



# Influence of environmental and individual characteristics on baseline stress levels, and consequences in a large mammal, the roe deer *Capreolus capreolus*

Jeffrey Carbillet Malherbe

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# THÈSE

## En vue de l'obtention du DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE

Délivré par l'Université Toulouse 3 - Paul Sabatier

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Présentée et soutenue par  
**Jeffrey CARBILLET MALHERBE**

Le 16 décembre 2019

**Influence des caractéristiques environnementales et individuelles  
sur le niveau de stress basal, et conséquences, chez un grand  
mammifère, le chevreuil *Capreolus capreolus***

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Ecole doctorale : **SEVAB - Sciences Ecologiques, Vétérinaires, Agronomiques et  
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Spécialité : **Ecologie, biodiversité et évolution**

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**CEFS - Laboratoire Comportement et Ecologie de la Faune Sauvage**

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*« It's not who I am underneath, but what I do that defines me »*

*- Batman -*





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## Chapter 1 -

**MS1: The Neutrophil to Lymphocyte ratio indexes individual variation in the behavioural stress response of wild roe deer across fluctuating environmental conditions.**

J. Carbillet, B. Rey, T. Lavabre, Y. Chaval, J. Merlet, F. Débias, C. Régis, S. Pardonnet, J. Duhayer, J.M. Gaillard, A.J.M. Hewison, J.F. Lemaître, M. Pellerin, B. Rannou, H. Verheyden, E. Gilot-Fromont

**MS2: Under cover of the night: context-dependency of anthropogenic disturbance on stress levels of wild roe deer *Capreolus capreolus*.**

J. Carbillet, B. Rey, R. Palme, N. Morellet, N. Bonnot, Y. Chaval, B. Cargnelutti, A.J.M. Hewison, E. Gilot-Fromont, H. Verheyden

## Chapter 2 -

**MS3: Human activities and behaviour influence salivary cortisol concentrations during early life of roe deer fawns**

J. Carbillet, B. Rey, N. Morellet, Y. Chaval, B. Cargnelutti, M. Hollain, C. Loiseau, A.J.M. Hewison, E. Gilot-Fromont, H. Verheyden

**MS4: Maternal stress during early gestation does not influence offspring phenotype in fawns of roe deer**

J. Carbillet, B. Rey, R. Palme, Y. Chaval, B. Cargnelutti, M. Hollain, N. Morellet, A.J.M. Hewison, E. Gilot-Fromont, H. Verheyden

## Chapter 3 -

**MS5: Into the wild of the stress-immunity relationship, a case study on roe deer *Capreolus capreolus***

M. Hollain, J. Carbillet, B. Rey, R. Palme, M. Pellerin, C. Régis, T. Lavabre, S. Pardonnet, J. Duhayer, F. Débias, J.M. Gaillard, H. Verheyden, J.F. Lemaître, E. Gilot-Fromont

**MS6: Co-variation between faecal cortisol metabolites, behaviour and immune parameters over time in captive roe deer**

J. Carbillet, B. Rey, R. Palme, L. Börger, T. Lavabre, M.L. Maublanc, N. Cebe, J.L. Rames, B. Coracin, E. Bideau, B. Lourtet, G. Le Loch, M. Wasniewski, B. Rannou, E. Gilot-Fromont, H. Verheyden

## Appendices -

### **Does body growth impair immune function in a large herbivore?**

L. Cheynel, F. Douhard, E. Gilot-Fromont, B. Rey, F. Débias, S. Pardonnet, J. Carbillet, H. Verheyden, A.J.M. Hewison, M. Pellerin, J.M. Gaillard and J.F. Lemaître.

### **Glucocorticoid level predicts short-term telomere dynamics in wild roe deer**

J.F. Lemaître<sup>1</sup>, J. Carbillet, B. Rey, R. Palme, H. Froy, R.V. Wilbourn, S.L. Underwood, L. Cheynel, J.M. Gaillard, A.J.M. Hewison, H. Verheyden, J.F. Débias, J. Duhayer, C. Régis, S. Pardonnet, M. Pellerin, D.H. Nussey, E. Gilot-Fromont

