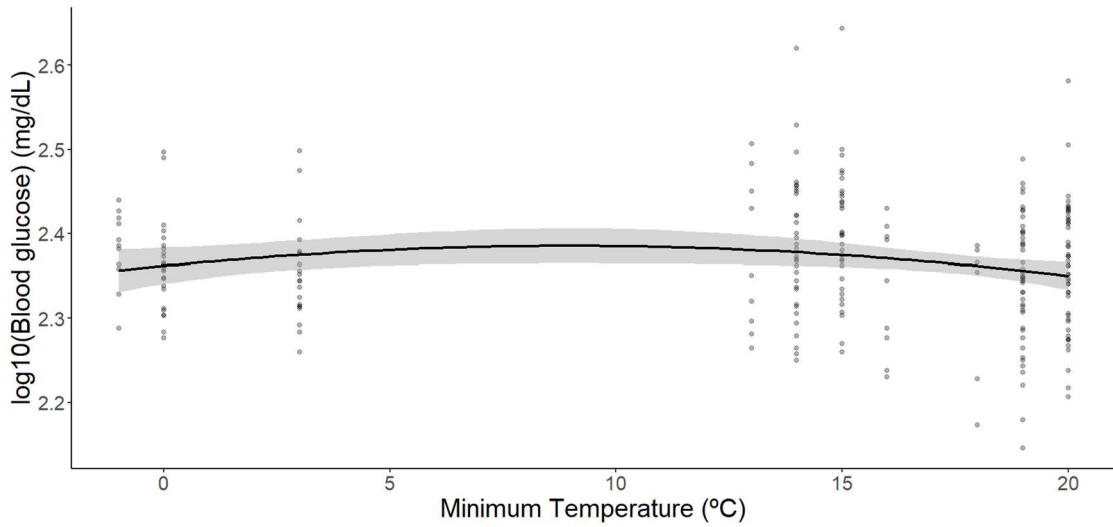
**Table 15.** Temperature effects on glucose. Bird ID and date are random factors.

	Estimate	Std. Error	df	t value	P-value
Intercept	2.4806105	0.0608198	12.5404823	40.786	$1.04 \times 10^{-14}$
Minimum Temperature	-0.0226522	0.0090057	28.7042546	-2.515	0.0178
Minimum Temperature <sup>2</sup>	0.0011766	0.0004568	33.5222776	2.576	0.0146
Temperature difference	-0.0335545	0.0141628	13.6343640	-2.369	0.0332
Temperature difference <sup>2</sup>	0.0019611	0.0007359	16.2846798	2.665	0.0168

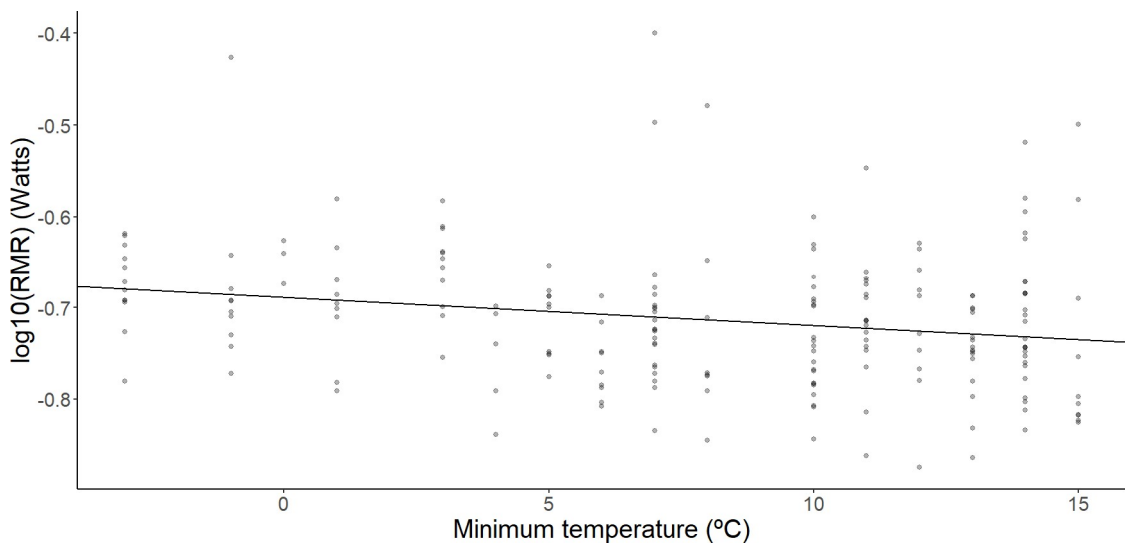
Shapiro-Wilk normality test

W = 0.98507, P-value = 0.0138





**Figure 22.** Blood glucose variation in function of **A.** daily minimum temperature and **B.** daily temperature amplitude. Quadratic relationships are represented. The grey area represents the 95 % confidence intervals.



**Figure 23.** Variation of birds RMR (log10 transformed) with daily minimum temperature experienced in the aviary the day before the respirometry measurement.

# Discussion

## **1. Effects of treatments on glucose, glycation and AGEs**

Despite treatments not affecting fasting plasma glucose values, glucose supplemented birds showed higher albumin glycation levels at the end of the experiment (August 2023). This is in line with previous dietary supplementation studies showing that birds' glucose levels are rarely affected by glucose supplementation (Basile et al. 2022), confirming that this trait is tightly controlled in birds. This may be attained, at least partially, by a compensatory mechanism consisting in a decreased food intake in the glucose supplemented group, as it was shown in our pilot study (see **Appendix 2**) or other mechanisms as increased energy expenditure by increasing their activity levels. Nevertheless, we cannot exclude higher postprandial glucose peaks due to the increased availability of glucose in the diet, leading to higher exposition of plasma proteins to glucose triggering higher glycation levels (see review in Aragno and Mastrocola 2017). Accordingly, an increase in methylglyoxal formation, from recently ingested glucose, has been observed during transient glucose peaks in oral glucose tolerance tests in humans (Xiaodi Zhang et al. 2023). Why the increase in albumin glycation is shown only at the end of the experiment, nevertheless, remains uncertain. A possibility would be that an increase in protein turnover associated to the cold induced increase in metabolism (see **section 3**) palliated such increase in glycation in February. Interestingly, whole blood glucose levels, for which more individuals were measured, did not show the same pattern, with both control and methylglyoxal supplementation group increasing their levels in May, to stay high only in the methylglyoxal group later in August, while the glucose group started with higher levels, but was then surpassed by the control in May and it finally dropped to lower levels in August, paralleling those of the control group. Seasonal variations may be influencing our measurements, as daily minimum temperature and temperature range in the outdoor aviaries were associated with whole blood glucose levels. A similar kind of variation has also been reported before for blood glucose levels in captive zebra finches housed outdoors in Europe, with higher glucose in colder and shorter days (Montoya et al. 2018). A similar seasonal variation has been reported for humans as well, with plasma glucose and glycated haemoglobin showing higher levels in winter and lower in summer, both with a negative relationship with temperature (Tseng et al. 2005; Sakura et al. 2010; Tanaka et al. 2018). Nevertheless, this seasonal variation is not found for human glycated albumin (Tanaka et al. 2018), and no studies on seasonal variation of protein glycation exist in birds. Interestingly, in Montoya et al. (2018), the reported temperatures ("ambient temperature") were higher than the range of minimum temperatures included in our model, which show a negative quadratic relationship suggesting no further increase in glucose with temperatures below a certain threshold (approx. 13 °C). All this evidence, however, does not support the reported increase in glucose levels of part of our birds during May, which was warmer and with longer days than February. Higher daily temperature ranges (that in Strasbourg tend to drastically increase towards the summer; see annual temperatures variation in **Appendix 3**) could, nevertheless, have influenced this, as we observe a positive quadratic relationship with blood glucose levels. Another possibility is that this increase was related to reproductive attempts, as both sexes were housed together in our aviaries. Despite not being allowed to raise chicks, as they are not provided with nest-boxes, some females laid eggs that were subsequently removed and some individuals may have mated, and also showed a higher activity related, for example, to nest building and courtship.

While plasma glucose values showed no intra-individual repeatability for our sample, whole blood glucose had a moderate repeatability of 19.8 %. This is lower to the 30% within year repeatability shown by Montoya et al. (2018) in zebra finches, although it can be related to the fact that they calculated the repeatability of the deviations from measurement session average, as these sessions (performed at different seasons) showed significantly different glucose values, and in our case we did it on raw glucose values, although controlling for all the variables used in our models, including the month. However, the same group also found a much higher repeatability of 54 % for their same population, handled in the same way, in another study (Montoya et al. 2020). In both studies, they had only two measurements for each individual, so that our overall variability may be higher. Albumin glycation had, interestingly, a higher repeatability (30.2 %), potentially due to the fact that blood glucose values, despite being a tightly regulated physiological parameter, vary more rapidly than glycation levels, which are thus a better representation of average glycaemic status over a medium-range period of time (Kim and Lee 2012). This is what makes assessing glycation a more reliable measure of overall glycaemic state than directly measuring glucose. Nevertheless, it is still relevant to consider that in birds, albumin turnover rates are much higher than in humans (Vanstone et al. 1957; Patterson et al. 1962; Bush and Marquardt 1977), with glycation levels potentially representing the glycaemic state shorter than the 2-3 weeks given for humans (see Guthrow et al. 1979; Yue et al. 1980; Dolhofer and Wieland 1980; Armbruster 1987). In our dataset, although glucose levels tended to decrease with sampling time, this variation was not significant when the animals had been overnight fasted instead of doing it so for only 1 hour, as in the pilot study (see **Appendix 2**).

Regarding the effects of the treatments on Advanced Glycation End-products (AGEs), the first that can be noticed is that, while methylglyoxal levels tended to decrease with time, with methylglyoxal supplementation apparently only dampening such a decrease (marginally), glyoxal is increased by both the glucose and methylglyoxal supplementation treatments at the end of the experiment (August 2023). Both methylglyoxal and glyoxal have similar endogenous origins, being mainly produced by either retroaldol condensation followed by auto-oxidation of monosaccharides, auto-oxidation of early glycation products, degradation of trioses phosphate (from glycolysis) and from the oxidation of lipids and other compounds (see e.g. Thornalley et al. 1999; Lange et al. 2012). Therefore, higher glucose intake would increase endogenous production of reactive dicarbonyls, such as glyoxal and methylglyoxal (Aragno and Mastrocola 2017; Xiaodi Zhang et al. 2023). Why only glyoxal is significantly increased by the treatments remains unknown. Glyoxal has previously been shown to be produced at a much higher rate and being more dependent on the redox state (through the presence of trace metal ions and phosphate) than methylglyoxal *in vitro* in the presence of glucose and N $\alpha$ -t-BOC-lysine. Nevertheless, this difference did not exist when exposing HSA (Human Serum Albumin) to glucose instead, suggesting that albumin arginine residuals would react with glyoxal and methylglyoxal, reducing their free plasma levels (Thornalley et al. 1999), suggesting this difference may not exist *in vivo*.

Glyoxal also seems to have been more important in AGE formation, as it positively correlates with plasma CEL levels, despite this latter compound not being increased in the treatment groups. CML, nevertheless, is increased by the treatments (significantly in the methylglyoxal group and marginally in the glucose group) in February, and only by the glucose supplementation in August, while also increasing with age. Interestingly, glyoxal also increases with age, but only in males. The correlation of glyoxal with CEL is unexpected, as this compound is derived from methylglyoxal (Ahmed et al. 1997). This is even more surprising given that the

levels of CML, which is actually derived from glyoxal, were not correlated with it. Therefore, the exact role of the different reactive dicarbonyl compounds on AGEs accumulation in birds remains to be further explored, with a focus on glyoxal. The increase of CML with age, nevertheless, has previously been reported for several tissues in humans (e.g. Dunn et al. 1989; Dunn et al. 1991), including plasma (Wang et al. 2024). In birds, the only studies, to our knowledge, that have shown an increase of AGEs with age, are done in skin, mainly with pentosidine (Iqbal et al. 1999; Klandorf et al. 1999; Fallon et al. 2006a; Fallon et al. 2006b; Cooley and Virginia 2008; Cooley et al. 2010; Dorr et al. 2017), although others did not find such correlation (Le Souëf 2012; Campion 2015; Rattiste et al. 2015; Labbé et al. 2019). It is remarkable that the levels of AGEs reported here in zebra finches (all measures pulled together, regardless of the treatment and month) are much lower than those observed in humans, with CML levels (mean  $\pm$  s.e.) of  $4.991 \pm 0.184$  ng/mL and CEL of  $4.006 \pm 0.429$  ng/mL, versus the 173.587 ng/mL and 196.051 ng/mL of CML reported for middle aged men and women respectively, and 80.752 ng/mL and 74.205 ng/mL of CEL for middle aged men and women respectively, in Wang et al. (2024). Other previously reported values for humans are also higher than what we see here for zebra finches (see e.g. Table 4 from Rankin et al. 2019). Finally, plasma CML is slightly higher than plasma CEL in zebra finches, in the same line as reported in humans (Wang et al. 2024), although at a much lower level. Other previously reported values for humans are also higher than what we see here for zebra finches (see e.g. Table 4 from Rankin et al. 2019). Plasma CEL, nevertheless, is higher than plasma CML in zebra finches, while the opposite is reported in humans (Wang et al. 2024). This could represent a higher ability of birds to resist AGEs accumulation, although more studies in other avian species and tissues are needed to confirm this. In this regard, Baker et al. (2022) reported higher levels of AGE-modified albumin in plasma of birds than that of mammals ( $\sim 40$   $\mu$ g/mL vs.  $\sim 15$   $\mu$ g/mL). Nevertheless, as already discussed in the general introduction (**Section 4.1.3**), they measured it with an ELISA kit using anti-AGE antibodies and a standard curve with AGE-BSA (Bovine Serum Albumin), which represent several drawbacks: first, this method is non-specific, as AGEs are multiple, so it is not clear how their used antibodies would bind to different kinds of them; second, the reference used is also non-specific and belongs to a mammalian species (bovine albumin), so that the signal observed on it may not be extrapolable to all the samples used in their study. Finally, they measure (or at least extrapolate their values to) AGEs bound to protein, whereas we measure free plasma AGEs, which may contribute to a lower comparability of the results.

## **2. Survival**

Only the glucose supplementation increased mortality hazard, with methylglyoxal treatment not affecting it. However, this effect cannot be explained by fasting plasma glucose levels as they did not increase with the treatment and only had a marginal and very weak (and also positive) effect on survival in our study. Whole blood glucose, which has previously been shown to negatively affect lifespan in zebra finches (Montoya et al. 2018), was not increased by the treatment, although it started at higher level in the glucose group. However, it was not found to affect mortality hazard in our study. The only measured variable that may be mediating such effect is CEL levels, which positively influenced mortality, but it was not affected by the supplementation, indicating that it was likely not the main driver of the high mortality observed in the glucose group. The mediator of glucose mortality thus remains unknown and further experiments are needed to determine its nature.

The effects of free plasma CEL on mortality remain controversial in the human literature. Some studies demonstrate they exist, even controlling for certain pathological processes that have

been related to AGEs accumulation (Nin et al. 2011), while others show they are not significant when correcting for certain other factors (Hanssen et al. 2013). It remains that the direct comparison of these studies is difficult, given that the factors included in the models and the health status of the individuals used in those studies vary. The possibility of glucose treatment increasing mortality through an increased glycation or oxidative stress (as plasma glucose positively correlates with d-ROMs levels, see below) are not consistent with the fact that none of those parameters was found to significantly affect survival, and the fact that glycation was increased only at the end of the treatment, while most dead events in this group happened during the first months. Some other possible explanations can account for this phenomenon. First, it is possible that the measuring intervals were too long to detect any change in parameters as blood glucose levels big enough to cause mortality events, i.e. birds that may have suffered a deregulation on their glycaemia would have died shortly after this happened, without being able to sustain chronic hyperglycaemia. Secondly, other non-measured parameters may have been the drivers of this increased mortality, as lower levels of certain nutrients due to a potential compensatory feeding behaviour (as the one reported in the pilot study) or an alteration of intestinal microbiota by the increased glucose intake, causing dysbiosis and an augmentation of associated inflammatory processes (see e.g. Do et al. 2018; Zhang et al. 2021; Kawano et al. 2022 for examples in mice). This is in line with previous studies in birds, showing the effects of intraspecific diet differences on microbiota composition and diversity (e.g. Teyssier et al. 2018; Schmiedová et al. 2022). Particularly, the supplementation with an “urban diet” with and increased sugar and decreased protein and fibre concentration decreased microbiota diversity and altered its metabolic functional composition in house sparrows (*Passer domesticus*) (Teyssier et al. 2020). This scenario would be vaguely supported by the observation of a certain number of birds that showed diarrhoea at death, although this happened as well in the methylglyoxal group. Despite this evidence being anecdotal, it suggest that future studies should consider this factor as a possible mediator of death risk in glucose supplementation treatments.

Apart from the previously discussed effects, there was an increase in mortality hazard with age, i.e. zebra finches show actuarial senescence (as previously reported by Briga et al. 2017a). Body mass was found to be generally a protective factor (i.e. the higher the body mass, the lower the mortality hazard), although its effect was reversed in the glucose group, with bigger individuals showing a higher death risk instead. This is in contrast to Montoya et al. (2022), that did not find effects of body mass on captive zebra finches mortality.