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# Introduction





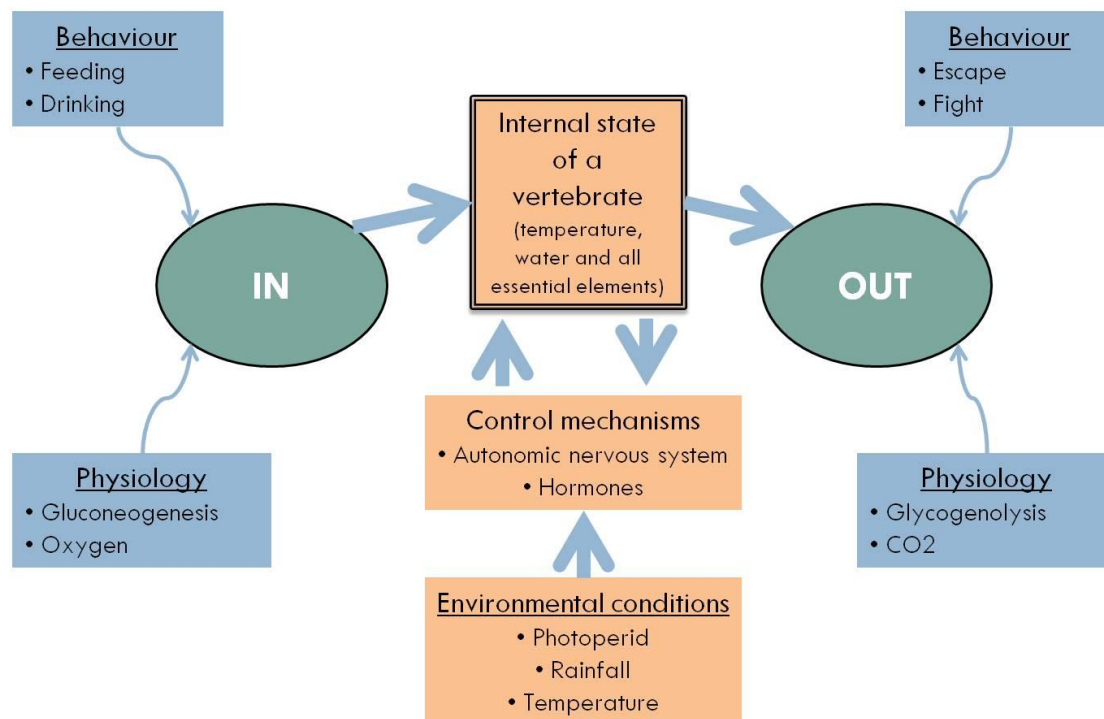
## 1- A dynamic environment

Most people may agree that there is little common ground between a human, a squirrel, a whale, a flamingo and a tortoise. But there is an obvious one, they all live in a dynamic environment. No single day is identical to the previous or the next. What makes their environment so dynamic? For instance, the interactions individuals have with other organisms, fluctuations in resources quantity, meteorological conditions or seasonal rhythms are among common factors making the environment dynamic. Some of these variations are predictable due to environmental cues, allowing individuals to adjust their behaviour and physiology, as is the case for diurnal rhythms and seasonal cycles. But most of the environmental changes are not predictable and individuals may not be able to pre-emptively adjust their behaviour and physiology to these events. These unpredictable events include (but are not restricted to) social interactions, competition for resources or sexual partners, predation, diseases, rapid modifications of habitats, or extreme climatic events. In recent centuries, and even more so in the last decades, unpredictable environmental fluctuations have increased dramatically, mainly because of the intensification of human activities (Sih et al., 2011; Hooke et al., 2012), and have become major selective forces for natural populations (Wingfield and Romero, 2001). Therefore, understanding the potential of wild animals to respond to these perturbations and their consequences on life history traits and population dynamics is receiving increasing attention in ecology and conservation biology. Throughout this manuscript, I will refer to “stressors”, as any changes perceived as a threat to homeostasis (Box 1) and that trigger a stress response in an individual. The “stress response” will be defined as a suite of physiological and behavioural responses whose coordination aim at neutralising the effects of the stressor and restoring homeostasis. If wild animals have adapted

evolutionarily to predictable stressors, they must rely on the stress response to adjust their physiology and behaviour to cope with unpredictable and rapid changes in their environment.

### Box 1 - What is homeostasis? (1/2)

According to Walter Cannon (1929), homeostasis is defined as “the coordinated physiological processes which maintain most of the steady states in the organism”. In other words, homeostasis, from the Greek words for "same" and "steady," refers to any process used to actively maintain stable internal conditions necessary for the survival of an organism. For instance, it refers to the processes that allow the maintenance of body temperature, the control of water balance, electrolytes (salt), carbohydrates (sugar), protein, lipids, and oxygen contents in tissues and biological fluids.

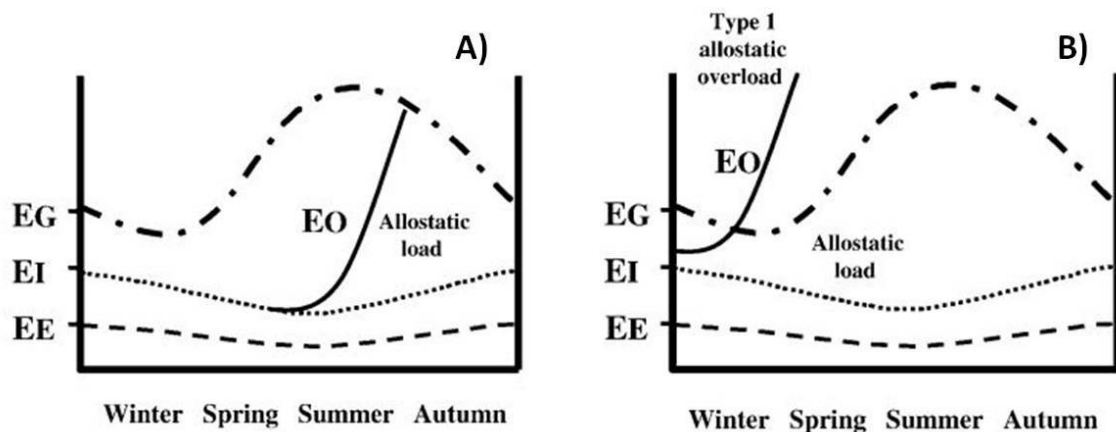


**Figure 1** - Extended representation of the concept of homeostasis, where the maintenance of the internal state is ensured by influxes and outfluxes brought through behavioural and physiological processes, and under the control of hormones, such as glucocorticoid hormones. The secretion of these hormones is also under the control of environmental conditions. Environmental conditions act as signals for the control mechanism to ensure that the levels of essential elements match the need of the organism according to the current external conditions. (Adapted from Bradshaw, 2003).



### Box 1 - What is homeostasis? (2/2)

The concept of homeostasis has been extended by McEwen and Wingfield (2003) to the concept of allostasis, which refers to the maintenance of homeostasis through change. Allostasis explicitly accounts for changes in environmental and life history stage, and therefore for changes in the different levels of essential elements required depending on the environmental context and life history stage of individuals. In other words, homeostatic set points can fluctuate through time. The concept of allostasis includes the state of allostatic load, defined as the addition of the current and anticipated energetic demands an individual is facing (McEwen and Wingfield, 2003), and the state of allostatic overload, defined as a condition where the allostatic load of an individual is exceeding the available resources (McEwen and Wingfield, 2003). Long-term exposure to stressors usually leads to the state of allostatic overload (often referred as chronic stress) that can have deleterious effects on individual's fitness.



**Figure 2** - Representation of the concept of allostasis, with allostatic load (A) and allostatic overload (B) situations. EE represents the energy required for basic homeostasis. EI represents the extra energy required for the individual to forage and assimilate food under favourable conditions and without stressor. EG represents the amount of energy (in food) available in the environment. EO represents the energy required to forage and assimilate nutrients under stressful conditions. In figure A) the energy required to forage and assimilate nutrients under stressful conditions (EO) does not exceed the amount of available energy (EG) and the individual therefore remain in a state of allostatic load. When EO exceeds EG, as it is the case in figure B), the individual reach the state of allostatic overload and remain in this state until the perturbation has stopped or the amount of available energy increased. (Figures from McEwen and Wingfield, 2003).

## 2- Behavioural adjustments

### 2-1 Short-term adjustments

In the face of unpredictable events, behavioural stress response serves to redirect an animal's attention from behaviours that can be resumed later (e.g. foraging) to those necessary to deal with the stressor that threatens immediate survival (e.g. escaping a predator, moving toward a refuge). These behavioural adjustments are part of what John Wingfield and his colleagues termed the "emergency life history stage (ELHS)" (1998), and imply that a current life history stage (e.g. breeding) may be postponed. Besides suppressing some behaviours such as territoriality, the ELHS triggers behavioural responses that have been divided in three components by John Wingfield and his colleagues: 1) Moving away from the perturbation; 2) Seeking refuge; 3) Seeking refuge and moving away. These behavioural responses may be particularly used in a context of prey-predator interactions as immediate or anticipatory responses. Indeed, individual may perceive cues from the presence of predators and adjust their behaviour accordingly. The concept of the landscape of fear has been developed by Laundré and colleagues (2001) on this basis and predicts that habitat use should differ in relation to variations in perceived risk. For instance, a higher level of perceived risk of predation should provoke an elevated level of vigilance from the prey. A famous test of this prediction was the study from John Laundré and his colleagues (2001), showing that after wolf reintroduction in the Yellowstone National Park, elk females exhibited significantly higher levels of vigilance in areas containing wolves than those without. But in the landscape of fear concept, not only vigilance, but also more global behavioural adjustments occur. For instance, wild animals exhibit more pronounced modifications in their spatial behaviour as the level of perceived threat increases. Accordingly, in the same elk-wolves system of the Yellowstone

National Park, Scott Creel and his colleagues (2005) showed that elk tend to move into wooded areas, which served as a refuge when wolves were present, therefore reducing their use of more nutritive grassland habitats but that presented higher risks of predation. These short-term behavioural adjustments might help individuals to mitigate direct exposure to stressors, and consequently, to limit the amount of energy allocated to the stress response.