### **3. Body mass, body condition and body composition**

Body mass was found to be slightly but significantly higher than the baseline level in the control group in August 2023 and marginally higher in November, with marginally lower levels for the treated groups in August 2023 and only the glucose group in November. This was paralleled by a lower fat score found in the treated birds at the end of the experiment (August 2023), potentially explaining the reduced body mass, but no effects on the muscle score were found. Fasting body mass seems thus quite stable (with an individual repeatability of 75 %) and resistant to the effects of the treatments. The observed variation could be linked to seasonal fluctuations, which have been reported before for both wild (Rozman et al. 2003) and captive-reared (Meijer et al. 1996; Rozman and Meijer 1998) zebra finches, although these show a decrease in mass in winter and an increase during longer days linked to reproduction, while our birds did not reproduce. This effect of reproduction is also less likely to affect our birds, as zebra finches do not present seasonal development of reproductive organs with the corresponding increase in

their mass, those being instead readily available for reproduction whenever conditions allow it (Sossinka 1975; 1980).

Finally, body mass was positively correlated with structural size, with a higher slope for head-beak length than for tarsus length, and significantly but slightly lower ( $-0.731 \pm 0.278$  g) in males than in females, a sexual dimorphism also shown before in some zebra finches populations (Zann 1996), although in our population this may have been influenced by the presence of heterogeneous pedigrees, including a few females of very big size. Body composition, as measured in the spontaneously dead individuals, was not affected by the treatments, contrary to what has been seen in glucose supplemented chickens, that augmented their fat deposition in different tissues (Shapira et al. 1978; Jiang et al. 2008). Our results may be partly biased by spontaneous deaths occurring mostly after depletion of internal resources had ensued and therefore homogenising body composition in dead individuals. The only effect found here was a lower pectoralis muscle lipid content in males, independently of the treatment. Although previous studies show no differences in fat composition (type) in pectoralis muscle between sexes in zebra finches (McCue et al. 2009), no reports on differences in total fat content (amount) exist to our knowledge. Water content was not different across the treatments in any of the tissues, inferring no dehydration effects induced by the potentially reduced drinking rates in the supplementation groups. This may be related to the capacity of zebra finches to survive during long periods of water deprivation (Sossinka 1972), being able to acclimate to water scarcity conditions by mechanisms as decreasing transepidermal water loss (G. K. Menon et al. 1989).

#### **4. Metabolism variation with season, body mass and glucose**

As expected, RMR was found to decrease with age, as a sign of ageing. This has been previously reported in zebra finches (e.g. Moe et al. 2009; Briga 2016; Briga and Verhulst 2021). Also, RMR increases with body mass linearly, discarding thus allometric effects within the observed range of body masses, which is likely to represent the whole range of the species (Zann 1995). Nevertheless, a transitional increase during the middle of the experiment in RMR in the supplemented groups during February (and marginal in the control group) and in general in RER in February and May suggests the existence of seasonal fluctuations in metabolism, previously reported for birds (Swanson 2010) and in zebra finches in particular (Briga and Verhulst 2021), with BMR increasing during spring and winter (see also Versteegh et al. 2012 for patterns similar to ours in stonechats). This is probably explained by the very low temperatures experienced in February, as the metabolism was clearly increased when minimum temperatures decreased. This increase in BMR/RMR in cold acclimated zebra finches is well established (e.g. Beamonte-Barrientos and Verhulst 2013; Stier et al. 2014; Briga and Verhulst 2017; Campbell et al. 2024). Seasonal changes in RER in birds remain nevertheless quite unexplored, with few examples reported, such as its decrease during migration (DeMoranville et al. 2019), when a higher proportion of lipids is used to sustain endurance flight (reviewed in Jenni-Eiermann 2017). The seasonal change in RER observed here is however very moderate, increasing from an initial value (taking the control as the reference group) of 0.69 during November (given that baseline levels from August 2022 are not available) to 0.75 in February and 0.72 in May. This change may imply a slight increase in the proportion of carbohydrates or proteins catabolized (although whole blood glucose did not predict RER variation), or just a change in the nature of the lipids used to fuel metabolism, with an augmentation of the number of unsaturation or a decrease of the length of fatty acids employed, for example (Price and Mager 2020).

The previously reported positive relationship between blood glucose and basal metabolic rate per gram of mass at the interspecific level (Lasiewski and Dawson 1967; Tomasek et al. 2019) could not be confirmed here at the intraspecific level. This is consistent with previous findings from zebra finches (Montoya et al. 2018) and may be related to the abovementioned lack of allometry between RMR and body mass within this small body mass range.

## **5. Oxidative stress varies with time and plasma glucose**

Here, we measured blood oxidative stress to determine how blood/plasma glucose levels and its consequences in terms of glycation and AGEs would affect a known pathophysiological process that is acknowledged, in other species, to be affected by glycaemia (e.g. Folmer et al. 2002; Menon et al. 2004; Vágási et al. 2020) and dicarbonylic compounds like methylglyoxal (see e.g. Chang and Wu 2006; Desai et al. 2010; Sena et al. 2012). For this, we checked both damage at the plasma (d-ROMs and protein carbonyl) and cell level (DNA damage), accompanied by plasma antioxidant protection, as oxidative status would be regulated differently at distinct blood portions (i.e. plasma versus cells). Low avian red blood cells permeability to glucose (Johnstone et al. 1998) would putatively provide them a higher protection against glucose led stress. The patterns that we observed were not consistent for all plasma damage variables. We saw both an increase in damage (d-ROMs) over time and a decrease in it (protein carbonyls) in plasma, while in cells (DNA), it decreased over time in the control group while increasing in the supplemented groups. Moreover, the change in d-ROMs was not equal in all groups, being faster in the control than in the methylglyoxal treatment, and starting at a higher level in the glucose group, potentially due to their higher glucose levels during the baseline. This is supported by our finding of plasma glucose being positively correlate with d-ROMs, and the fact that baseline blood glucose levels have previously been reported to positively relate to a plasma marker of oxidative damage (malondialdehyde) at the intraspecific level (house sparrows, *Passer domesticus*; Vágási et al. 2020), although not at the interspecific level (51 European songbird species; Vágási et al. 2024). Nevertheless, it must be pointed out that only whole blood and not plasma glucose was higher in the control group during the baseline, and that whole blood glucose was not found to influence oxidative stress in our models. Oxidative damage levels could be influenced not only by the treatments, but also by both ROS production (see **section 6**) and antioxidant levels. For plasma, total non-enzymatic antioxidants (OXY) showed a complex relationship with oxidative damage, starting with a trend towards a negative relationship with d-ROMs in the control group, which changed to a positive relationship in the following months. The opposite was observed in the treated groups, i.e. starting with a positive relationship and becoming negative later (from February on for the methylglyoxal group, and only in August for both). Plasma antioxidant status has also a negative relationship with protein carbonyl levels. All of this could be explained as an antioxidant protective barrier effect in the cases in which we find a negative relationship. It could, nevertheless, represent the effects of a defence response elicited when levels of oxidative damage are increased, and therefore antioxidant levels also increase to protect the organism from further damage (Costantini and Verhulst 2009). This scenario is partly supported by the finding of such positive relationships appearing when the levels of d-ROMs are actually increased over time in the control group, and by the fact that methylglyoxal group showed lower d-ROMs levels in February, associated with a negative relationship with OXY. Also, glucose group shows a higher d-ROMs level at the baseline measurement, associated with a positive relationship with OXY. Nevertheless, it does not explain neither why we observe the positive relation during the baseline then turning to negative in the methylglyoxal group, nor why it also turns negative in August for the glucose group. These complex and changing nature on the relationship patterns between oxidative

damage and defences are frequently reported in the literature, and it has been discussed that they may appear due to factors as differential nutritional or energy states that may constraint/modulate the responses (Costantini and Verhulst 2009; Costantini 2019). In this sense, our treatments would potentially alter the expected relationship by a paired increase in energy availability and nutritional quality deficiency in the glucose group (see **section 1**).

Our results also show that the macromolecule in which oxidative damage is more clearly increased by the treatments is DNA, which is unexpected given the hypothesized lower (erythrocytes) intracellular exposition to glucose. Still, the absence of significant repeatability of this parameter ( $R=0 \pm 0.054$ ;  $CI_{95}[0, 0.183]$ ), and the low between-plate repeatability, suggests its interpretation should be more cautious than some of the others, as protein carbonyls, for which the repeatability is moderate ( $R=0.213 \pm 0.109$ ;  $CI_{95}[0, 0.417]$ ). The considerable repeatability of OXY ( $R=0.201 \pm 0.097$ ;  $CI_{95}[0, 0.387]$ ;  $P\text{-value} = 0.003$ ) and lack of repeatability for d-ROMs ( $R=0.082 \pm 0.085$ ;  $CI_{95}[0, 0.291]$ ;  $P\text{-value} = 0.215$ ) is similar to what has been reported for a wild population of collared flycatcher (*Ficedula albicollis*; OXY:  $R=0.128\text{--}0.581$ , d-ROMs:  $R=0\text{--}0.061$ ; (Récapet et al. 2019), but not with a previous example on zebra finches (Beamonte-Barrientos and Verhulst 2013), in which the pattern was reversed (OXY:  $R \pm \text{s.e.}=0.124 \pm 0.111$ , d-ROMs:  $R \pm \text{s.e.}=0.418 \pm 0.093$ ). Finally, it is worth mentioning that no effects of any of the parameters on survival was observed (see **section 2**), and that whole body oxygen consumption did not affect oxidative status. These findings are in contrast with a previous study showing and increase in both protein carbonyl and 8-OH-d-Guanosine in captive zebra finches after one year, even in the control group, with effects on survival of the latter (Marasco et al. 2017). It is worth noting, however, that they measured protein carbonyl in cells and 8-OH-d-Guanosine in plasma, the opposite of us, which may reflect more tightly the survival probabilities, as free plasma 8-OH-d-Guanosine could reflect cell death and release of DNA, a worse scenario than DNA damage found in the cells. Finally, they showed that the effects on age-related damage accumulation and of this damage on survival is context-dependent, with harsh foraging environment suppressing survival effects (see Constantini 2014 for a review on oxidative stress effects on the wild). For the effects of metabolism on oxidative stress in zebra finches, Beamonte-Barrientos and Verhulst (2013) reported an absence of effects of acute cold exposure induced increase in metabolic rate on OXY and d-ROMs levels in zebra finches, while (Stier et al. 2014) showed a similar absence of a significant increase in oxidative damage on plasmatic markers of both with an acute and a chronic cold induced increase in metabolic rate, but an increase in DNA damage, although only with acute but not chronic cold exposure, which would be more comparable to our case (for the observed increase in RMR in February). However, they showed a decrease in OXY with chronic cold exposure, which does not replicate in our experiment. These complex and diverse results may reflect tissue-dependent effects (see discussion in Costantini 2019), or rather be related to the difficulty of predicting the relationship between oxygen consumption and oxidative status without accounting for factors such as the mitochondrial uncoupling state (Hou et al. 2021; Stier et al. 2014). Measures more integrative than RMR, such as SMR (Standard Metabolic Rate, i.e. metabolism at a certain ambient temperature out of the thermoneutral zone) or DEE (Daily Energy Expenditure) may potentially be more relevant in the estimation of costs and constraints in ecologically realistic conditions (potentially for our birds, housed in outdoors aviaries exposed to natural environmental variation). Such measures should be considered in the future in this kind of experiments (see discussion in Briga and Verhulst 2017).



## **6. Apoptosis and mitochondrial superoxide production**

### **6.1. Apoptosis**

Another consequence of blood glucose levels is the effect it may have on cell survival, with cell death induced by several mechanisms, mainly related to glycoxidative damage (reviewed in Allen et al. 2005). Methylglyoxal has also been shown to induce eryptosis (erythrocyte apoptosis) in humans (Nicolay et al. 2006), and some of the mechanisms of methylglyoxal-induced apoptosis have been reported to be mediated by superoxide production (e.g. Du et al. 2001). This higher apoptosis level can induce anaemia and circulatory problems as increased thrombosis risk in diabetes patients (reviewed in Williams et al. 2023).

In birds, it is evident that this should not happen with the same probability for a given glucose level, even if such levels would be pathological for humans, as their glycaemia is naturally higher. Also, some of the mechanisms participating into the induction of apoptosis due to hyperglycaemia in mammals are probably not present here. Indeed, the low reliance on glucose of avian erythrocytes (Johnstone et al. 1998) would convey them a higher resistance to hyperglycaemia-induced apoptosis. Nevertheless, exploring if our supplementation treatments, by increasing the cell exposure to glucose, dicarbonylic compounds (methylglyoxal) and the resulting AGEs, affect blood cell apoptosis in relation to prooxidative state (i.e. superoxide production) may inform us on the potential physiological mechanism mediating the consequences of these products on birds health. Indeed, previous studies have shown that high glucose levels can be related with anaemia in birds (Minias 2014).

For what we could observe, we can outline that, while apoptosis levels were much higher in November, the levels in treated birds remained below those of the control birds. This pattern was reversed in all the following months. Both methylglyoxal and glucose supplemented birds showed a higher ratio of early apoptotic red blood cells over healthy living cells in February, May, and August. Thus, except in the first session, when general apoptosis levels were also very high and variable, this is in line with the expected outcomes. It is possible that the first session was affected by a higher measurement error, including the higher overall cell mortality. It is also worth noting that November was a high mortality period, which could partly be related with this increased cell death (see further discussion in **section 6.2**).

Apoptosis levels 24 hours after the first measure were more homogeneous, increasing in the control group and decreasing in the treated groups in comparison to the first measure in both February and May, while November and August values did not differ from the initial measurement. This homogenisation process could be explained by a similar environment of the cells *ex vivo*, suggesting that the observed differences in cell death were related to the different cell surrounding environments they were experiencing *in vivo*, and therefore they would present a considerable resilience. It is interesting to point out that bird erythrocytes have nucleus, which would make them able to repair their components and thus be more resilient than mammal red blood cells in this regard (see Stier et al. 2015). Finally, it is worth noting that early apoptotic to living cell ratio was found to be highly repeatable in our population ( $R = 0.637 \pm 0.052$ ;  $CI_{95}[0.516, 0.721]$ ;  $P\text{-value} = 0.0003$ ), which indicates this trait could be further considered as a reliable individual trait to be explored in future studies.

### **6.2. Superoxide**

In terms of the possible mechanisms underlying the observed differences, mitochondrial superoxide production is known to actively participate in cell death in different cell models

studied (e.g. Stowe and Camara 2009; Kanamori et al. 2010; Ma et al. 2011; Fujii et al. 2022), and the observed patterns were consistent with that, with much higher values observed in early apoptotic than in living cells. Moreover, while superoxide production decreased after November in living cells, it increased in early apoptotic cells, although only from May on (with a temporal decrease in February for the treated groups; see below). Its pattern of variation is also complex and associated to a rather low individual repeatability (around 10% and 12% for living and early apoptotic cells, respectively). The patterns of change in superoxide production after 24 hours were also inconsistent, with both increases and decreases depending on the treatments, type of cells and month. Moreover, none of these values were found to correlate with whole body O<sub>2</sub> consumption, although this kind of correlation is difficult to establish when changing scales (i.e. in this example from blood cells to whole body), as the subset of cells measured do not necessarily represent a good proxy of whole-body metabolism and vice versa (see Suarez 2012 for a general discussion). Also, the respirometry measurements are performed in a different day, and represent the RMR, which may not be the most reliable indicator of the superoxide production levels measured here. Finally, without controlling for coupling status, the relationship between O<sub>2</sub> consumption and O<sub>2</sub><sup>-</sup> production is not straightforward (see Hou et al. 2021 for a general discussion, and Stier et al. 2014a; Stier et al. 2014b for experimental results on zebra finches).

As a general description of the patterns observed in the experiment, first we can see how higher superoxide production per living cell was reported for cells from the treated birds than from the control in February. This effect disappears after 24 hours, when both treated groups decrease their superoxide production. However, lower values are found in methylglyoxal supplemented birds in May. Finally, only trends are observed in August (higher levels for methylglyoxal and lower for glucose). In general, superoxide production per living cell increased in the control group after 24 hours, significantly or marginally, except in November, when it marginally decreased. The latter effect may be due to a regression to the mean event, as superoxide production levels were much higher during this month, while in the other cases it may actually be reflecting the effect of the higher oxygen levels at which cells are exposed *ex vivo* (see discussion in Palacio-Castañeda et al. 2022). All of these patterns suggest a certain homogenization across treatments after 24 hours, similar to what we see for apoptosis as well, and potentially linked to this phenomenon, as a mechanism underlying it (i.e. superoxide production leading to apoptosis; reviewed in Fujii et al. 2022).

Regarding early apoptotic cells, superoxide production per cell rose after February, with methylglyoxal treated birds always showing lower levels (only marginally in August). Glucose treated birds, however, show significantly lower level than the control only in February. For the values after 24 hours, the patterns were inconsistent, with an increase (November), decrease (February and August) or no change (May) in the control group, depending on the month, and no change for the treated groups. Given that after 24 hours the cells which were observed to be in early apoptosis may correspond to a new “cohort” of cell death events, influenced by the new *ex vivo* environment, the low significance of these changes, and the multiple comparisons made, making interpretations on these patterns doubtful.