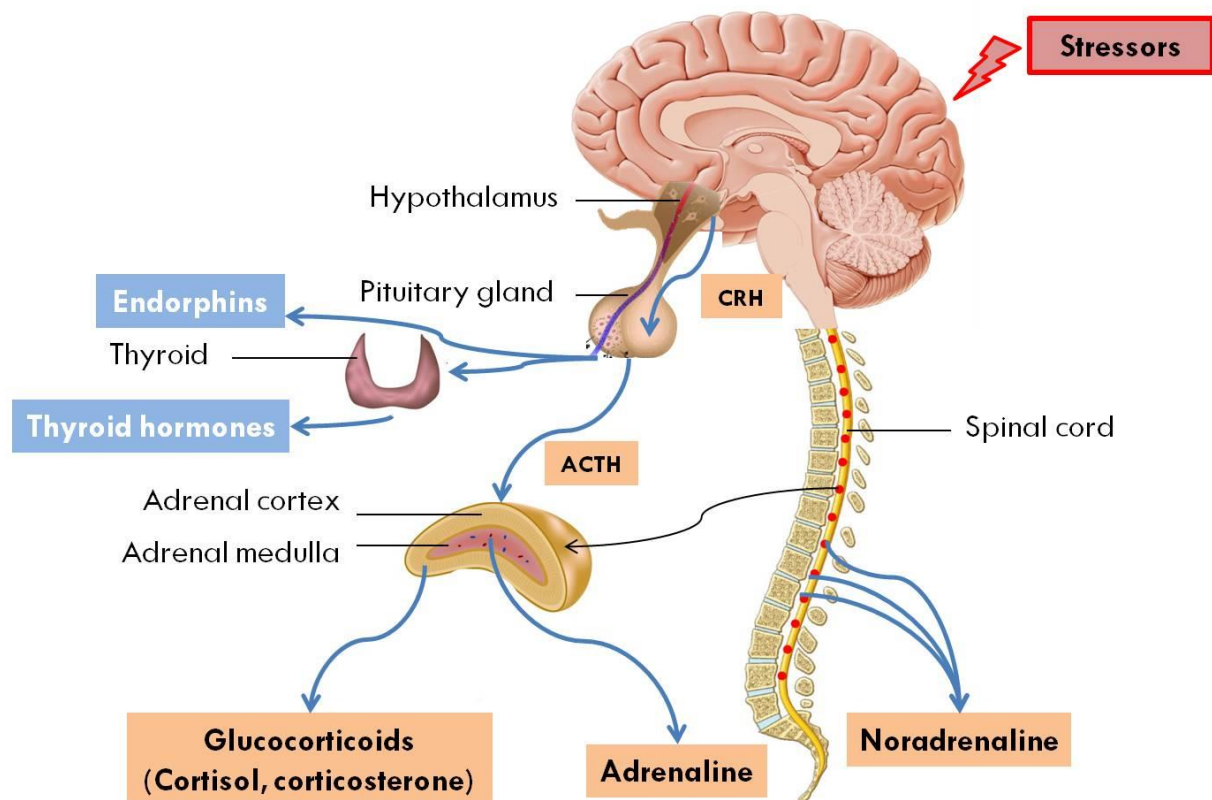
## **2-2 Long-term adjustments**

As mentioned earlier, part of the environmental variations are predictable, and individuals have evolved a large suite of behaviours allowing them to respond to environmental cues of predictable variations occurring across a long-term timescale. First, to face scarcity of resources at a given time of the year, individuals may move away a habitat A toward a habitat B for a certain amount of time in order to find a higher quantity and/or quality of resources, i.e., migration (Levey et al., 1992). Another behavioural tactic that is widely adopted by wild animals is dispersal, the movement of an individual from its site of birth to a site of reproduction, or between sites of reproduction (Clobert et al., 2012). Compared to migration, this process is not seasonal and can, among others, allow individuals to escape local unfavourable conditions (e.g. poor quality or quantity of resources or sexual partners, competition with other individuals) and therefore avoid potential costs (in terms of fitness) associated with stressful situations.

## **3- Physiological adjustments**

Along with behavioural responses, physiological processes are also stimulated to cope with a stressor. The two most important, in terms of consequences for individuals, and the most studied physiological stress responses in vertebrates are the stimulation of the sympathetic nervous system (SNS) and that of the hypothalamic-pituitary-adrenal (HPA) axis, or

hypothalamic-pituitary-interrenal (HPI) axis in fish (Reeder and Kramer, 2005). Other pathways are also activated to cope with stressors, and most of these pathways involve the mobilisation of resources to allow the individual to secure or restore homeostasis (Figure 3). Examples of these other physiological responses include thyroid hormones, that amplify baseline metabolism, and endorphins that mitigate the perception of pain. Throughout this manuscript, I will consider only the SNS and the HPA axis.



**Figure 3** - Representation of the physiological stress responses in vertebrates. In the face of stressors, diverse physiological pathways are activated, including the SNS that results in the secretion of catecholamines (adrenaline and noradrenaline), and the HPA axis that results in the secretion of glucocorticoid hormones. Endorphins are also secreted by the pituitary gland along with thyroid hormones. CRH stands for corticotrophin-releasing hormone and ACTH for adrenocorticotropin hormone. The back arrow corresponds to the neuronal stimulation of the adrenal cortex.

### **3-1 Catecholamine neurohormones**

Few seconds after the brain identified a stressor, the SNS is activated, resulting in the rapid secretion of catecholamine neurohormones, namely, adrenaline and noradrenaline (Reeder and Kramer, 2005). These two neurohormones increase heart rate and provide the required amount of energy to deal with the current stressor by promoting glycogenolysis (the release of glucose from the stored glycogen) and lipolysis (the release of fatty acid through breakdown of triglycerids). This activation of the SNS is part of the so called “fight, flight or freeze” response firstly describe by Walter Cannon (1929) and the secretion of catecholamines support these behavioural responses. Secretion of catecholamines is the first physiological response to stressors, but the release of these neurohormones lasts for no longer than a few minutes. Due to their rapid onset and disappearance kinetics (instantaneous rise and a short half-life), measuring these neurohormones is technically very challenging in free ranging mammals. Therefore, the focus has been on glucocorticoid hormones to study the ecology of stress.

### **3-2 Glucocorticoid hormones**

Simultaneously with the SNS, the endocrine system and, more specifically, the HPA axis is also activated under stressful situations (Möstl and Palme, 2002). Activation of this axis results in the secretion of glucocorticoid hormones, which are mainly corticosterone in birds, amphibians, reptiles and some rodents, but mainly cortisol in fish and most mammals (some species produce the two hormones in significant proportions) (Palme, 2019). After exposure to a stressor, glucocorticoid levels increase markedly and this elevation, usually detectable within 3 to 5 minutes in vertebrates plasma (De Kloet et al., 2005), lasts from a few minutes to several hours (Sapolsky, 2000; Wingfield and Romero, 2001). Glucocorticoid levels can also be detectable in other matrices such as faeces, urine, saliva, feathers or hair (see Appendix 1

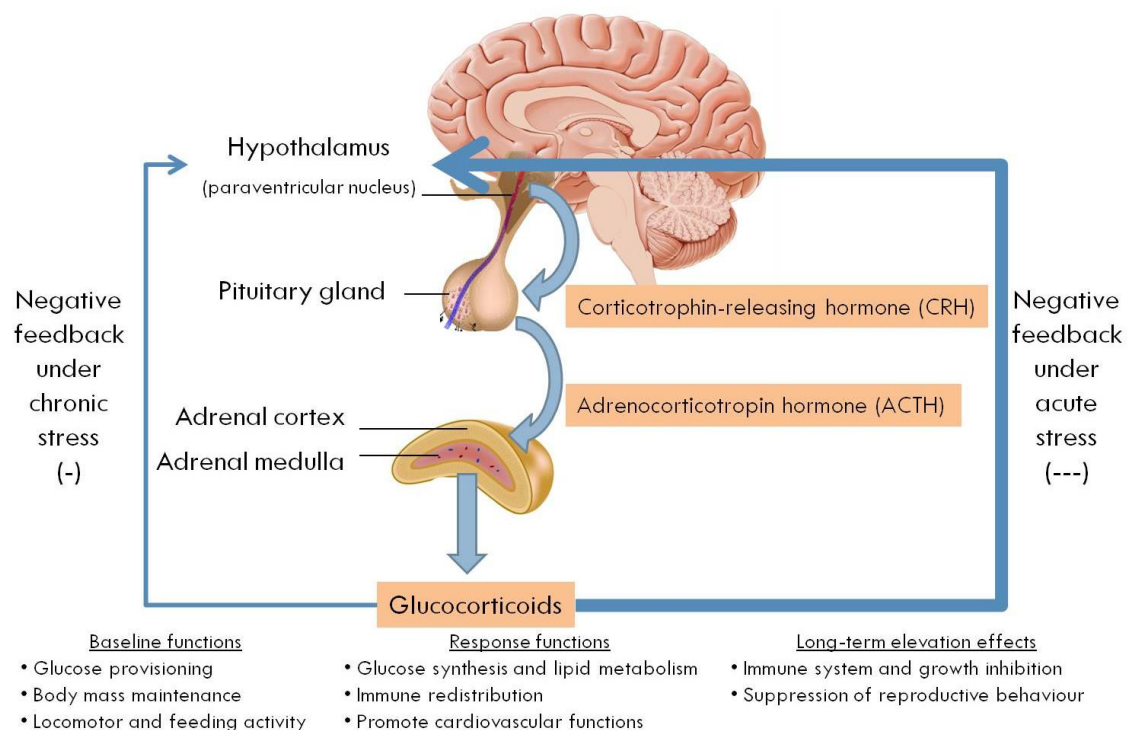
for more details and a comparison). Thereafter I will refer to the increase of glucocorticoids following exposure to a stressor as “response levels”, in contrast to “baseline levels”. The relative ease of measuring glucocorticoids compared to catecholamines, the fact that glucocorticoids promote or suppress the expression of about 10% of the genome (Le et al., 2005), and that the suppression of these hormones leads to death (Darlington et al., 1990) led to call the HPA axis, the stress axis. The measurement of the HPA axis activity is considered as a true window “to see under the surface of the animal into the functional mechanisms it uses to cope” with environmental stressors (Boonstra, 2013).

### ***3-2-1 HPA axis and glucocorticoids secretion***

The HPA axis is a hormonal cascade (see Box 2 for details) resulting in the secretion of glucocorticoid hormones. The secretion of these hormones supports the action of catecholamines by inhibiting energy storage to mobilise lipids, glucose, promote cardiovascular functions, increase locomotor activity (Sapolsky et al., 2000), and orchestrate immune redistribution (Dhabhar and McEwen, 1997). These processes help to cope with the current stressor by making stored energy available, and preparing the organism for a possible immunologic challenge (e.g. wounding or infection). As glucocorticoid levels increase, the hormone exerts a negative feedback on its own secretion (Wingfield and Sapolsky, 2003; Sheriff et al., 2011) so that glucocorticoid levels return to baseline.