The extent to which the observed temporal patterns may reflect seasonal variation through effects like temperature acclimation (Pacheco et al. 2023), and if they may as well relate to the measured oxidative damage markers, remains to be explored. Although it has not been tested in the present models, the effects of age on reduced mitochondrial quality (Dawson and Salmón

2020) and (tissue-dependent) increased superoxide production (Salmón et al. 2022) in zebra finches can also be influencing some of the results.

## **7. Heterophil-to-lymphocyte ratio**

Heterophil-to-lymphocyte ratio (H/L ratio), a parameter used to indicate inflammation status in birds (e.g. Ewenson et al., 2001; Lentfer et al., 2015; Skwarska, 2018), was higher in methylglyoxal supplemented birds than in the control group in February and May, with a trend to be increased in August, also shown for the glucose group. However, in November, in which H/L ratio was much higher than in the other months, methylglyoxal treated birds had lower levels than the control instead.

The higher levels in November are possibly related to what we observe for both apoptosis and superoxide production per living cell, from which it can be concluded that birds generally suffered a higher levels of homeostasis disruption during that period. In the light of this, and if the mentioned relationship reflects some underlying causal link, what we observe in this parameter (H/L ratio) would confirm that the reported effects are not caused (at least not mainly) by some condition experienced by the cells during the processing of the samples, but by relevant biological processes occurring *in vivo*. This is so as the proportion of immune cells in a sample, that were measured on the same day and on the same samples as apoptosis and superoxide production, is less likely to be modified *ex vivo* as the latter two.

The patterns observed suggest that methylglyoxal supplementation, and with less certainty glucose supplementation, contribute to the inflammatory process, likely through the increase in AGE production (i.e. CML is partly increased by the treatments, see **section 1**). This is in line with a previous study showing an increase in inflammatory response in chicken in relation to increased plasma AGEs (Wein et al. 2020), which also suggests galactin 3 as a potential receptor for AGEs in birds, something that has remained elusive for a long time and as such was traditionally proposed as a mechanism for birds' resistance to the deleterious effects of AGEs (Szwergold and Miller 2014). Although it remains also possible that this increased H/L ratio was an effect of dehydration derived from an expected lower drinking rate in the methylglyoxal group, as it has recently been reported for zebra finches with limited water accessibility (Colominas-Ciuró et al. 2024). That said, our birds, as previously discussed (see **section 3**) do not show different water content in any of the tissues assessed, suggesting this not to be a likely effect of the treatment.

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# General discussion, conclusion and perspectives





## General discussion, conclusion and perspectives

This thesis explored how glycaemia and its consequences affect birds fitness and ageing and how this may relate to life-history evolution in the context of the Pace of Life Syndrome hypothesis (POLS). For this, three tasks were set up, each with a different particular approach: first, a comparative analysis of albumin glycation across the birds tree, including plasma glucose levels, and aimed at exploring how these parameters vary across taxa (i.e. are different birds equally prone to glycation?) and how they may have coevolved with life-history traits and species specific diet; secondly, a correlational study on a wild species trying to relate glucose and glycation to fitness, by measuring both reproductive outcome and survival; and third, a laboratory experiment with a model passerine species trying to induce hyperglycaemia and/or higher glycation and AGE production, in order to explore the consequences on survival and ageing, mainly through other parameters that have been more widely explored in the past. From these, a summary of the main conclusions and a description of the perspectives, some based on research currently ongoing in our team, are described in the following sections.

### 1. Conclusions

#### 1.1. Comparative analysis

The first chapter of this thesis showed how plasma glucose and albumin glycation rates vary across a wide variety of bird taxa, many of which had never been assessed. Some birds, like flamingos (Phoenicopteriformes) or geese and ducks (Anseriformes), show a lower glycaemia, comparable to that of some mammals, while others, like songbirds (Passeriformes) or swifts (Apodiformes), show much higher levels. Glucose levels were also positively related to albumin glycation, but in an allometric fashion, i.e. glycation linearly increased with  $\log_{10}$  transformed glucose, which means that birds with a higher glycaemia show comparatively lower glycation rates. Also, we showed that some groups have species with very diverse glycation rates, as it is the case of Procelariiformes, with some species showing the highest glycation levels for the entire dataset, while others show more moderate levels.

In terms of POLS, this study confirms the previously reported negative relationship between body mass and glucose levels (Umminger 1975; Kjeld and Ólafsson 2007; Braun and Sweazea 2008; Tomášek et al. 2019; Szarka and Lendvai 2024), potentially caused by the positive relationship between glucose and BMR *per* gram that has previously been demonstrated (Lasiewski and Dawson 1967; Tomášek et al. 2019), as higher body mass associates with lower metabolic rate *per* gram. Nevertheless, the previously reported positive relationship between glucose and body mass and phylogeny-corrected average species clutch mass (as an index of species-specific reproductive investment) in Passeriformes (Tomášek et al. 2019) did not come out in our analyses. Moreover, the previously non-significant relationship of glucose with maximum lifespan was here found to be significant for both the linear and the quadratic term, suggesting an increase in mass and phylogeny-adjusted lifespan with glucose, up to a threshold.

Finally, although we failed to find significant differences in glucose levels between the diet groups, as other recent studied did (Szarka and Lendvai 2024), we report higher albumin glycation levels in terrestrial carnivores than in omnivores, something that was interestingly not found in aquatic predators, for which we suggest some differences in dietary components between terrestrial and aquatic ecosystems, as proportion of different polyunsaturated fatty acids, could play a role (see **perspectives**).

## **1.2. Correlative data on the wild**

The second chapter shows the first study on variation in albumin glycation levels in a wild bird, and one of the firsts relating protein glycation with fitness. Here, we used adult females from a population of age known Alpine swifts (*Tachymarptis melba*), a bird relatively long-lived for its body mass, as a model for the study of glycation in wild birds. We demonstrated that both plasma glucose and albumin glycation levels varied during a specific reproductive season, with glucose increasing together with body mass, after reproduction, while glycation decreased, being also negatively related with body mass. The dynamics of these variables across the reproductive period (i.e. how much they change) was also related to reproduction through clutch size, with females laying more eggs lowering more their glycation levels while gaining less mass.

Although we could not find any cost of reproduction expressed through the post-reproductive values of these parameters, while statistically controlling for the pre-reproductive values, we did find a relationship of pre-reproduction glucose levels with fledging success (proportion of chicks hatched that fledged), suggesting mothers' glucose increases reproductive success up to a level (around 307 mg/dL) above which it does not improve anymore. This, together with the finding of glucose after reproduction predicting higher survival to the next year, while albumin glycation measured at the same instance relate negatively with survival, although only when controlling for age, suggest that glucose can act as a mediator of an antagonistic pleiotropy shaping ageing.

Finally, albumin glycation levels were shown to vary in a bell-shaped way with age, reaching a peak at 5 to 6 years, similarly to other traits studied in this species (Moullec et al. 2023), although in a cross-sectional manner, which limits our ability to interpret the source of this variation (i.e. ageing or selective appearance/disappearance).

## **1.3. Experimental work on ageing in captivity**

In the third chapter, we report a laboratory experiment with captive zebra finches (*Taeniopygia guttata*), a captive-model bird species, in which a supplementation with either glucose or methylglyoxal (a reactive dicarbonyl compound) is provided to the birds in order to explore how this may affect ageing-related processes, hypothetically by increasing glycation, AGEs, oxidative stress, systemic inflammation or changes in body composition, which in turn could induce damage in the form of telomere attrition, cell death or reduced body condition, ultimately negatively impacting survival.

The results show a clearly increased mortality hazard in glucose-supplemented birds, which is not observed in methylglyoxal-treated ones. The reasons for this are subsequently discussed by looking at the mechanisms that may mediate this mortality outcome. First, we detected higher albumin glycation levels in the glucose group, but only at the end of the experiment, after one year of treatment. Since glycation levels do not explain mortality hazard in our models, it suggests that glycated albumin might not be the driver of the observed higher mortality in glucose treated birds. Second, we observed an increase in glyoxal, but not in methylglyoxal, at the end of the experiment in both supplemented groups, with this glyoxal effects being sex-biased (i.e. it increases with age only in males, which also show higher levels within the methylglyoxal supplementation group). This, together with the fact that CEL, an AGE compound, positively correlates with glyoxal levels, and negatively influences survival, seems to point towards an important line of causation. Nevertheless, as CEL was not increased by the

treatments, while CML, that did not influence mortality, but increased with age, did increase in the treated groups, although in a complex and time dependent way, makes interpretations more difficult, especially when we consider that CML is a recognized product of glyoxal metabolism, while CEL is so from methylglyoxal. Furthermore, this would still not explain why the mortality observed in the glucose group is not observed in the methylglyoxal group as well.

For the remaining parameters, both supplemented groups increased DNA damage at the end of the experiment, and red blood cell apoptosis throughout the experiment, except in the first measurement, while the inflammatory marker (H/L ratio) was mostly increased by the methylglyoxal treatment. Moreover, plasma glucose levels were found to correlate with general oxidative damage (d-ROMs marker) in the control baseline state, while the effects of the treatments on mitochondrial superoxide production were seasonally varying and context-dependent (for example, the treatments increased superoxide production in early apoptotic, but decreased it in healthy living cells, during February, while acting differently and in opposing directions in other months). All of this, together with the inability of the treatments to influence metabolism and body composition, except from a reduced fat score at the end of the experiment, makes us again unable to determine what caused the increased mortality in the glucose group.

Finally, in this line, some other possible causes of the observed mortality are discussed, such as the fact that the glucose group started the experiment with higher blood glucose levels, which nevertheless did not predict mortality, the potential effects of higher glucose intake on the microbiota or the deficiency of certain nutrients that could have been caused by compensatory feeding behaviour in glucose-supplemented birds.

## **2. Perspectives**

### **2.1. Comparative analysis**

Given the results obtained with the present study, we can use this information now to decide which species would be the most interesting to explore further. For example, comparing species that, for comparable glycaemia levels, exhibit either higher-than-expected glycation levels or low glycation levels could be a way to search for traits that may constitute adaptations against glucose induced damage. Also, the fact that groups like Procelariiformes, which are generally long-lived, present species with both high and low glycation levels, may mean that different approaches exist for sustaining glucose levels that would be pathological for other organisms without suffering the consequences. For example, while some species could be better able to avoid glycation, others may have higher glycation levels but be more resistant to AGE formation or to the proinflammatory and/or oxidative stress consequences that follow.

It is also worth considering that some groups were underrepresented in our dataset, such as herbivorous, which also showed very high glycaemia variation, with geese (Anseriformes), which are characterised by low glycaemia, and pigeons (Columbiformes), within which we find the highest glucose value reported in our dataset. Also, we could not properly distinguish between granivorous and frugivorous species, since in our case most of them had both components relatively equal in their diets, with the exception of the fully granivorous zebra finches. This may have prevented us from finding significant differences in glycaemia that had been reported previously (Szarka and Lendvai 2024). Considering other lifestyle variables, such as flying mode and migration, could also help to discern the ecological pressures moulding glycaemia across species in the future. The particular case of non-flying versus flying species is very interesting to



help understanding the reasons why birds show comparatively high glucose levels. This, nevertheless, can be difficult to test, as in our case, most of the species sampled are flying ones, with the few that are not showing correlated traits like very high body mass or belong to very specific groups (e.g. cassowaries or penguins). This would be more easily tested by comparing closely related species with similar ecologies and body masses, only differing in their flying ability (although losing flying ability may be related to an increase in weight), as it is the case for many species that have colonized oceanic islands and lost their flying ability, but have mainland relatives which have not, as it is common among pigeons (Columbidae) or rails (Rallidae).

Moreover, collecting more data, and of better quality for life history traits (e.g. age reached by a certain percentage, like 90 %, of the individuals or age at onset and rate of actuarial senescence, instead of maximum lifespan; see Kremenetz al. 1989; Ronget and Gaillard 2020), would help future studies to perform these analyses over more species. Collecting species based on previously available information may also be a strategy to consider. Exploring the possible relationship between plasma glucose and glycation levels and other physiological traits such as metabolic rate in such a variety of birds would also help to better understand the evolution of glycation resistance in terms of POLS. For food guilds, more detailed classifications could be made considering not only the type of food but also its composition (i.e. the percentage of different components, as fibres, antioxidants,  $\omega$ -3 versus  $\omega$ -6 polyunsaturated long chain fatty acids or glucose versus fructose or other carbohydrates).

Finally, acquiring more information on protein turnover rates and albumin primary (aminoacids sequences) and tertiary (degree of exposition of the glycation prone residues; see Zuck et al. 2017) structures across species would help to inform on mechanisms contributing to differential resistance profiles shown by diverse groups.

Currently, our team is also working on more detailed intraspecific studies on glycation levels on different species in which enough individuals from known age, sex, and other interest variables are available, as it is the case for king penguins (*Aptenodytes patagonicus*) and flamingos (*Phoenicopterus roseus* and *P. ruber*). These studies will help to better understand how glycation varies within and between individuals in a population, in a similar way as the study on Alpine swifts, which is needed to determine if and how this trait may evolve (see **section 2.2.**).

## **2.2. Correlative data in the wild**

Our current results on glycaemia and albumin glycation in swifts indicate they can predict survival and at least one parameter of reproductive success (fledging success in the case of glucose), i.e. they correlate with fitness. Therefore, the logical next step in order to determine if they can evolve by natural selection, and therefore contribute to species physiological adaptations to their mode of life, would be to determine the heritability of the traits. This is possible given that there is good information on pedigrees for the studied population, and many individuals can be sampled and their early life conditions controlled and even manipulated, such that experiments including cross fostering can be performed to disentangle environmental parental and nest effects from genetic effects. The main current caveat for that is that the number of samples needed to perform animal models is quite big (see Wilson et al. 2010), and limitations exist in the number of samples that can be processed at a time with the mass spectrometry technique, something that limited sample size in our current study. However, while this poses a problem for glycation, glucose, although more variable, is much easier to measure, allowing for this kind of analyses to be performed.

On another issue, for the study of age-related variation of glycation, longitudinal set ups would be preferable, as this would allow us to separate the effects of age from those of selection. Nevertheless, preliminary analyses performed by our team on old samples have shown that it may not be possible to obtain reliable glycation levels on samples that have not been conserved in a very strict way (-80 °C instead of -20 °C, as they are currently being conserved). This would rule out the possibility of using samples from the last 20 years of long-term study, and make research on age variation of glycation much harder, at least in regard to this population.

It also remains to be tested if the obtained results can be repeated in different years, as the dynamics of glucose, glycation and body mass change, and how they reflect body condition/individual quality may vary from year to year, depending on the annual variation on resources availability. Including also more detailed temporal resolution could help to better understand the explored dynamics. Finally, experimental manipulation of reproductive costs, such as by clutch or brood size manipulations, or through decreased foraging efficiency, by procedures artificially increasing the birds' energy demands, by adding supplemental weights or modifying flying feathers, could help on determining how ecological conditions affect glycation levels, for example by inducing chronically increased glycaemia, as it has similarly been done in zebra finches before (Briga 2016), although without knowing the consequential effects on glycation. Experiments altering glycation and/or AGE levels directly could also be performed, to study their effects on reproduction and survival, although this is more complicated to do in a predictable way, as it is shown by the results of the zebra finches experiment. Other body condition (e.g. fat scores) or physiological status-related variables, such as plasma triglycerides or corticosterone levels, could help to better understand to which extent glycation constitutes a body condition measure in this species, and if its effects on fitness are direct or the detected correlation is mediated by other underlying common cause.

## **2.3. Experimental work on ageing in captivity**

### **2.3.1. Ongoing research: integrative traits and omics**

The first thing to consider in this section is that the results shown within this thesis are only a part of what has been measured during this experiment. The first next step, thus, will be to interpret the coloration results, and analyse the telomere and haematology data. Flying ability, as a functional ageing parameter, has to be determined as well, as not all the videos have been processed yet. Also, more complex statistical procedures, such as pathway analyses, can be applied to better understand the chain of influences of the currently assessed traits, and thus better assess the mechanisms which are more relevant for the observed outcomes, mainly the higher mortality rate in the glucose but not the methylglyoxal group. Another of the main following steps will be to perform proteomics and metabolomics analyses on the tissues collected from the subset of individuals sacrificed at the end of the experiment, to explore the mechanisms used by birds to cope with glycaemic stress, notably how they resist glucose and glycation related damage. This will be done in the context of the fourth axis of the ANR grant which funds this project (AGEs – ANR21-CE02-0009). In this line, some of the candidate targets are galectin 3, a possible RAGE (Receptor of Advanced Glycation End-products), FN3K (Fructosamine 3 Kinase, a deglycation enzyme), glyoxalase I and glyoxalase II (which detoxify glyoxal by converting it to glycolate and methylglyoxal by transforming it to lactate). Other measurements are under consideration within this part of the project, such as assessing the exact glycation sites of the plasma proteins explored in zebra finches, mainly albumin, and try to relate this to potential alterations in protein function (see e.g. Henning et al. 2022)

### **2.3.2. Planned research: hormones**

Other measures initially planned but not yet addressed are to determine the effects of the treatments on hormonal levels, i.e. glucocorticoids (in the collected feathers, via collaborations already established) and insulin/glucagon in plasma at different points of the treatments. These key glucoregulatory hormones have never been measured in zebra finches, and determining their status and influence on glucose levels, especially in comparison with a literature that is currently biased towards Galloanserae (chicken, quails, ducks...) would help to broaden our knowledge about glucose regulation in birds (see Braun and Sweazea 2008; Sweazea 2022).

### **2.3.3. Future targets: microbiota, specific defence mechanisms and senescence patterns**

Other things that have not been considered here and that could also be included in future studies are the effects of glucose supplementation on the microbiota, as this is discussed as one of the mechanisms potentially affecting survival. In fact, gut microbiota is well known to affect and to be affected by glucose-diet in humans, and is recognized as a key factor in the prevalence of diabetes and obesity in our modern society (e.g. Greiner and Bäckhed 2011; Nuli et al. 2019). Also, similar but longer studies, or under more controlled conditions, could be performed, trying to see what are the consequences on the long term, or under different environmental conditions, perhaps avoiding the seasonal effects observed here. Targeting the variables that have been found to be relevant in this experiment, e.g. focusing on glyoxal more than methylglyoxal as an AGE formation agent, or measuring the change in expression on particular genes, by RT-qPCR or Western blots for proteins, that may be found to be altered on our currently ongoing wide omics analyses, could help to facilitate the process in terms of workload and budget. Also, exposing the birds deliberately to different conditions can help to understand how the effects of glycation and AGEs on survival and ageing patterns may dependent upon external factors. Using younger birds and for longer periods would also allow to better explore senescence patterns in relation with glycation and AGEs accumulation (i.e. onset and rate of ageing for these traits).

### **2.3.4. Effects on functional ageing parameters**

The effects of glycation and AGEs on functional ageing in passerine birds (i.e. performance related measurements) could also be explored using this model, helping to determine which mechanisms constitute relevant links between the formation/accumulation of these compounds and survival in these animals. This could help determine if there are aspects of their physiology which are more resistant to the common deleterious effects of glucose, glycation and AGEs in mammalian models (i.e. vascular, renal, neurological and visual dysfunctions, for instance). For example, in a similar way as flying test, things like neurological status, with tasks such as memory tests, could be assessed without too much difficulty. Exploring how glucose (as the main energy fuel of the brain) or glycation affect cognition is of prime interest in the recently developing context of studying cognition as a modulator of individual fitness (see e.g. Ashton et al. 2018; Fichtel et al. 2023). Something also interesting in this line would be to test how an increase in glycation and AGE like the ones seen here could affect glucose tolerance, and its changes with age in birds. A protocol for intraperitoneal glucose tolerance test (GTT) has previously been developed for zebra finches (Montoya et al. 2020), with a higher glucose tolerance being related with increased survival in old individuals (Montoya et al. 2022). Another similar integrative measurement that could be useful is metabolic flexibility, understood as the ability to change fuel usage when the organism is submitted to a stimulus, environmental

challenge or any condition that would normally imply it, e.g. the change occurring from fasted to fed state, from rest to exercise or when a stress (i.e. homeostasis threatening condition) is posed to it (see Saltin and Goldnick 1983; Kelley and Mandarino 2000; Galgani et al. 2008). This is mostly measured by calculating the change in RQ (Respiratory Quotient) or its proxy, RER (Respiratory Exchange Ratio), over a certain treatment. Most commonly, feeding is used to assess how RQ increases, with diabetes and obese patients normally showing lower metabolic flexibility, i.e. lower RQ change, associated also with a higher fasting RQ (see Galgani and Fernández-Verdejo 2020), and constituting a marker of ageing (Curl et al. 2024). This is easy to measure within our set up, and the change of birds' metabolic flexibility after a dietary supplementation can be measured controlling for body mass, fat scores, and even blood glucose and glycation levels, to determine how these traits may influence metabolic health in birds. Indeed, a similar experiment is currently undergoing with hummingbirds, known to have a high metabolic flexibility, within one of my collaborations with the University of Toronto (Pr. Kenneth Welch), to explore if a dietary supplementation like the one within this thesis can affect this crucial capacity in nectarivorous birds.

### **2.3.5. Metabolic ageing**

On a side note, the information gathered in this experiment on seasonal metabolic and glucose variations can be further explored. For instance, there seems to be a metabolic fluctuation characterised by an increase in RMR during winter and spring (February and May), followed by a decrease back in summer (August). Interestingly, these fluctuations are less evident in the older birds, indicating they may show a lower metabolic flexibility, understood here as phenotypic plasticity in the metabolic rate (McKechnie 2008), and therefore potentially a lower capacity to face environmental fluctuations. This hypothesis, nevertheless, remains to be formally addressed with statistical tests, including the effects of treatments on it.

## **3. Summary**

As a summary, we can conclude that this work gives us a further look into the still underexplored field of the effects of glucose and its consequential damages (glycation, AGE) on fitness and ageing in birds, and its coevolution with life-history and ecological pressures like diet. We first provide a wide report of how plasma albumin glycation varies across diverse avian taxa and in relation to glucose, confirming a higher resistance to glycation in species with higher glucose levels, and a higher glycation rate in terrestrial carnivorous birds. At the same time, we provide criteria and examples for future selection of model species for the study of this phenomenon. In this line, we also suggest ways to use the comparative approach to help disentangle the evolutionary cause of birds comparatively high glucose levels within vertebrates. Then, we determine the contrasting effects of glucose and glycation on fitness, using a population with individuals with known ages and pedigrees, which could be useful for future determinations of heritability and therefore the suitability of these traits to evolve by natural selection and thus contribute to the set of adaptations hypothesized to be part of POLS, as explored in the comparative study. Differential and more or less direct or indirect consequences of glucose on fitness, including the apparent age dependent effects of some of them, like glycation, make it an interesting trait to be explored as an antagonistic pleiotropic factor modulating ageing phenotypes in the wild. Moreover, our experimental approach showed that dietary glucose and dicarbonyl supplementation have an effect on birds glycation and glyoxal formation, which in turn affects the formation of certain AGEs, like CEL (Carboxy-Ethyl-Lysine), which affects survival. Also, the supplementation can increase erythrocyte apoptosis and DNA damage, with

proinflammatory stress appearing more impacted by methylglyoxal, while glucose clearly affects mortality. The exact reasons for these latter effects remain to be answered in detail, and many functional ageing parameters and physiological decline proxies are being studied currently or suggested to be explored as a way to improve our knowledge on the effects of glucose, glycation and AGE on birds ageing. Last, but not least, omics explorations are planned to help us unveil the mechanisms underlying bird responses to glucose related stress, which could also give us useful information on the ways that may be predominant for them to cope with physiological blood sugar levels that, despite being normal for them, would be lethal for similarly sized mammals. This is of particular interest given the high mass-adjusted longevity of birds in comparison to mammals, and contributes to pave an important way towards the pursued horizon of a general better understanding of the evolution of ageing.

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## Version française

### **Discussion générale, conclusion et perspectives**

Cette thèse a étudié comment la glycémie et ses conséquences affectent le fitness et le vieillissement des oiseaux et comment cela peut être lié à l'évolution des traits d'histoire de vie dans le contexte de l'hypothèse des syndromes du rythme de vie (POLS). Pour ce faire, trois tâches ont été mises en place, chacune avec une approche particulière différente : premièrement, une analyse comparative de la glycation de l'albumine à travers l'arbre des oiseaux, y compris les niveaux de glucose plasmatique, et visant à explorer comment ces paramètres varient à travers les taxons (c'est-à-dire, différents oiseaux sont-ils également enclins à la glycation ?) et comment ils ont pu coévoluer avec les traits d'histoire de vie et la diète spécifique à chaque espèce ; deuxièmement, une étude corrélacionnelle sur une espèce sauvage essayant de relier le glucose et la glycation au fitness, en mesurant à la fois les résultats reproductives et la survie ; et troisièmement, une expérience de laboratoire avec une espèce de passereau modèle essayant d'induire une hyperglycémie et/ou une production plus élevée de glycation et d'AGE, afin d'explorer les conséquences sur la survie et le vieillissement, principalement par le biais d'autres paramètres liés au vieillissement qui ont été plus largement explorés dans le passé. A partir de là, un résumé des principales conclusions et une description des perspectives, dont certaines sont basées sur des recherches actuellement en cours dans notre équipe, sont décrits dans les sections suivantes.

#### **1. Conclusions**

##### **1.1. Analyse comparative**

Le premier chapitre de cette thèse a montré comment les taux de glycation du glucose plasmatique et de l'albumine varient dans une grande variété de taxons d'oiseaux, dont beaucoup n'ont jamais été évalués. Certains oiseaux, comme les flamants (Phoenicopteriformes) ou les oies et les canards (Anseriformes), présentent une glycémie plus faible, comparable à celle de certains mammifères, tandis que d'autres, comme les passereaux (Passeriformes) ou les martinets (Apodiformes), affichent des taux beaucoup plus élevés. Les niveaux de glucose étaient également positivement



liés à la glycation de l'albumine, mais de manière allométrique, c'est-à-dire que la glycation augmentait linéairement avec le glucose  $\log_{10}$  transformé, ce qui signifie que les oiseaux ayant une glycémie plus élevée présentent des taux de glycation comparativement plus faibles. Nous avons également montré que certains groupes ont des espèces avec des taux de glycation très divers, comme c'est le cas des Procelariiformes, avec certaines espèces montrant les niveaux de glycation les plus élevés pour l'ensemble des données, tandis que d'autres montrent des niveaux plus modérés.

En termes de PLOS, cette étude confirme la relation négative précédemment rapportée entre la masse corporelle et les niveaux de glucose (Umminger 1975 ; Kjeld et Ólafsson 2007 ; Braun et Sweazea 2008 ; Tomášek et al. 2019 ; Szarka et Lendvai 2024), potentiellement causée par la relation positive entre le glucose et le BMR par gramme qui a été précédemment démontrée (Lasiewski et Dawson 1967 ; Tomášek et al. 2019), étant donné qu'une masse corporelle plus élevée est associée à un taux métabolique par gramme plus faible. Néanmoins, la relation positive précédemment rapportée entre le glucose et la masse corporelle et la masse de la cuvette moyenne de l'espèce corrigée par la phylogénie et la masse corporelle (en tant qu'indice de l'investissement reproductif spécifique à l'espèce) chez les Passeriformes (Tomášek et al. 2019) n'est pas ressortie dans nos analyses. De plus, la relation précédemment non significative du glucose avec la durée de vie maximale s'est ici révélée significative à la fois pour le terme linéaire et le terme quadratique, suggérant une augmentation de la longévité maximale corrigée par la phylogénie et la masse corporelle avec le glucose, jusqu'à un seuil.

Finalement, bien que nous n'ayons pas trouvé de différences significatives dans les niveaux de glucose entre les groupes de diète, comme l'ont fait d'autres études récentes (Szarka et Lendvai 2024), nous rapportons des niveaux de glycation de l'albumine plus élevés chez les carnivores terrestres que chez les omnivores, ce qui, de manière intéressante, n'a pas été trouvé chez les prédateurs aquatiques, pour lequel nous suggérons que certaines différences dans les composants alimentaires entre les écosystèmes terrestres et aquatiques, comme la proportion de différents acides gras polyinsaturés, pourraient jouer un rôle (voir les **perspectives**).

## **1.2. Données corrélatives dans la nature**

Le deuxième chapitre présente la première étude sur la variation des niveaux de glycation de l'albumine chez un oiseau sauvage, et l'une des premières à établir un lien entre la glycation des protéines et le fitness. Nous avons utilisé des femelles adultes d'âge connu d'une population de martinets alpins (*Tachymarptis melba*), un oiseau qui vit relativement longtemps pour sa masse corporelle, comme modèle pour l'étude de la glycation chez les oiseaux sauvages. Nous avons démontré que les niveaux de glycation de l'albumine et du glucose plasmatique variaient au cours d'une saison reproductive spécifique, le glucose augmentant au même temps que la masse corporelle après la reproduction, tandis que la glycation diminuait, étant également liée négativement à la masse corporelle. La dynamique de ces variables au cours de la période de reproduction (c'est-à-dire l'ampleur de leur changement) était également liée à la reproduction à travers de la taille de la couvée, les femelles pondant plus d'œufs réduisant davantage leurs niveaux de glycation, en gagnant aussi moins de masse.

Bien que nous n'ayons pas trouvé de coût de la reproduction exprimé par les valeurs post-reproductives de ces paramètres, en contrôlant statistiquement les valeurs pré-reproductives, nous avons trouvé une relation entre les niveaux de glucose pré-reproductives et le succès de l'envol (proportion de poussins éclos qui se sont envolés), suggérant que le glucose des mères augmente le succès de la reproduction jusqu'à un niveau (autour de 307 mg/dL) au-delà duquel il ne s'améliore plus. Ce résultat, associé à la découverte que le glucose après la reproduction prédit une survie plus élevée l'année suivante, alors que la glycation de l'albumine mesurée au même moment est liée négativement à la survie, bien que seulement en contrôlant pour l'âge, suggère que le glucose peut agir en tant que médiateur d'une pléiotropie antagoniste façonnant le vieillissement.

Finalement, les niveaux de glycation de l'albumine varient en forme de cloche avec l'âge, atteignant un pic entre 5 et 6 ans, comme d'autres traits étudiés chez cette espèce (Moullec et al. 2023), mais de manière transversale, ce qui limite notre capacité à interpréter la source de cette variation (vieillesse ou apparition/disparition sélective).

### **1.3. Travaux expérimentaux sur le vieillissement en captivité**

Dans le troisième chapitre, nous rapportons une expérience de laboratoire avec des diamants mandarins (*Taeniopygia guttata*), une espèce d'oiseau modèle en captivité, dans laquelle une supplémentation en glucose ou en méthylglyoxal (un composé dicarbonyle réactif) est fournie aux oiseaux afin d'explorer la manière dont cela peut affecter les processus liés au vieillissement, hypothétiquement en augmentant la glycation, les AGE, le stress oxydatif, l'inflammation systémique ou la composition corporelle, qui à leur tour pourraient induire des dommages sous forme de raccourcissement des télomères, de mort cellulaire ou de réduction de la condition corporelle, ce qui aurait en fin de compte un impact négatif sur la survie.

Les résultats montrent un risque de mortalité clairement accru chez les oiseaux supplémentés en glucose, ce qui n'est pas observé chez les oiseaux traités avec méthylglyoxal. Les raisons de ce phénomène sont ensuite discutées en examinant les mécanismes qui peuvent être à l'origine de cette mortalité. Tout d'abord, nous avons détecté des niveaux de glycation de l'albumine plus élevés dans le groupe traité avec glucose, mais seulement à la fin de l'expérience, après un an de traitement. Comme les niveaux de glycation n'expliquent pas le risque de mortalité dans nos modèles, cela suggère que l'albumine glyquée n'est peut-être pas la cause de la mortalité plus élevée observée chez les oiseaux traités avec glucose. Deuxièmement, nous avons observé une augmentation du glyoxal, mais pas du méthylglyoxal, à la fin de l'expérience dans les deux groupes supplémentés, les effets du glyoxal étant liés au sexe (c'est-à-dire qu'ils augmentent avec l'âge uniquement chez les mâles, qui présentent également des niveaux plus élevés dans le groupe supplémenté en méthylglyoxal). Ceci, associé au fait que la CEL, un composé AGE, présente une corrélation positive avec les niveaux de glyoxal et influence négativement la survie, semble indiquer une ligne de causalité importante. Néanmoins, le fait que la CEL n'ait pas été augmentée par les traitements, alors que la CML, qui n'a pas influencé la mortalité mais a augmenté avec l'âge, a augmenté dans les groupes traités, bien que de manière complexe et dépendante du temps, rend les interprétations plus difficiles, surtout si l'on considère que la CML est un produit reconnu du métabolisme du glyoxal, alors que la CEL l'est à partir du méthylglyoxal. En outre, cela

n'expliquerait toujours pas pourquoi la mortalité observée dans le groupe glucose n'est pas observée dans le groupe méthylglyoxal également.

Pour les autres paramètres, les deux groupes supplémentés ont augmenté les dommages à l'ADN à la fin de l'expérience et l'apoptose des globules rouges tout au long de l'expérience, sauf lors de la première mesure, tandis que le marqueur inflammatoire (rapport H/L) a été principalement augmenté par le traitement au méthylglyoxal. En outre, les niveaux de glucose plasmatique se sont avérés corrélés avec les dommages oxydatifs généraux (marqueur d-ROMs) dans l'état de base du group contrôle, tandis que les effets des traitements sur la production de superoxyde mitochondrial variaient selon les saisons et le contexte (par exemple, les traitements ont augmenté la production de superoxyde dans les cellules dans une processus d'apoptose, mais l'ont diminuée dans les cellules vivantes saines, au cours du mois de février, tout en agissant différemment et dans des directions opposées au cours d'autres mois). Tous ces éléments, ainsi que l'incapacité des traitements à influencer le métabolisme et la composition corporelle, à l'exception d'une réduction du taux de graisse à la fin de l'expérience, nous empêchent une fois de plus de déterminer la cause de la mortalité accrue dans le groupe traité avec glucose.

Finalement, dans cette ligne, d'autres causes possibles de la mortalité observée sont discutées, comme le fait que le groupe glucose a commencé l'expérience avec des niveaux de glucose sanguin plus élevés, ce qui n'a toutefois pas prédit la mortalité, les effets potentiels d'un apport plus élevé en glucose sur le microbiote ou la carence en certains nutriments qui aurait pu être causée par un comportement alimentaire compensatoire chez les oiseaux supplémentés en glucose.