## Box 2 - Physiology of the HPA axis

The HPA axis is a hormonal cascade involving the hypothalamic paraventricular nucleus (PVN), the anterior pituitary gland, and the adrenal cortex (Figure 4). When a stressor is perceived, the PVN is stimulated, which causes the release of corticotrophin-releasing hormone (CRH) into the hypophyseal portal system that connects the hypothalamus and the anterior pituitary. The CRH then stimulates the anterior pituitary gland, resulting in the secretion of adrenocorticotropin hormone (ACTH). ACTH is then released into the blood stream and stimulates the secretion of glucocorticoid hormones by the adrenal cortex. As glucocorticoid levels increase, the hormone exerts a negative feedback on its own secretion (Wingfield and Sapolsky, 2003; Sheriff et al., 2011). This inhibits the initial steps of this hormonal cascade due to interactions between glucocorticoids and their receptors in the hippocampus, hypothalamus and pituitary gland (De Kloet et al., 1998). Glucocorticoid hormones exert their actions through binding to two main types of receptors: the intracellular glucocorticoid receptors (GR) and the mineralocorticoid receptors (MR). At baseline levels, glucocorticoids preferentially bind to MR since they exhibit a higher affinity for these receptors. At high glucocorticoid concentrations, such as those observed after exposure to a stressor, glucocorticoids bind to the low-affinity GRs (Sapolsky et al., 2000). When an individual reaches a state of chronic stress, the MR and GR receptors both bind to glucocorticoids even at baseline levels (Romero, 2004).



**Figure 4** - Representation of the hypothalamic-pituitary-adrenal axis resulting in the secretion of glucocorticoid hormones. These hormones then support different functions at baseline and stress-induced levels.

In the case of acute stress, and depending on the severity of the stressor and on the species, glucocorticoid levels generally return to initial baseline level within 1 to 2 hours (De Kloet et al., 2005) and ensures the maintenance of the energy balance according to an individual's needs (see part 3-2-2 for more details). However, when the stressor is persistent (e.g., a long period of harsh conditions or poor quantity of food) or repeated regularly (e.g., being subject to daily hunting attempts), the negative feedback becomes less effective and glucocorticoids secretion is maintained at high levels for a longer period of time. This disruption of negative feedback under chronic stress inducing an overall increase in glucocorticoids is one of the reasons why assaying these hormones is more and more used as physiological indices of stress levels in research (Bonier et al., 2009). However, glucocorticoid hormones are not the only hormones secreted under stressful situations and it is important to keep in mind that measuring glucocorticoid levels does not equate to measuring stress hormones (see MacDougall-Shackleton et al., 2019 for a discussion on this topic).

### ***3-2-2 Baseline glucocorticoids functions***

Glucocorticoid hormones are not only secreted in response to a stressor, but are primarily metabolic hormones. At baseline levels they play a major role in diverse physiological functions, and ensure the regulation of an individual's energy through acquisition, storage and mobilisation (Landys et al., 2006; Busch and Hayward, 2009), for instance by increasing available energy through gluconeogenesis (synthesis of glucose from non-sugar precursors), by decreasing glucose utilisation and protein and fat metabolism. Under normal conditions (i.e., without stressors), the negative feedback of glucocorticoids on their own secretion is efficient and allows the regulation of the normal circadian rhythm of glucocorticoids release in the blood stream (De Kloet et al., 1998; Wingfield and Sapolsky, 2003). This regulation results from a long-term adjustment of individuals' physiology. Peak concentrations of



glucocorticoids are usually found just prior to an individual's activity peaks, so that they provide the amount of energy needed for the behaviours that occur when individuals begin their daily activities, such as an increased locomotor activity, and foraging behaviour (McEwen et al., 1988, Landys et al., 2006).

#### **4- Among-individual differences in glucocorticoid levels**

Baseline as well as response levels of glucocorticoids are dynamic and exhibit changes throughout the life of an individual. These changes may depend on intrinsic and extrinsic factors, such as the life history stage of an individual or the availability of resources. For instance, when resources are scarce baseline levels of glucocorticoids might increase and the ability to respond efficiently to an unpredictable stressor might be impaired (Romero, 2004). It is therefore important to consider as many biologically relevant factors as possible when studying variations in glucocorticoid hormones. Here I will only focus on some of these factors in the following sections 4-1 and 4-2, but recent reviews discuss several other factors such as reproductive state, social status and interactions or predation (Hau et al., 2016; Palme, 2019).

##### **4-1 Individual sources of variations in glucocorticoid levels**

###### **4-1-1 Age**

Effects of advancing age on glucocorticoid levels are different between response and baseline levels. Response glucocorticoid levels appear to be lowered with increasing age in many species (Reeder and Kramer, 2005). Alongside this decrease in the reactivity of the HPA axis with age, impairment in the efficacy of the negative feedback described earlier has been documented (Romero, 2004). While the effect of age on stress-induced concentrations of glucocorticoids is very consistent among species, effect of age on baseline levels is not. It is established that glucocorticoid levels are low in the first few weeks of life in many vertebrate

species (period of hyporesponsiveness of the HPA axis). Most likely, such hyporesponsiveness period protect growing neurons from the potential adverse effects of high glucocorticoid levels during growth (Sapolsky and Meaney, 1986). It is less clear how patterns of glucocorticoids changes with age at adult stages. While some studies found an increase of baseline glucocorticoid level with age (Sapolsky et al., 1983; Dellu et al., 1996; Bauer, 2005), some others did not (van der Ohe et al., 2004; MacBeth et al., 2010; Terwissen et al., 2013). These discrepancies among studies highlight the need to take into account age of individuals in every studies seeking to evaluate glucocorticoid variations in species where the age effects remain unknown.