## **2. Perspectives**

### **2.1. Analyse comparative**

Compte tenu des résultats obtenus dans le cadre de la présente étude, nous pouvons maintenant utiliser ces informations pour décider quelles espèces seraient les plus intéressantes à explorer davantage. Par exemple, la comparaison d'espèces qui, pour des niveaux de glycémie comparables, présentent soit des niveaux de glycation plus élevés que prévu, soit des niveaux de glycation faibles, pourrait être un moyen de

rechercher des traits qui pourraient constituer des adaptations contre les dommages induits par le glucose. En outre, le fait que des groupes comme les Procelariiformes, qui ont généralement une longue durée de vie, présentent des espèces avec des niveaux de glycation à la fois élevés et faibles, peut signifier qu'il existe différentes approches pour maintenir des niveaux de glucose qui seraient pathologiques pour d'autres organismes sans néanmoins souffrir les conséquences. Par exemple, alors que certaines espèces pourraient être mieux à éviter la glycation, d'autres pourraient avoir des niveaux de glycation plus élevés mais être plus résistantes à la formation d'AGE ou aux conséquences pro-inflammatoires et/ou de stress oxydatif qui s'ensuivent.

Il faut également tenir compte du fait que certains groupes étaient sous-représentés dans notre ensemble de données, comme les herbivores, qui ont également montré une très forte variation de la glycémie, avec les oies (Anseriformes), qui sont caractérisées par une faible glycémie, et les pigeons (Columbiformes), au sein desquels nous trouvons la valeur de glucose la plus élevée rapportée dans notre ensemble de données. En outre, nous n'avons pas pu distinguer correctement les espèces granivores et frugivores, puisque dans notre cas, la plupart d'entre elles avaient les deux composantes relativement égales dans leur diète, à l'exception des diamants mandarins, entièrement granivores. Cela nous a peut-être empêchés de trouver des différences significatives dans la glycémie qui avaient été rapportées précédemment (Szarka et Lendvai 2024). La prise en compte d'autres variables liées au mode de vie, telles que le mode de vol et la migration, pourrait également aider à discerner les pressions écologiques qui façonnent la glycémie entre les espèces dans le futur. Le cas particulier des espèces non volantes par rapport aux espèces volantes est très intéressant pour comprendre les raisons pour lesquelles les oiseaux présentent des niveaux de glucose comparativement élevés. Néanmoins, cela peut être difficile à tester, car dans notre cas, la plupart des espèces échantillonnées sont des espèces volantes, les quelques espèces non volantes présentant des caractéristiques corrélées comme une masse corporelle très élevée ou appartenant à des groupes très spécifiques (par exemple, les casoars ou les manchots). Il serait plus facile de vérifier cette hypothèse en comparant des espèces étroitement

phylogénétiquement liées ayant une écologie et une masse corporelle similaires et ne différant que par leur capacité de vol (bien que la perte de cette capacité puisse être liée à une augmentation du poids), comme c'est le cas pour de nombreuses espèces qui ont colonisé des îles océaniques et perdu leur capacité de vol, mais qui ont des parents continentaux qui ne l'ont pas perdue, comme c'est souvent le cas chez les pigeons (Columbidae) ou les râles (Rallidae).

En outre, la collecte de données plus nombreuses et de meilleure qualité pour les traits d'histoire de vie (par exemple, l'âge atteint par un certain pourcentage, comme 90 % des individus, ou l'âge de rentrée et le taux de sénescence actuarielle, au lieu de la durée de vie maximale ; voir Kremenetz al. 1989 ; Ronget et Gaillard 2020), aiderait les études futures à effectuer ces analyses sur un plus grand nombre d'espèces. La collecte d'espèces sur la base d'informations précédemment disponibles peut également être une stratégie à envisager. L'exploration de la relation possible entre les niveaux de glucose et de glycation plasmatiques et d'autres traits physiologiques tels que le taux métabolique chez une telle variété d'oiseaux aiderait également à mieux comprendre l'évolution de la résistance à la glycation en termes de POLS. Pour les groupes de diète, des classifications plus détaillées pourraient être établies en tenant compte non seulement du type d'aliment mais aussi de sa composition (c'est-à-dire le pourcentage de différents composants, tels que les fibres, les antioxydants, les acides gras polyinsaturés à longue chaîne  $\omega$ -3 par rapport aux acides gras polyinsaturés à longue chaîne  $\omega$ -6 ou le glucose par rapport au fructose ou à d'autres carbohydrates).

Finalement, l'acquisition de plus d'informations sur les taux de renouvellement des protéines et les structures primaires (séquences d'acides aminés) et tertiaires (degré d'exposition des résidus sujets à la glycation ; voir Zuck et al. 2017) de l'albumine à travers les espèces aiderait à informer sur les mécanismes contribuant aux profils de résistance différentiels montrés par divers groupes.

Actuellement, notre équipe travaille également sur des études intraspécifiques plus détaillées sur les niveaux de glycation chez différentes espèces pour lesquelles on dispose d'un nombre suffisant d'individus dont l'âge, le sexe et d'autres variables d'intérêt sont connus, comme c'est le cas pour les manchots royaux (*Aptenodytes patagonicus*) et les flamants (*Phoenicopterus roseus* et *P. ruber*). Ces études

permettront de mieux comprendre comment la glycation varie au sein des individus d'une population, de la même manière que l'étude sur les martinets alpins, qui est nécessaire pour déterminer si et comment ce trait peut évoluer (voir **section 2.2.**).

## **2.2. Données corrélatives dans la nature**

Nos résultats actuels sur la glycémie et la glycation de l'albumine chez les martinets indiquent qu'ils peuvent prédire la survie et au moins un paramètre du succès de la reproduction (le succès de l'envol dans le cas du glucose), c'est-à-dire qu'ils sont en corrélation avec le fitness. Par conséquent, l'étape suivante logique pour déterminer s'ils peuvent évoluer par sélection naturelle, et donc contribuer à l'adaptation physiologique des espèces à leur mode de vie, serait de déterminer l'héritabilité des traits. Cela est possible si l'on dispose de bonnes informations sur les pedigrees de la population étudiée, si l'on peut prélever des échantillons de nombreux individus et si l'on peut contrôler, voire manipuler, leurs conditions de vie précoces, de sorte que l'on puisse réaliser des expériences, y compris des croisements de familles, afin de distinguer les effets environnementaux, des parents et du nid des effets génétiques. La principale mise en garde actuelle est que le nombre d'échantillons nécessaires pour réaliser des modèles animaux (animal models) est assez important (voir Wilson et al. 2010) et qu'il existe des limites au nombre d'échantillons qui peuvent être traités à la fois avec la technique de spectrométrie de masse, ce qui a limité la taille de l'échantillon dans notre étude actuelle. Cependant, si cela pose un problème pour la glycation, le glucose, bien que plus variable, est beaucoup plus facile à mesurer, ce qui permettrait de réaliser ce type d'analyses.

Par ailleurs, pour l'étude de la variation de la glycation liée à l'âge, il serait préférable de procéder à des études longitudinales, ce qui permettrait de séparer les effets de l'âge de ceux de la sélection. Néanmoins, des analyses préliminaires effectuées par notre équipe sur des échantillons anciens ont montré qu'il n'est peut-être pas possible d'obtenir des niveaux de glycation fiables sur des échantillons qui n'ont pas été conservés de manière très stricte (-80 °C au lieu de -20 °C, comme ils le sont actuellement). Cela exclurait la possibilité d'utiliser des échantillons des 20 dernières années d'une étude à long terme et rendrait la recherche sur la variation de la

glycation en fonction de l'âge beaucoup plus difficile, du moins en ce qui concerne cette population.

Il reste également à vérifier si les résultats obtenus peuvent être répétés sur plusieurs années, étant donné que la dynamique du glucose, de la glycation et de la masse corporelle, et la manière dont elle reflète la condition corporelle/la qualité individuelle, peuvent varier d'une année à l'autre, en fonction de la variation annuelle de la disponibilité des ressources. L'inclusion d'une résolution temporelle plus détaillée pourrait aussi aider à mieux comprendre les dynamiques explorées. Finalement, la manipulation expérimentale des coûts de reproduction, par exemple par la manipulation de la taille de la couvée ou de la nichée, ou par la diminution de l'efficacité de la recherche et obtention de nourriture (foraging), par des procédures augmentant artificiellement les demandes énergétiques des oiseaux, en ajoutant des poids supplémentaires ou en modifiant les plumes de vol, pourrait aider à déterminer comment les conditions écologiques affectent les niveaux de glycation, par exemple en induisant une glycémie chroniquement élevée, comme cela a été fait chez les diamants mandarins auparavant (Briga 2016), bien que sans connaître les effets consécutifs sur la glycation. Des expériences modifiant directement les niveaux de glycation et/ou d'AGE pourraient également être réalisées, afin d'étudier leurs effets sur la reproduction et la survie, bien que cela soit plus compliqué à réaliser de manière prévisible, comme le montrent les résultats de l'expérience sur les diamants mandarins. D'autres variables liées à la condition corporelle (par exemple les scores de graisse) ou à l'état physiologique, telles que les triglycérides plasmatiques ou les niveaux de corticostérone, pourraient aider à mieux comprendre dans quelle mesure la glycation constitue une mesure de la condition corporelle chez cette espèce, et si ses effets sur le fitness sont directs ou si la corrélation détectée est médiée par d'autres causes communes sous-jacentes.

## **2.3. Travaux expérimentaux sur le vieillissement en captivité**

### **2.3.1. Recherche en cours : traits intégratifs et omiques**

La première chose à considérer dans cette section est que les résultats présentés dans cette thèse ne sont qu'une partie de ce qui a été mesuré au cours de cette



expérience. La première étape consistera donc à interpréter les résultats de la coloration et à analyser les données relatives aux télomères et à l'hématologie. La capacité de vol, en tant que paramètre fonctionnel du vieillissement, doit également être déterminée, car toutes les vidéos n'ont pas encore été traitées. Par ailleurs, des procédures statistiques plus complexes, telles que les pathway analyses, peuvent être appliquées pour mieux comprendre la chaîne d'influence des caractéristiques actuellement évaluées, et ainsi mieux évaluer les mécanismes qui sont plus pertinents pour les résultats observés, principalement le taux de mortalité plus élevé dans le groupe glucose mais pas dans le groupe méthylglyoxal. Une autre des principales étapes suivantes consistera à effectuer des analyses protéomiques et métabolomiques sur les tissus prélevés sur le sous-ensemble d'individus sacrifiés à la fin de l'expérience, afin d'explorer les mécanismes utilisés par les oiseaux pour faire face au stress glycémique, notamment la façon dont ils résistent aux dommages liés au glucose et à la glycation. Ceci sera fait dans le contexte du quatrième axe de la bourse ANR qui finance ce projet (AGEs - ANR21-CE02-0009). Dans cet axe, certaines des cibles candidates sont la galectine 3, un possible RAGE (Receptor of Advanced Glycation End-products), la FN3K (Fructosamine 3 Kinase, une enzyme de déglycation), la glyoxalase I et la glyoxalase II (qui détoxifient le glyoxal en le convertissant en glycolate et le méthylglyoxal en le transformant en lactate). D'autres mesures sont envisagées dans cette partie du projet, comme l'évaluation des sites exacts de glycation des protéines plasmatiques explorées chez le diamant mandarin, principalement l'albumine, et la tentative de relier ça à des altérations potentielles de la fonction des protéines (voir par ex. Henning et al. 2022).

### **2.3.2. Recherche prévue : hormones**

D'autres mesures initialement prévues mais pas encore abordées consistent à déterminer les effets des traitements sur les niveaux hormonaux, c'est-à-dire les glucocorticoïdes (dans les plumes collectées, grâce à des collaborations déjà établies) et l'insuline/glucagon dans le plasma à différents moments des traitements. Ces hormones clés de la glucorégulation n'ont jamais été mesurées chez le diamant mandarin, et la détermination de leur statut et de leur influence sur les niveaux de glucose, surtout en comparaison avec une littérature qui est actuellement biaisée en

faveur des Galloanserae (poulets, cailles, canards...) aiderait à élargir nos connaissances sur la régulation du glucose chez les oiseaux (voir Braun et Sweazea 2008 ; Sweazea 2022).

### **2.3.3. Cibles futures : microbiote, mécanismes de défense spécifiques et patterns de sénescence**

D'autres éléments qui n'ont pas été pris en compte ici et qui pourraient également être inclus dans de futures études sont les effets de la supplémentation en glucose sur le microbiote, étant donné qu'il s'agit de l'un des mécanismes susceptibles d'affecter la survie. En fait, il est bien connu que le microbiote intestinal affecte et est affecté par le régime de glucose chez les humains, et il est reconnu comme un facteur clé dans la prévalence du diabète et de l'obésité dans notre société moderne (par exemple Greiner et Bäckhed 2011 ; Nuli et al. 2019). Par ailleurs, des études similaires mais plus longues, ou dans des conditions plus contrôlées, pourraient être réalisées, en essayant de voir quelles sont les conséquences sur le long terme, ou dans des conditions environnementales différentes, en évitant peut-être les effets saisonniers observés ici. Cibler les variables qui se sont avérées pertinentes dans cette expérience, par exemple en se concentrant sur le glyoxal plutôt que sur le méthylglyoxal en tant qu'agent de formation d'AGE, ou en mesurant le changement d'expression de gènes particuliers, par RT-qPCR ou Western blots pour les protéines, qui pourraient être trouvés d'être modifiées à travers de nos analyses omiques actuellement en cours, pourrait contribuer à faciliter le processus en termes de charge de travail et de budget. En outre, l'exposition délibérée des oiseaux à différentes conditions peut aider à comprendre comment les effets de la glycation et des AGE sur la survie et les schémas de vieillissement peuvent dépendre de facteurs externes. L'utilisation d'oiseaux plus jeunes et pendant des périodes plus longues permettrait également de mieux explorer les patterns de sénescence en relation avec la glycation et l'accumulation d'AGE (c'est-à-dire le renchérissement et le taux de vieillissement pour ces traits).

### **2.3.4. Effets sur les paramètres de vieillissement fonctionnel**

Les effets de la glycation et des AGE sur le vieillissement fonctionnel des passereaux (c'est-à-dire les mesures liées aux performances) pourraient également être étudiés à

l'aide de ce modèle, ce qui permettrait de déterminer quels mécanismes constituent des liens pertinents entre la formation/accumulation de ces composés et la survie de ces animaux. Cela pourrait aider à déterminer s'il existe des aspects de leur physiologie qui sont plus résistants aux effets délétères communs du glucose, de la glycation et des AGE dans les modèles de mammifères (c'est-à-dire les dysfonctionnements vasculaires, rénaux, neurologiques et visuels, par exemple). Par exemple, de la même manière que le test de vol, l'état neurologique, avec des tâches telles que des tests de mémoire, pourrait être évalué sans trop de difficultés. L'étude de la manière dont le glucose (principal carburant énergétique du cerveau) ou la glycation affectent la cognition est d'un intérêt primordial dans le contexte récemment développé de l'étude de la cognition en tant que modulateur du fitness individuelle (voir par exemple Ashton et al. 2018 ; Fichtel et al. 2023). Quelque chose d'également intéressant dans cette ligne serait de tester comment une augmentation de la glycation et des AGE comme ceux observés ici pourrait affecter la tolérance au glucose, et ses changements avec l'âge chez les oiseaux. Un protocole de test de tolérance au glucose (GTT) intrapéritonéal a déjà été développé pour les diamants mandarins (Montoya et al. 2020), une tolérance au glucose plus élevée étant liée à une survie accrue chez les individus âgés (Montoya et al. 2022). Une autre mesure intégrative similaire qui pourrait être utile est la flexibilité métabolique, comprise comme la capacité à modifier l'utilisation du substrat lorsque l'organisme est soumis à un stimulus, à un défi environnemental ou à toute condition qui l'impliquerait normalement, par exemple le changement se produisant de l'état de jeun à l'état nourri, du repos à l'exercice ou lorsqu'un stress (c'est-à-dire une condition menaçant l'homéostasie) est posé à l'organisme (voir Saltin et Goldnick 1983; Kelley et Mandarino 2000; Galgani et al. 2008). Ce phénomène est généralement mesuré en calculant la variation du QR (Quotient Respiratoire) ou de son équivalent, le RER (Rapport d'Échange Respiratoire), au cours d'un traitement donné. Le plus souvent, l'alimentation est utilisée pour évaluer l'augmentation du QR, les patients diabétiques et obèses présentant normalement une flexibilité métabolique plus faible, c'est-à-dire un changement de QR plus faible, associé également à un QR à jeun plus élevé (voir Galgani et Fernández-Verdejo 2020), et constituant un marqueur de vieillissement (Curl et al. 2024). Ceci est facile à mesurer dans notre laboratoire, et le

changement de la flexibilité métabolique des oiseaux après une supplémentation alimentaire peut être mesuré en contrôlant la masse corporelle, les scores de graisse, et même les niveaux de glucose et de glycation dans le sang, afin de déterminer comment ces traits peuvent influencer la santé métabolique chez les oiseaux. En effet, une expérience similaire est actuellement en cours avec des colibris, connus pour leur grande flexibilité métabolique, dans le cadre d'une de mes collaborations avec l'Université de Toronto (Pr. Kenneth Welch), afin d'explorer si une supplémentation alimentaire comme celle de cette thèse peut affecter cette capacité cruciale chez les oiseaux nectarivores.

### **2.3.5. Vieillesse métabolique**

Par ailleurs, les informations recueillies dans le cadre de cette expérience sur les variations saisonnières du métabolisme et du glucose peuvent être plus approfondies. Par exemple, il semble y avoir une fluctuation métabolique caractérisée par une augmentation de la RMR pendant l'hiver et le printemps (février et mai), suivie d'une diminution en été (août). Il est intéressant de noter que ces fluctuations sont moins évidentes chez les oiseaux plus âgés, ce qui indique qu'ils pourraient faire preuve d'une plus faible flexibilité métabolique, entendue ici comme une plasticité phénotypique du taux métabolique (McKechnie 2008) et donc potentiellement une plus faible capacité à faire face aux fluctuations environnementales. Cette hypothèse, néanmoins, reste à être formellement abordée avec des tests statistiques, y compris les effets des traitements sur cette hypothèse.

## **3. Résumé**

En résumé, nous pouvons conclure que ce travail nous donne un aperçu supplémentaire du domaine encore peu exploré des effets du glucose et de ses dommages consécutifs (glycation, AGE) sur le fitness et le vieillissement chez les oiseaux, et de sa coévolution avec les traits d'histoire de vie et les pressions écologiques telles que la diète. Nous présentons tout d'abord un vaste rapport sur la manière dont la glycation de l'albumine plasmatique varie dans divers taxons aviaires et en fonction du glucose, confirmant une plus grande résistance à la glycation chez les espèces ayant des niveaux de glucose plus élevés, et un taux de glycation plus élevé

chez les oiseaux carnivores terrestres. Dans le même temps, nous fournissons des critères et des exemples pour la sélection future d'espèces modèles pour l'étude de ce phénomène. Dans cette optique, nous suggérons également des moyens d'utiliser l'approche comparative pour aider à démêler la cause évolutive des niveaux de glucose comparativement élevés chez les vertébrés. Ensuite, nous déterminons les effets contrastés du glucose et de la glycation sur le fitness, en utilisant une population dont les individus ont un âge et un pedigree connus, ce qui pourrait être utile pour de futures déterminations de l'héritabilité et donc de l'aptitude de ces traits à évoluer par sélection naturelle et donc à contribuer à l'ensemble des adaptations supposées faire partie du POLS, tel qu'exploré dans l'étude comparative. Les conséquences différentielles et plus ou moins directes ou indirectes du glucose sur le fitness, y compris les effets apparemment dépendants de l'âge de certains d'entre eux, comme la glycation, en font un caractère intéressant à explorer en tant que facteur constituant une pléiotropie antagoniste modulant les phénotypes de vieillissement dans la nature. De plus, notre approche expérimentale a montré que la supplémentation alimentaire en glucose et en dicarbone a un effet sur la glycation des oiseaux et la formation de glyoxal, qui à son tour affecte la formation de certains AGE, comme la CEL (Carboxy-Ethyl-Lysine), qui affecte la survie. De plus, la supplémentation peut augmenter l'apoptose des érythrocytes et les dommages à l'ADN, le stress pro-inflammatoire semblant plus affecté par le méthylglyoxal, alors que le glucose affecte clairement la mortalité. Les raisons exactes de ces derniers effets n'ont pas encore été élucidées, et de nombreux paramètres de vieillissement fonctionnel et proxies de déclin physiologique sont actuellement étudiés ou suggérés pour améliorer nos connaissances sur les effets du glucose, de la glycation et des AGE sur le vieillissement des oiseaux. Finalement, des explorations omiques sont prévues pour nous aider à dévoiler les mécanismes qui sous-tendent les réponses des oiseaux au stress lié au glucose, ce qui pourrait également nous donner des informations utiles sur les moyens qui peuvent être prédominants pour eux de faire face à des niveaux physiologiques de sucre dans le sang qui, bien qu'ils soient normaux pour eux, seraient mortels pour des mammifères de taille similaire. Cette étude est particulièrement intéressante compte tenu de la longévité élevée des oiseaux, ajustée à la masse, par rapport aux mammifères, et contribue à ouvrir une voie

importante vers l'objectif poursuivi d'une meilleure compréhension générale de l'évolution du vieillissement.



# Appendages





# Appendix 1

## Birds from the zebra finches' experiment

**Table 1.** Zebra finches forming part of the experiment, with their sex (assigned by visual inspection of the plumage), allocated treatment, age in years, body mass in grams and morphometric variables (in cm).

Bird_ID	Sex	Treatment	Tarsus	Head_beak	Wing	Age_years	BM
19	female	Methylglyoxal	14,5	24,3	57	5,884931507	14,60
35	female	Methylglyoxal	14,2	22,9	58	4,18630137	13,90
049	female	Glucose	15,3	26,1			18,20
60	male	Control	14,6	23,7	57	6,178082192	13,40
086	male	Methylglyoxal	14,4	24	59	4,016438356	16,30
102	female	Control	16,1	26,3			20,35
119	female	Control	17	26	62	4,726027397	21,90
127	female	Control	14,6	24,3	60	3,04109589	15,70
132	male	Methylglyoxal	15	25,7		3,005479452	18,45
139	male	Control	14,65	23,95	60	2,969863014	15,30
147	male	Glucose	14,5	23,9	60	2,969863014	20,60
154	male	Glucose	14,9	24,1	58	2,969863014	15,70
156	male	Glucose	15,7	25,5	60	2,969863014	18,10
165	male	Glucose	14,8	23,3	58	2,649315068	12,20
177	female	Glucose	16,2	26,1	61		19,50
618	male	Control	14	24,9	56	4,123287671	13,25
840	male	Control	14,5	23,1	53	3,761643836	11,35
1008	male	Methylglyoxal	13,9	23,5	56	2,183561644	11,95
1052	male	Methylglyoxal	14,5	23,3	55	2,156164384	13,20
010 K18	male	Methylglyoxal	14,4	24,8	61	4,306849315	14,85
013 K18	male	Glucose	14,1	24,6	59	4,304109589	17,00
019 K18	female	Methylglyoxal	14,3	23,8	57	4,304109589	13,35
020 K18	female	Glucose	13,9	23	59	4,306849315	16,35
022 K18	female	Methylglyoxal	14,8	23,1	62	4,304109589	13,05
029 K18	female	Control	14,3	23,4	56,5	4,304109589	14,80
035 K18	female	Glucose	14,4	23,8	57,5	4,304109589	12,95
044 K18	female	Glucose	13,7	23,4	56	4,298630137	14,05
045 K18	female	Methylglyoxal	14,6	24,2	58	4,298630137	13,60
047 K18	male	Control	13,4	23,5	62	4,298630137	15,65
049 K18	male	Methylglyoxal	14,6	24,5	59	4,295890411	14,25
053 K18	female	Methylglyoxal	14,4	24,5	62	4,295890411	15,00
054 K18	female	Methylglyoxal	13,9	24	62	4,295890411	15,05
059 K18	male	Glucose	13,5	24,2	60	4,293150685	12,65
061 K18	male	Glucose	13,4	23	61	4,290410959	14,40
071 K18	male	Methylglyoxal	13,8	23	59	4,287671233	15,25

073 K18	female	Control	13,9	22,7	59	4,282191781	11,85
076 K18	female	Control	14,6	23,6	61	4,24109589	14,20
079 K18	male	Methylglyoxal	13,9	24,9	59	4,235616438	13,95
088 K18	female	Control	13,7	23	57	4,210958904	14,30
089 K18	female	Methylglyoxal	14,4	23,3	57,5	4,208219178	14,35
099 K18	female	Control	14,5	24,4	57	4,194520548	14,20
10 16D	female	Control	15,3	22,8	59	6,391780822	13,25
104 16D	male	Control	15,5	24,1	61	6,260273973	15,35
104 S18	female	Control	14,7	23,5	58	4,197260274	12,80
106 16D	male	Control	14,4	23,8	57	6,254794521	15,65
107 S18	female	Methylglyoxal	14,7	21,8	58	4,2	13,05
109 S18	male	Methylglyoxal	13,7	23,7	57	4,194520548	12,80
11 16D	male	Methylglyoxal	13,9	24	59	6,391780822	15,10
112 K18	male	Glucose	15,1	23,5	59	4,194520548	14,40
115 S18	female	Control	13,4	23	61	4,191780822	13,45
119 16D	male	Glucose	14	23,9	60	6,22739726	13,20
119 K19	female	Methylglyoxal	14,7	23,3	59	3,309589041	13,85
119 S18	female	Glucose	15,9	23	59	4,189041096	14,10
12 16D	male	Control	13,9	23,7	60	6,391780822	15,25
121 K18	male	Control	14,8	25,9		4,194520548	12,35
127 K18	female	Glucose	14,2	23,8	58	4,191780822	13,25
129 K18	female	Glucose	14,7	24,2	61	4,191780822	15,40
132 S18	female	Methylglyoxal	14,5	22,9	60	4,156164384	13,30
135 K18	male	Methylglyoxal	14,5	23	60	4,183561644	13,60
135 K19	female	Glucose	14,6	23,1	55	3,306849315	14,90
137 K18	female	Methylglyoxal	13,4	22,8	55	4,183561644	13,70
145 K18	male	Methylglyoxal	14,7	23,3		4,145205479	13,05
145 S18	female	Control	14,4	23,5	57	4,139726027	13,75
148 S18	male	Glucose	15,4	22,7	58	4,131506849	10,60
151 K18	male	Control	14,6	24,1	57	4,131506849	14,35
153 K18	female	Glucose	15,7	23,8	58	4,134246575	13,65
158 K18	female	Glucose	13,3	22,3	57	4,101369863	11,45
173 16D	female	Methylglyoxal	13,9	23,5	58	5,991780822	14,40
173 K19	male	Glucose	13,5	23,5	57	3,298630137	13,55
175 16D	male	Control	14,3	24,4	62	5,994520548	14,30
194 16D	male	Control	14,5	23,9	61	5,947945205	13,80
196 16D	male	Control	14,3	23,9	60	5,942465753	14,45
205 16D	male	Glucose	14,1	23,7	58	5,926027397	13,35
220 16D	male	Methylglyoxal	14,9	24,1	58,5	5,882191781	12,50
222 16D	male	Control	14,8	24,2	61	5,882191781	12,70
230 16D	female	Glucose	14,5	23,6	60	5,873972603	12,95
234 16D	male	Glucose	13,8	24,1	60	5,849315068	14,53
235 16D	female	Control	14,5	23,4	58	5,846575342	14,20

242 16D	female	Glucose	14,3	23,9	60	5,81369863	15,40
254 16D	male	Glucose	15,3	24,3	60	5,769863014	17,25
27 16D	female	Glucose	14,7	23,6	59	6,38630137	15,25
29 16D	male	Control	15,1	24,2	58	6,383561644	15,45
295 K19	male	Glucose	14,4	23,8	57	3,180821918	12,65
37 16D	female	Methylglyoxal	14,7	23,9	58	6,378082192	14,80
4 16D	female	Control	14,4	23,8	60	6,4	15,00
70 16D	male	Methylglyoxal	14,8	23,8	58	6,290410959	15,30
71 16D	female	Glucose	14,8	24	59	6,287671233	15,75
78 16D	male	Methylglyoxal	14,5	23,6	59	6,279452055	15,10
89 17S	female	Methylglyoxal	14,3	23,5	62	5,142465753	14,35
S520F/008	female	Control	17	26,5	65		22,30

BM=body mass in grams

Tarsus, Head\_beak and wing correspond to body measurements in cm.

Age\_years= age in years of the birds the day of start of the experiment (see **Material and methods**). The age was not known for 4 individuals.

## **Provenance of the birds**

**Table 2.** Zebra finches forming part of the experiment, with their origin.

Bird_ID	Origin
19	Max-Planck-Institute for Ornithology
35	Max-Planck-Institute for Ornithology
049	Pet shop (2022)
60	Max-Planck-Institute for Ornithology
086	DEPE, originally from pet shop
102	Pet shop (2022)
119	DEPE, originally from pet shop
127	DEPE - Strasbourg
132	DEPE - Strasbourg
139	DEPE, originally from pet shop
147	DEPE, originally from pet shop
154	DEPE, originally from pet shop
156	DEPE, originally from pet shop
165	DEPE - Strasbourg
177	Pet shop (2022)
618	Bielefeld University
840	Bielefeld University
1008	Bielefeld University
1052	Bielefeld University
010 K18	Max-Planck-Institute for Ornithology
013 K18	Max-Planck-Institute for Ornithology
019 K18	Max-Planck-Institute for Ornithology

020 K18	Max-Planck-Institute for Ornithology
022 K18	Max-Planck-Institute for Ornithology
029 K18	Max-Planck-Institute for Ornithology
035 K18	Max-Planck-Institute for Ornithology
044 K18	Max-Planck-Institute for Ornithology
045 K18	Max-Planck-Institute for Ornithology
047 K18	Max-Planck-Institute for Ornithology
049 K18	Max-Planck-Institute for Ornithology
053 K18	Max-Planck-Institute for Ornithology
054 K18	Max-Planck-Institute for Ornithology
059 K18	Max-Planck-Institute for Ornithology
061 K18	Max-Planck-Institute for Ornithology
071 K18	Max-Planck-Institute for Ornithology
073 K18	Max-Planck-Institute for Ornithology
076 K18	Max-Planck-Institute for Ornithology
079 K18	Max-Planck-Institute for Ornithology
088 K18	Max-Planck-Institute for Ornithology
089 K18	Max-Planck-Institute for Ornithology
099 K18	Max-Planck-Institute for Ornithology
10 16D	Max-Planck-Institute for Ornithology
104 16D	Max-Planck-Institute for Ornithology
104 S18	Max-Planck-Institute for Ornithology
106 16D	Max-Planck-Institute for Ornithology
107 S18	Max-Planck-Institute for Ornithology
109 S18	Max-Planck-Institute for Ornithology
11 16D	Max-Planck-Institute for Ornithology
112 K18	Max-Planck-Institute for Ornithology
115 S18	Max-Planck-Institute for Ornithology
119 16D	Max-Planck-Institute for Ornithology
119 K19	Max-Planck-Institute for Ornithology
119 S18	Max-Planck-Institute for Ornithology
12 16D	Max-Planck-Institute for Ornithology
121 K18	Max-Planck-Institute for Ornithology
127 K18	Max-Planck-Institute for Ornithology
129 K18	Max-Planck-Institute for Ornithology
132 S18	Max-Planck-Institute for Ornithology
135 K18	Max-Planck-Institute for Ornithology
135 K19	Max-Planck-Institute for Ornithology
137 K18	Max-Planck-Institute for Ornithology
145 K18	Max-Planck-Institute for Ornithology
145 S18	Max-Planck-Institute for Ornithology
148 S18	Max-Planck-Institute for Ornithology
151 K18	Max-Planck-Institute for Ornithology
153 K18	Max-Planck-Institute for Ornithology
158 K18	Max-Planck-Institute for Ornithology
173 16D	Max-Planck-Institute for Ornithology

173 K19	Max-Planck-Institute for Ornithology
175 16D	Max-Planck-Institute for Ornithology
194 16D	Max-Planck-Institute for Ornithology
196 16D	Max-Planck-Institute for Ornithology
205 16D	Max-Planck-Institute for Ornithology
220 16D	Max-Planck-Institute for Ornithology
222 16D	Max-Planck-Institute for Ornithology
230 16D	Max-Planck-Institute for Ornithology
234 16D	Max-Planck-Institute for Ornithology
235 16D	Max-Planck-Institute for Ornithology
242 16D	Max-Planck-Institute for Ornithology
254 16D	Max-Planck-Institute for Ornithology
27 16D	Max-Planck-Institute for Ornithology
29 16D	Max-Planck-Institute for Ornithology
295 K19	Max-Planck-Institute for Ornithology
37 16D	Max-Planck-Institute for Ornithology
4 16D	Max-Planck-Institute for Ornithology
70 16D	Max-Planck-Institute for Ornithology
71 16D	Max-Planck-Institute for Ornithology
78 16D	Max-Planck-Institute for Ornithology
89 17S	Max-Planck-Institute for Ornithology
S520F/008	Pet shop (2022)



## Appendix 2

### Pilot study results

#### Methylglyoxal

Water drunk by all groups was lower than in the control group (**Table 1; Figure 1**).

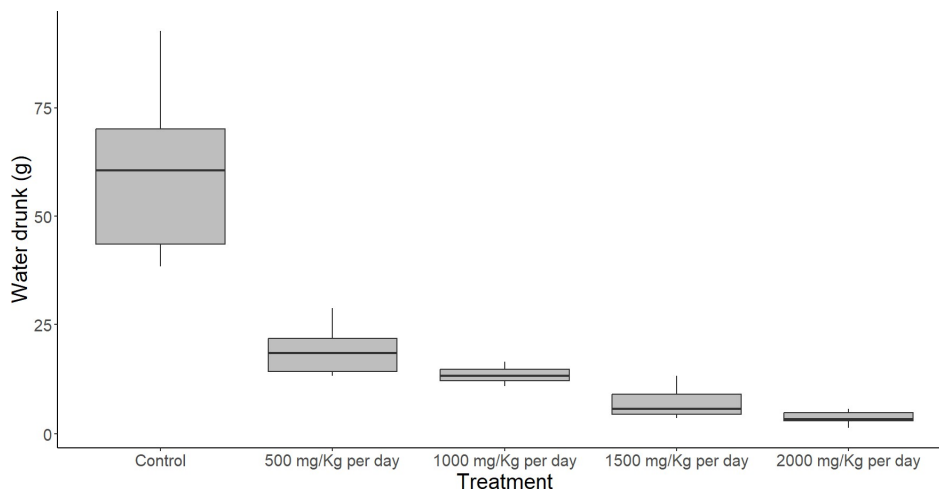
**Table 1.** Drinking rate

	Estimate	Std. Error	t value	P-value
Intercept	64.265	4.199	15.305	$<2*10^{-16}$
Group1	-44.102	5.938	-7.427	$1.29*10^{-9}$
Group2	-49.473	5.938	-8.332	$5.13*10^{-11}$
Group3	-56.344	5.938	-9.489	$9.12*10^{-13}$
Group4	-59.408	5.938	-10.005	$1.58*10^{-13}$

Residual standard error: 13.93 on 50 degrees of freedom

Multiple R<sup>2</sup>: 0.7258, Adjusted R<sup>2</sup>: 0.7038

F-statistic: 33.08 on 4 and 50 DF, p-value:  $1.718*10^{-13}$



**Figure 1.** Average water drunk per day in grams by birds in each group in the methylglyoxal supplementation pilot study.