#### **4-1-2 Sex**

Variations in glucocorticoid levels between males and females are not consistently biased toward one sex (Touma & Palme 2005). Some studies found higher glucocorticoid levels in males (in Steller sea lion (*Eumetopias jubatus*), domestic chicken (*Gallus gallus*), Hunt et al. 2004; Rettenbacher et al. 2004), or in females (in arctic ground squirrels (*Spermophilus parryii*), Boonstra et al., 2001). No difference between sexes were found in wolves (*Canis lupus*) or black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceros (Sands and Creel, 2004; Brown et al., 2001). The high interaction between the HPA axis and HPG (hypothalamic-pituitary-gonadal) axis has been proposed to explain sex differences in glucocorticoid levels. In particular, actions of sex steroids on the HPA axis vary by sex, and patterns differ across taxa (Handa and Weiser, 2013; Toufexis et al., 2014).

#### **4-1-3 Body condition**

Because of their metabolic function, it is not surprising that glucocorticoid levels have been closely linked to body condition. Most studies on this relationship have been conducted on

birds (but see Moore et al., 2000 for an example on reptile), with results showing a negative relationship between baseline corticosterone levels and body condition indices (Kitayski et al., 1999; Sockman and Schwabl, 2001; Angelier et al., 2009; Fokidis et al., 2011). The tight link between body condition and baseline glucocorticoid levels is probably one of the reasons why these hormones are increasingly used as physiological indices of the relative condition and health of individuals and populations (Walker et al., 2005; Wikelski and Cooke, 2006). In contrast, patterns are less clear for response corticosterone levels, which appeared to be either positive (Fokidis et al., 2011) or negative (Angelier et al., 2009) depending on studies. This diversity of patterns have been hypothesised to be due to the environmental context, but also the life-history stage of the individual.

#### **4-1-4 Behaviour**

It is a fact that marked differences in behaviour exist among individuals. In the past two decades, the study of animal personality (Box 3) has attracted many biologists and has led to a growing body of literature on the subject (Gosling, 2001; Sih et al., 2004a; Dingemanse and Réale, 2005; Réale et al., 2007; Réale et al., 2010; Stamps and Groothuis, 2010; DiRienzo and Montiglio, 2015; Roche et al., 2016). Interestingly, this large literature showed that personality can be linked to fitness. For example, boldness is positively related to the lifetime reproductive success (Réale et al., 2009) and survival (Réale and Festa-Bianchet, 2003) of bighorn sheep (*Ovis canadensis*). However this relationship may be modulated by the environmental context, so that some behavioural phenotypes may perform better in some contexts than in others (Dingemanse et al., 2004). In addition, personality has been linked to the HPA axis, and more precisely to glucocorticoid levels under laboratory conditions (Koolhaas et al., 1999) and in the wild (Montiglio et al., 2012; Montiglio et al., 2015). Typically, individuals exhibiting high baseline and response glucocorticoid levels are more likely to be shy, non-aggressive,

thorough explorers, and particularly attentive to changes in their environment. Conversely, individuals exhibiting low glucocorticoid levels are more likely to be bold, aggressive, actively explore their environment and follow routines (Koolhaas et al., 1999; Sih et al., 2004a). The existence of a relationship between the HPA axis and behavioural traits has been found in diverse taxa including mammals (Koolhaas et al., 1999), birds (Groothuis and Carere, 2005), fish and reptiles (Øverli et al., 2007), suggesting that the relationship has been conserved through evolutionary times. As stated above, stress response is composed of behavioural and physiological responses. Therefore the existence of a close relationship between these two is not surprising and the manner an individual faces stressful situation on both of these responses forms what Jaap Koolhaas and his colleagues (1999) have qualified: a coping style (Box 3). However, this framework has been recently questioned, and Jaap Koolhaas and his colleagues (2010) proposed a second framework suggesting the relationship between physiological and behavioural responses to stressors would be more complex. This recent “two-tier model” of coping styles suggests that the behavioural response to stressor would be primarily linked to the sympathetic stress response (release of catecholamines), but would be independent from the HPA axis activity (release of glucocorticoids). Although recent studies seem to support this alternative view of coping styles in wild populations (Qu et al., 2018; Westrick et al., 2019), further work is needed to assess the importance of these two models in the wild.

### **Box 3 - Behavioural type, coping style, personality... a glossary please**

**Animal personality:** Consistent among-individual differences in behaviour over time and/or across contexts (Réale et al., 2007).

**Behavioural syndrome:** Consistent co-variation between a suite of behaviours across contexts (e.g. predator avoidance or reaction to novelty) or situations (e.g. different levels of predation) (Sih et al., 2004a).

**Behavioural type:** Within a behavioural syndrome (e.g. boldness, aggressiveness), individuals have a behavioural type (e.g. shy versus bold, more versus less aggressive) (Sih et al., 2004b).

**Coping style:** Characterises the association between the behavioural and physiological responses of individuals to stressful situations (Koolhaas et al., 1999). The coping style continuum ranges from reactive to proactive individuals, with reactive individuals being typically shy and exhibiting a high reactivity of the HPA axis. Conversely, proactive individuals are typically bold and exhibit a low reactivity of the HPA axis (Koolhaas et al., 1999; Sih et al., 2004a). An alternative view states that a close relationship exist between reactive-proactive behavioural response and SNS axis (Koolhaas et al. 2010).