Plasma methylglyoxal levels ( $\log_{10}$  transformed derived compound) were significantly higher than in the control group in the medium dosages (3 and 4) during the second week of treatment (**Table 2; Figure 2**):



**Table 2.** Methylglyoxal levels.

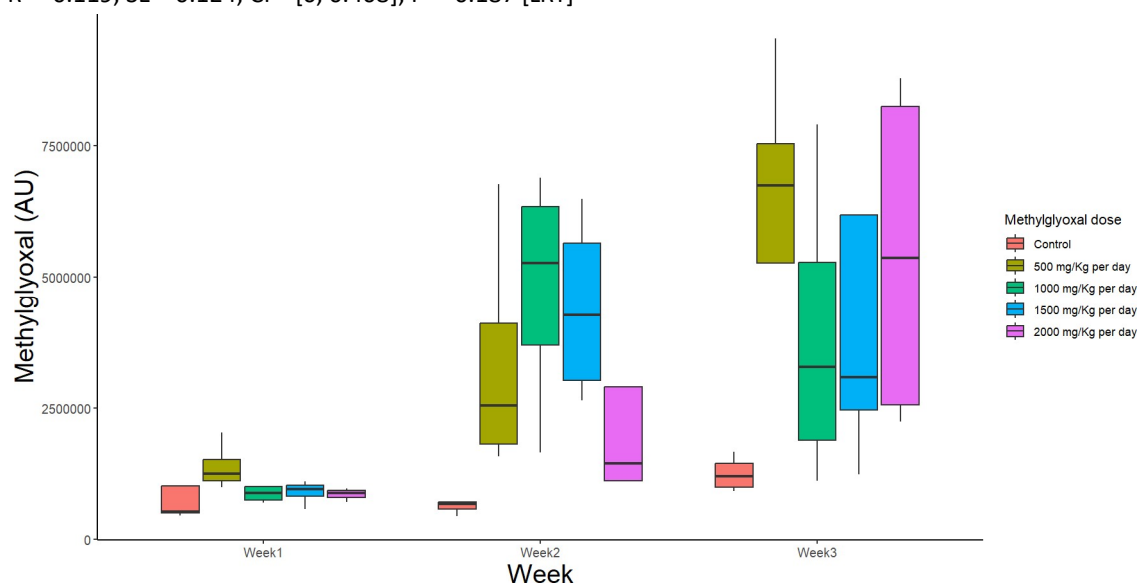
	Estimate	Std. Error	df	t value	P-value
<b>Intercept</b>	<b>5.87383</b>	<b>0.13576</b>	<b>43.77009</b>	<b>43.266</b>	<b>&lt; 2*10<sup>-16</sup></b>
Cage1	0.24894	0.19199	43.77009	1.297	0.20156
Cage2	0.05991	0.19199	43.77009	0.312	0.75650
Cage3	0.06625	0.19199	43.77009	0.345	0.73169
Cage4	0.05758	0.19199	43.77009	0.300	0.76568
WeekWeek2	-0.08429	0.18026	30.00000	-0.468	0.64345
WeekWeek3	0.20962	0.18026	30.00000	1.163	0.25403
Cage1:WeekWeek2	0.41400	0.25492	30.00000	1.624	0.11483
<b>Cage2:WeekWeek2</b>	<b>0.77199</b>	<b>0.25492</b>	<b>30.00000</b>	<b>3.028</b>	<b>0.00502</b>
<b>Cage3:WeekWeek2</b>	<b>0.75957</b>	<b>0.25492</b>	<b>30.00000</b>	<b>2.980</b>	<b>0.00567</b>
<i>Cage4:WeekWeek2</i>	<i>0.43872</i>	<i>0.25492</i>	<i>30.00000</i>	<i>1.721</i>	<i>0.09555</i>
Cage1:WeekWeek3	0.34584	0.25492	30.00000	1.357	0.18501
Cage2:WeekWeek3	0.33609	0.25492	30.00000	1.318	0.19734
Cage3:WeekWeek3	0.41027	0.25492	30.00000	1.609	0.11801
<i>Cage4:WeekWeek3</i>	<i>0.51521</i>	<i>0.25492</i>	<i>30.00000</i>	<i>2.021</i>	<i>0.05227</i>

Shapiro-Wilk normality test

W = 0.98216, p-value = 0.5266

Repeatability for Bird\_ID

R = 0.119; SE = 0.124; CI = [0, 0.408]; P = 0.187 [LRT]



**Figure 2.** Plasma methylglyoxal levels in arbitrary units (AU) of derived compound (they were not referred to a standard, as the absolute value was not deemed relevant here) from each treatment group and week from the methylglyoxal supplementation pilot study.

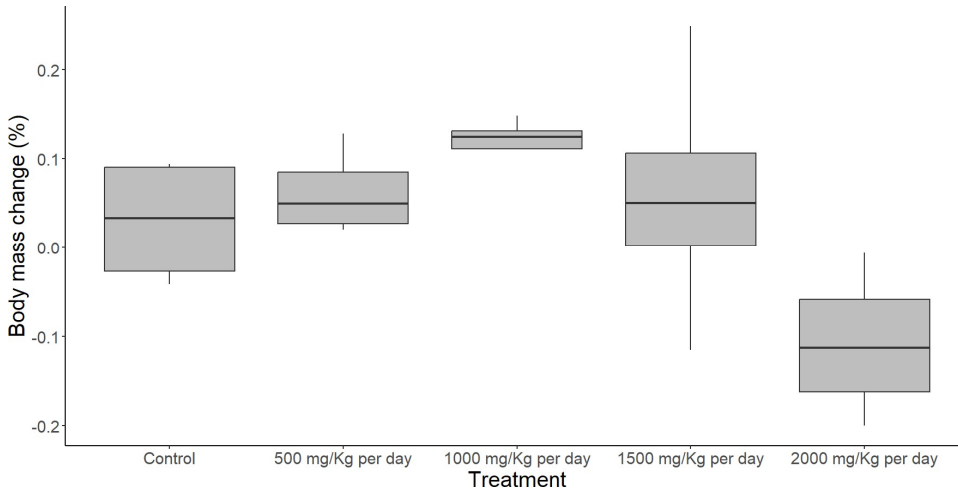
Body mass was lost from week 1 to week 3 in the group with highest methylglyoxal dosage (Table 3; Figure 3):

**Table 3.** Percentage of body mass change.

	Estimate	Std. Error	t value	P-value
Intercept	0.02982	0.04354	0.685	0.5039
Cage1	0.03242	0.06158	0.526	0.6063
Cage2	0.08826	0.06158	1.433	0.1723
Cage3	0.02874	0.06158	0.467	0.6474
<b>Cage4</b>	<b>-0.13783</b>	<b>0.06158</b>	<b>-2.238</b>	<b>0.0408</b>

Residual standard error: 0.08708 on 15 degrees of freedom  
 Multiple R<sup>2</sup>: 0.5018, Adjusted R<sup>2</sup>: 0.3689  
 F-statistic: 3.776 on 4 and 15 DF, p-value: 0.02563

Shapiro-Wilk normality test  
 W = 0.97154, p-value = 0.787



**Figure 3.** Percentage of body mass change from week 1 to week 3 of treatment at different methylglyoxal dosages.

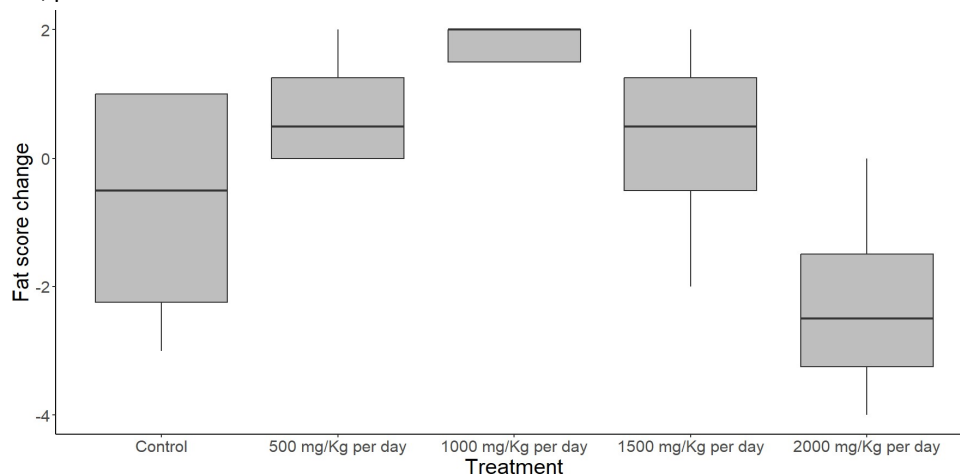
Fat score is marginally increased in the group 2 (1000 mg/Kg per day; **Table 4; Figure 4**):

**Table 4.** Fat score.

	Estimate	Std. Error	t value	P-value
Intercept	-0.7500	0.7746	-0.968	0.3483
Cage1	1.5000	1.0954	1.369	0.1911
Cage2	2.2500	1.0954	2.054	0.0578
Cage3	1.0000	1.0954	0.913	0.3757
Cage4	-1.5000	1.0954	-1.369	0.1911

Residual standard error: 1.549 on 15 degrees of freedom  
 Multiple R<sup>2</sup>: 0.4842, Adjusted R<sup>2</sup>: 0.3467  
 F-statistic: 3.521 on 4 and 15 DF, p-value: 0.03229

Shapiro-Wilk normality test  
 W = 0.95342, p-value = 0.422



**Figure 4.** Change in fat score (see **body condition** section from Material and methods) from week 1 to week 3 of treatment depending on methylglyoxal dosage.

Muscle score is decreased in the group 4 (2000 mg/Kg per day; **Table 5; Figure 5**):

**Table 5.** Muscle score.

	Estimate	Std. Error	t value	P-value
Intercept	0.3750	0.2700	1.389	0.185
Cage1	-0.3750	0.3819	-0.982	0.342
Cage2	0.2500	0.3819	0.655	0.523
Cage3	-0.1250	0.3819	-0.327	0.748
<b>Cage4</b>	<b>-1.1250</b>	<b>0.3819</b>	<b>-2.946</b>	<b>0.010</b>

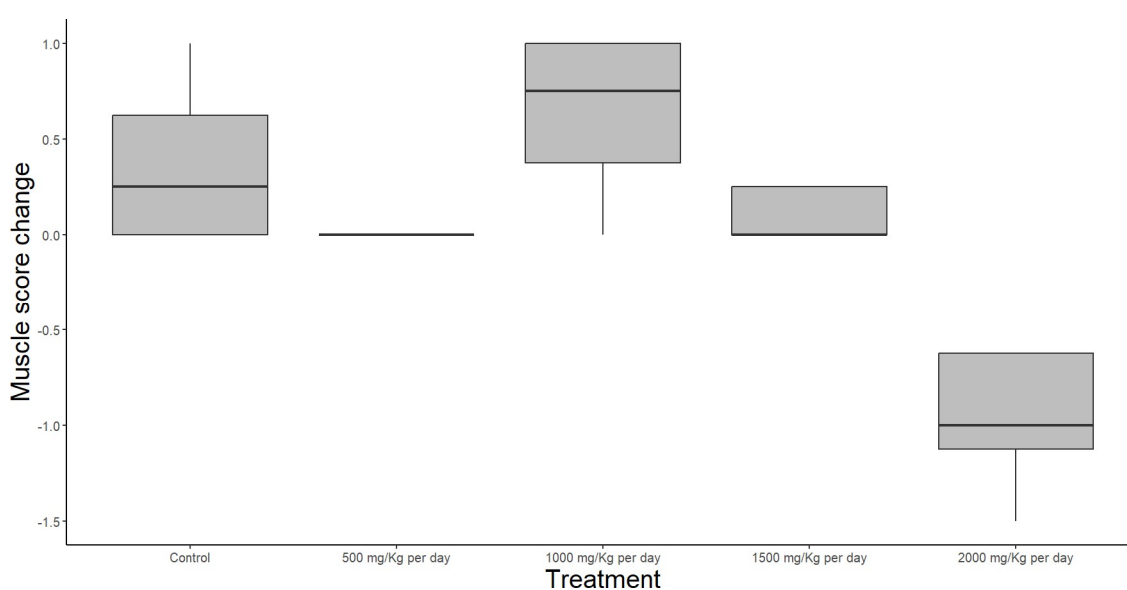
Residual standard error: 0.5401 on 15 degrees of freedom

Multiple R<sup>2</sup>: 0.5028, Adjusted R<sup>2</sup>: 0.3703

F-statistic: 3.793 on 4 and 15 DF, p-value: 0.02526

Shapiro-Wilk normality test

W = 0.92533, p-value = 0.1255



**Figure 5.** Change in Muscle score (see **body condition section** from Material and methods) from week 1 to week 3 of treatment depending on methylglyoxal dosage.

## Glucose

Food consumption is reduced by the higher glucose supplementation levels (**Table 6; Figure 6**):

**Table 6.** Food consumption.

	Estimate	Std. Error	t value	P-value
<b>Intercept</b>	<b>9.1150</b>	<b>0.3830</b>	<b>23.800</b>	<b>&lt; 2*10<sup>-16</sup></b>
Group1	0.1008	0.5416	0.186	0.852707
Group2	-0.9200	0.5416	-1.699	0.092663
<b>Group3</b>	<b>-2.1267</b>	<b>0.5416</b>	<b>-3.927</b>	<b>0.000163</b>
<b>Group4</b>	<b>-2.3833</b>	<b>0.5416</b>	<b>-4.400</b>	<b>2.82e-05</b>

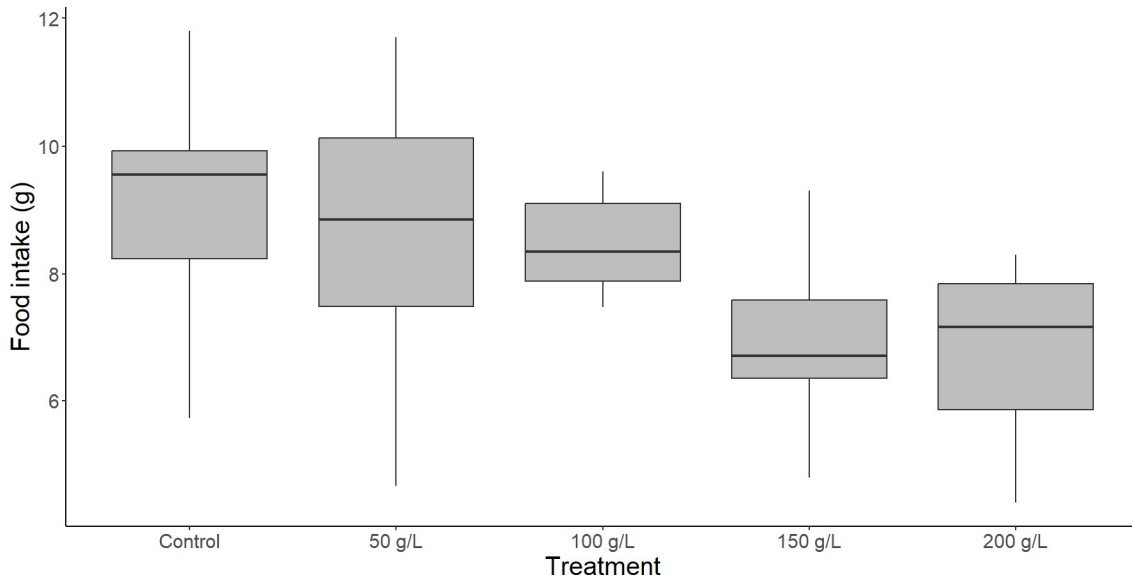
Residual standard error: 1.713 on 95 degrees of freedom

Multiple R<sup>2</sup>: 0.2785, Adjusted R<sup>2</sup>: 0.2482

F-statistic: 9.169 on 4 and 95 DF, p-value: 2.62\*10<sup>-6</sup>

Shapiro-Wilk normality test

W = 0.96304, p-value = 0.006662



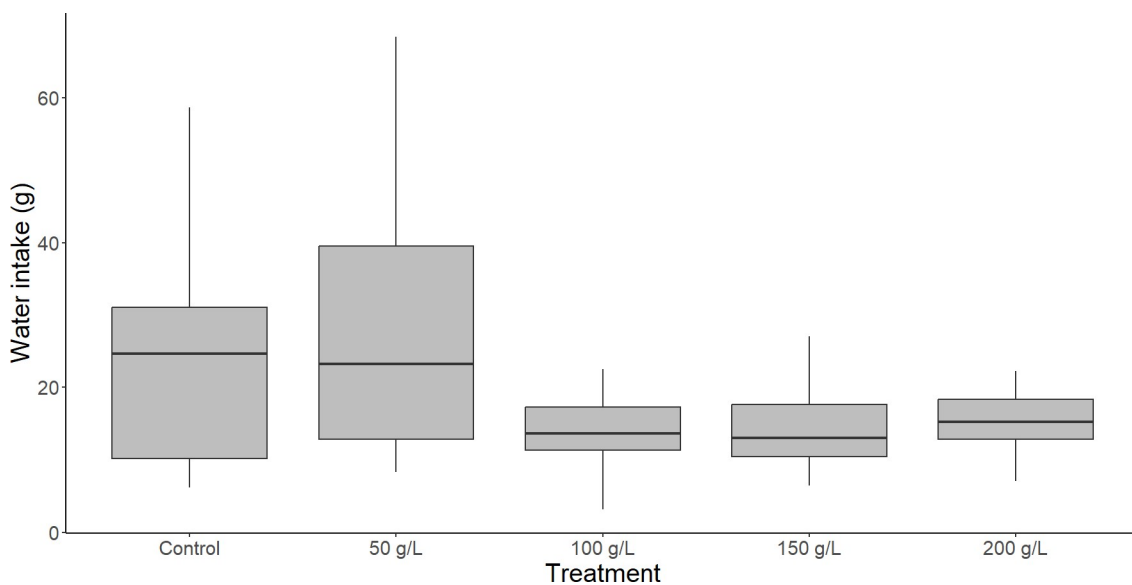
**Figure 6.** Average water drunk per day in grams by birds in each group in the methylglyoxal supplementation pilot study.

Water intake is reduced by the medium (150 g/L and marginally for 200 g/L) glucose supplementation levels (**Table 7; Figure 7**):

**Table 7.** Water intake.

	Estimate	Std. Error	df	t value	P-value
<b>Intercept</b>	<b>1.30348</b>	<b>0.08207</b>	<b>2.35534</b>	<b>15.882</b>	<b>0.00189</b>
Group1	0.03809	0.07671	94.00000	0.497	0.62066
<b>Group2</b>	<b>-0.20198</b>	<b>0.07671</b>	<b>94.00000</b>	<b>-2.633</b>	<b>0.00989</b>
Group3	-0.14037	0.07671	94.00000	-1.830	0.07041
Group4	-0.11682	0.07671	94.00000	-1.523	0.13111

Shapiro-Wilk normality test  
W = 0.9856, p-value = 0.3508



**Figure 7.** Average water drunk per day in grams by birds in each group in the glucose supplementation pilot study.

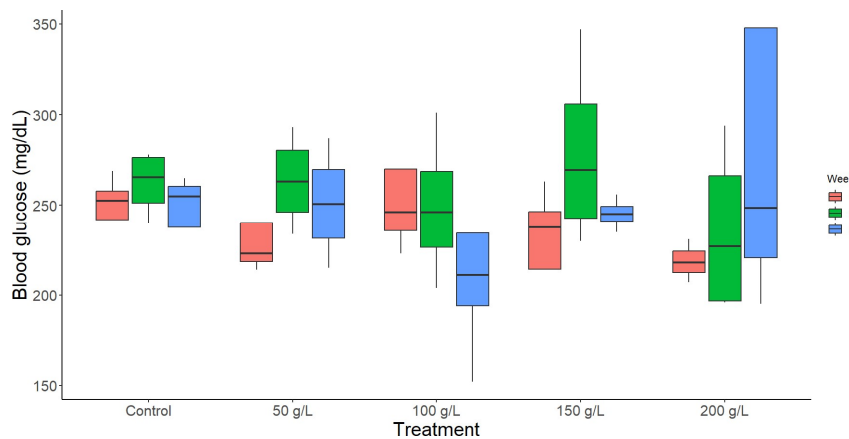
Whole blood glucose levels are not affected by the treatment (**Table 8; Figure 8**), but decrease with fasting time (**Figure 9**):

**Table 8.** Glucose model.

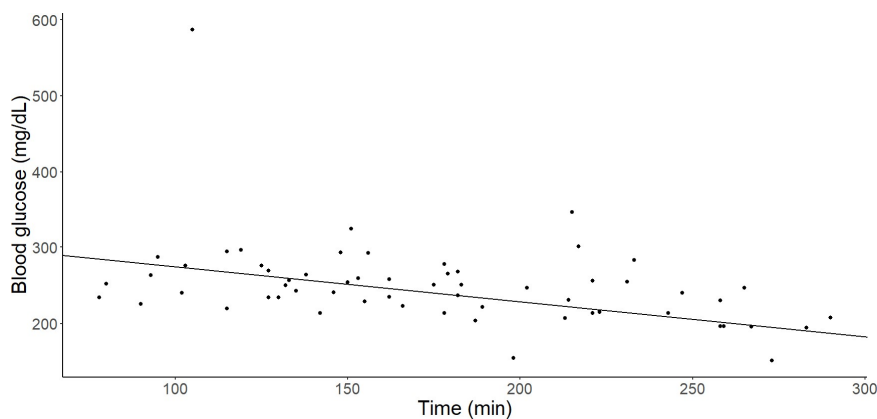
	Estimate	Std. Error	df	t value	P-value
<b>Intercept</b>	<b>2.5029144</b>	<b>0.0472696</b>	<b>42.6563824</b>	<b>52.950</b>	<b>&lt; 2*10<sup>-16</sup></b>
Group1	-0.0316443	0.0516986	34.9273366	-0.612	0.54444
Group2	-0.0053989	0.0520394	35.2316544	-0.104	0.91796
Group3	-0.0637494	0.0517439	34.9681461	-1.232	0.22616
Group4	-0.0172262	0.0524693	35.6052796	-0.328	0.74460
Week2	0.0518045	0.0420105	28.7697940	1.233	0.22751
Week3	0.0139223	0.0419032	28.7121607	0.332	0.74211
<b>Timespan</b>	<b>-0.0007006</b>	<b>0.0001883</b>	<b>39.9856757</b>	<b>-3.721</b>	<b>0.00061</b>
Group1:Week2	-0.0144892	0.0594714	28.7923493	-0.244	0.80924
Group2:Week2	-0.0442650	0.0586444	28.4744745	-0.755	0.45656
Group3:Week2	0.1056494	0.0593011	28.7278154	1.782	0.08539
Group4:Week2	-0.0358507	0.0594481	28.7835467	-0.603	0.55119
Group1:Week3	0.0232218	0.0587368	28.5105549	0.395	0.69553
Group2:Week3	-0.0240205	0.0602983	29.0990068	-0.398	0.69327
Group3:Week3	0.0624239	0.0586591	28.4802360	1.064	0.29619
Group4:Week3	0.0890343	0.0597224	28.8865635	1.491	0.14685

Shapiro-Wilk normality test

W = 0.92262, p-value = 0.0009789

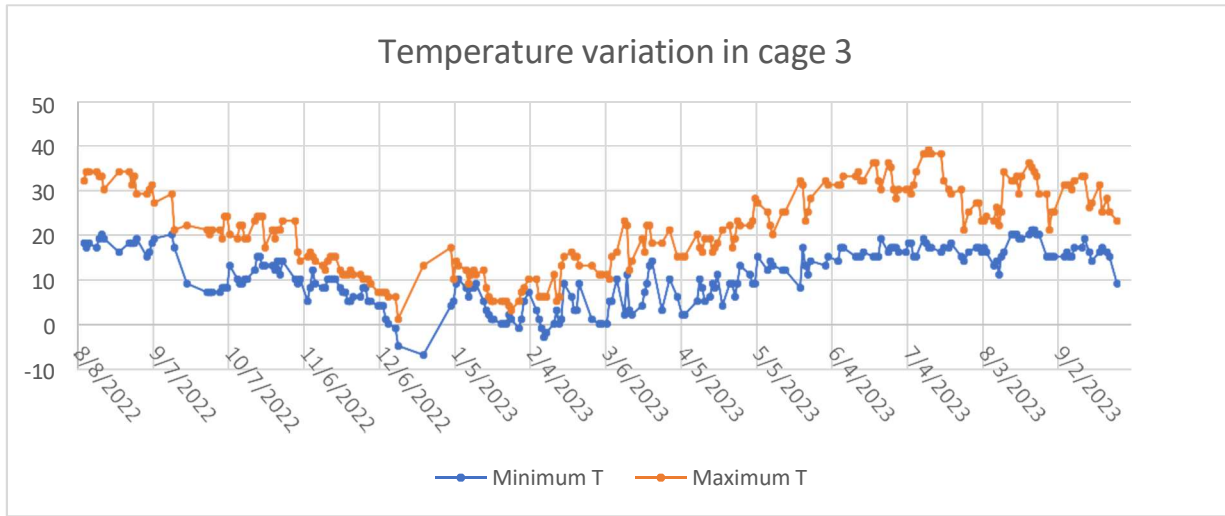


**Figure 8.** Whole blood glucose in mg/dL of birds in each group across the experiment weeks in the glucose supplementation pilot study.

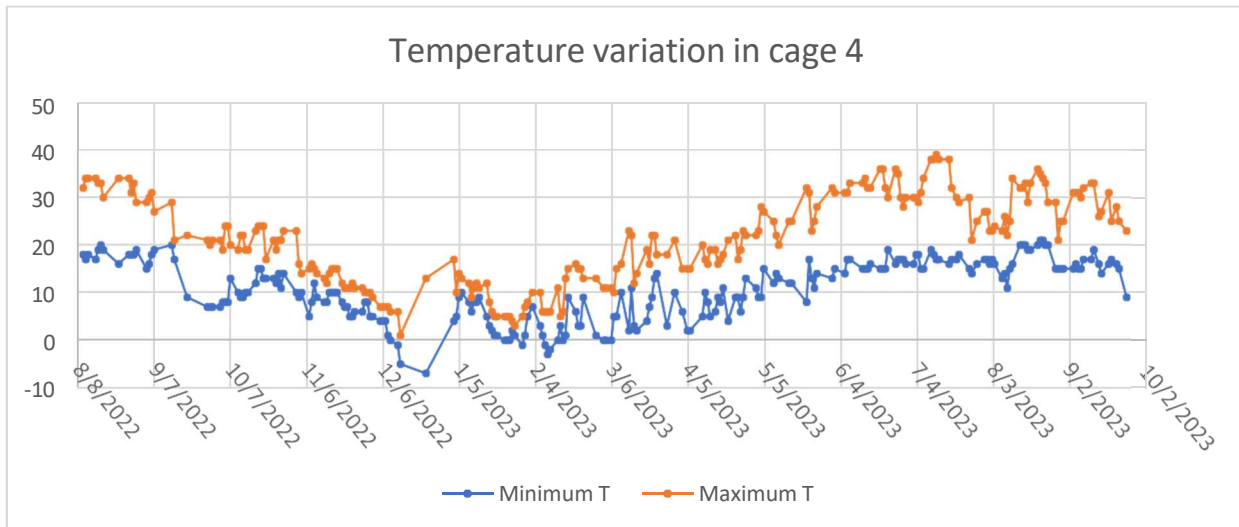


**Figure 9.** Whole blood glucose levels variation in function of the time in which it was measured counting from the start of the fasting period in the glucose supplementation pilot study.

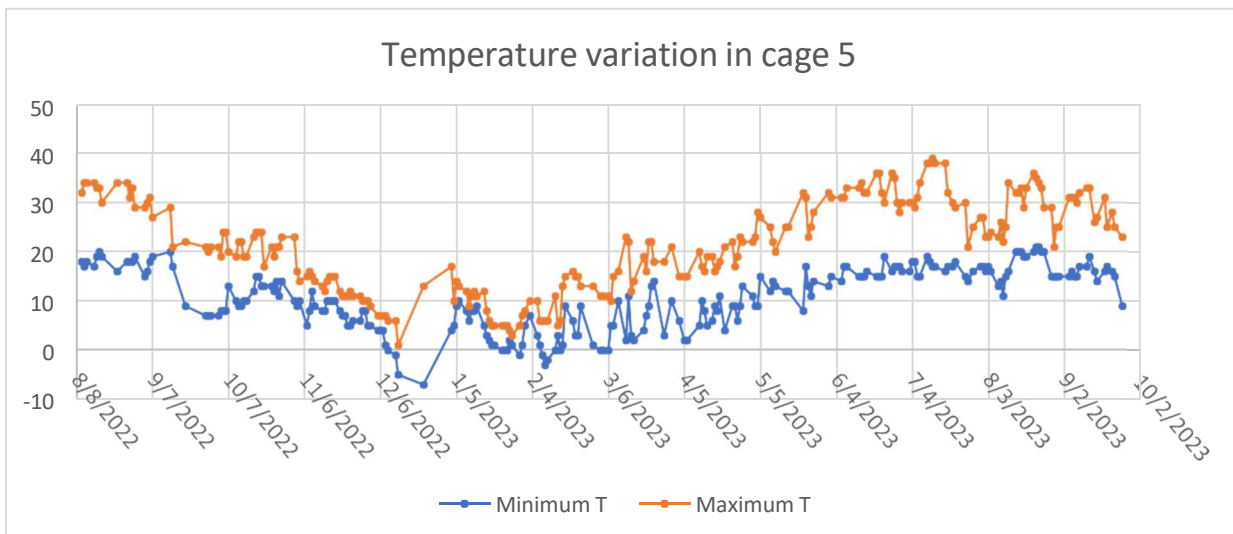
# Appendix 3



**Figure 1.** Minimum and maximum temperature variation along the year of the experiment in cage 3 (methylglyoxal supplementation group).



**Figure 2.** Minimum and maximum temperature variation along the year of the experiment in cage 4 (glucose supplementation group).



**Figure 3.** Minimum and maximum temperature variation along the year of the experiment in cage 5 (control group).







## Glucose and glycation in birds: phylogenetic and ecological factors, physiological mechanisms and individual fitness links

### **Abstract**

Glucose plays a central role in metabolism, but it also affects ageing by enhancing the non-enzymatic glycation of proteins and the formation of Advanced Glycation End-products (AGE) whose accumulation into tissues leads to pro-oxidative and pro-inflammatory effects. This aspect of ageing process, however, has been largely neglected from the eco-evolutionary perspective. However, birds appear to successfully manage chronically comparatively high diabetes-like blood glucose levels, showing yet a higher mass-adjusted lifespan in comparison with mammals. This thesis aims to generate new knowledge on glucose and glycation in birds, through (i) a comparative approach assessing plasma glucose and glycation levels along the avian phylogenetic tree in relation with diet and life-history, (ii) an ecological approximation on its link with fitness in wild populations and (iii) an experimental approach in captivity exploring the adverse effects of glucose, glycation and AGE levels on individual senescence markers. With this research, we showed considerable variation in both glucose and glycation levels across avian taxa, with terrestrial carnivores having higher albumin glycation than omnivores. We also demonstrated that both glucose and glycation affect fitness through survival effects, and parents' glucose seems to influence their reproductive outcomes in a wild bird. Finally, chronic glucose supplementation reduced survival in zebra finches, inducing glycation and AGEs production, with some of those showing an age increase and a negative impact on survival.

Key words: Ageing, glucose, glycation, AGEs, birds, fitness, life-history, evolution

### **Résumé**

Le glucose joue un rôle central dans le métabolisme, mais il affecte également le vieillissement en favorisant la glycation non enzymatique des protéines et la formation de produits finaux de glycation avancée (AGE) dont l'accumulation dans les tissus entraîne des effets pro-oxydants et pro-inflammatoires. Cet aspect du processus de vieillissement a toutefois été largement négligé du point de vue éco-évolutif. Cependant, les oiseaux semblent gérer avec succès des niveaux de glucose sanguin chroniquement et comparativement hauts, comparables à ceux du diabète, tout en présentant une durée de vie ajustée en fonction de la masse plus élevée que celle des mammifères. Cette thèse vise à générer de nouvelles connaissances sur le glucose et la glycation chez les oiseaux, à travers (i) une approche comparative évaluant les niveaux de glucose et de glycation plasmatiques le long de l'arbre phylogénétique des oiseaux en relation avec la diète et les traits d'histoire de vie, (ii) une approximation écologique sur son lien avec la fitness dans les populations sauvages et (iii) une approche expérimentale en captivité explorant les effets adverses du glucose, de la glycation et des niveaux d'AGE sur des marqueurs de la sénescence des individus. Cette recherche a montré une variation considérable des niveaux de glucose et de glycation entre les taxons des oiseaux, les carnivores terrestres ayant une glycation de l'albumine plus élevée que les omnivores. Nous avons également démontré que le glucose et la glycation affectent le fitness par des effets sur la survie, et que le glucose des parents semble influencer leurs résultats en matière de reproduction chez un oiseau sauvage. Finalement, une supplémentation chronique en glucose a réduit la survie des diamants mandarins, induisant la glycation et la production d'AGEs, certains d'entre eux montrant une augmentation avec l'âge et un impact négatif sur la survie.

Mots clés : Vieillissement, glucose, glycation, AGEs, oiseaux, fitness, histoire de vie, évolution