## **4-2 Environmental sources of variations**

### ***4-2-1 Quantity and quality of resources***

Food availability and quality have been demonstrated to influence glucocorticoid levels and act as stressors in vertebrates (Kitaysky et al., 2007). Numerous studies have reported elevated baseline glucocorticoid levels in individuals exposed to poor food availability in some years (Hellgren et al., 1993), adverse weather conditions (Huber et al., 2003), or poor overall habitat quality (Marra and Holberton, 1998). In addition, this relationship between environmental context in terms of resources and glucocorticoid levels is not necessarily linked to body condition (Fokidis et al., 2012).

#### **4-2-2 Predation**

The effects of predation on glucocorticoid levels in vertebrates have been reviewed by Michael Clinchy and his colleagues (2013) and usually lead to a negative effect. For example, on a study of wild European rabbits (*Oryctolagus cuniculus*), Raquel Monclús and her colleagues (2008) showed that glucocorticoid levels were positively correlated with indexes of presence of mammalian carnivore predators. In a previous experimental study, the same authors showed that not only presence of predators, but also simulated presence of a predator through odour cues resulted in an increase in glucocorticoid levels (Monclús et al., 2006). Similarly, Rudy Boonstra and his colleagues (1998) showed that snowshoe hare (*Lepus americanus*) were chronically stressed by predation risk, as indicated by higher levels of free cortisol and reduced maximum corticosteroid-binding capacity during periods of population decline (high predation risk), compared to population low (low predation risk).

#### **4-2-3 Social interactions**

Social interactions with conspecifics are another factor that has been shown to influence glucocorticoid levels (see Creel, 2001 for a review). Numerous studies have investigated the relationship between glucocorticoid levels and the dominant-subordinate status and found contradictory results. While most of the primary works in the field showed higher glucocorticoid levels in subordinates (see Sapolsky, 1992 for a review), others found no relationship (Bercovitch and Clarke, 1995), or higher glucocorticoid levels in dominant individuals (Creel, 2005). A possible explanation to these mixed observations is the stability of the hierarchy in the group and associated predictability of stressors. In groups with stable hierarchies, high-ranking individuals usually have control over a wide range of situations, leading to predictable changes and lower glucocorticoid levels for them, compared to subordinates that do not have control and face unpredictable situations. Conversely, in groups

with unstable hierarchies, the dominance position may be regularly threatened, leading to an absence of control, high rate of agonistic interactions with other individuals, and consequently higher glucocorticoid levels.

#### ***4-2-4 Human disturbance***

In the last centuries, and even more during the last few decades, human activities have intensified and brought Crutzen and Stoemer (2000) to name the modern area the “Anthropocene”. Through the change in land use that has fragmented more than 50% of the earth’s land surface (Hooke et al., 2012), or recreational activities such as hunting or snowmobile activity, human induced disturbance is now widespread and have become a major selective force in natural populations (Wingfield and Romero, 2001). Several studies have focused on the effects of this kind of stressor on baseline glucocorticoid levels and consistently showed clear elevations in response to human disturbance (e.g., Dantzer et al., 2014; Rehnus et al., 2014; Jachowski et al., 2015; Formenti et al., 2018). For instance, Bateson and Bradshaw (1997) showed that blood cortisol concentrations were 70% higher in a group of red deer (*Cervus elephus*) that underwent prolonged chases during hunts compared to a group of non-hunted. Another example of the effects of recreational activities is the association between areas and times of heavy snowmobile use on cortisol levels of wolves and red deer of the Yellowstone National Park (Creel et al., 2002).

Despite the obvious effect of the different environmental and individual factors mentioned above on glucocorticoid levels, very few studies are able to consider these factors together in the same study due to field constraints. Consequently, taking them into account when evaluating glucocorticoid level variations, and their potential effects on diverse functions, remains a challenge in wild populations.

## **5 - Consequences of stress**

If individuals are transiently exposed to stressors, glucocorticoid levels do not remain elevated in the blood. On the opposite, when individuals are persistently or regularly exposed to stressors, glucocorticoid levels remain elevated in the blood. This difference may lead to different consequences. When individuals reach the state of allostatic overload, also referred to as a chronic stress state, several negative consequences are commonly observed such as decreased immune response and increased susceptibility to disease (Dhabhar, 2014), reduced growth and a decrease in reproductive performance (Sapolsky et al., 2000). These adverse effects impact individual fitness (Romero et al., 2009), have long-lasting consequences on the offspring through maternal stress effect, and, ultimately, affect population dynamics (Wingfield and Sapolsky, 2003).

### **5-1 Immune function**

Pathogens occupy an important place in living systems, and all organisms have been, are, or will be infected at least once in their life. Consequently, pathogens represent a major selective force affecting hosts fitness (Anderson and May, 1982; Møller et al., 1990). To resist or eliminate pathogens, organisms have evolved barriers (such as skin) and a physiological mechanism, the immune system. The immune system is particularly elaborated and complex in vertebrates. It is composed of numerous cells and effectors, and can be divided in two complementary arms, the innate and the adaptive immune system (Box 4). However, this efficient system is costly in terms of energy (Lochmiller and Deerenberg, 2000; Demas, 2004), even if the costs appear arm-specific. Roughly, innate immunity is thought to have low developmental costs, but a high cost of use (Klasing and Leshchinsky, 1999), while adaptive



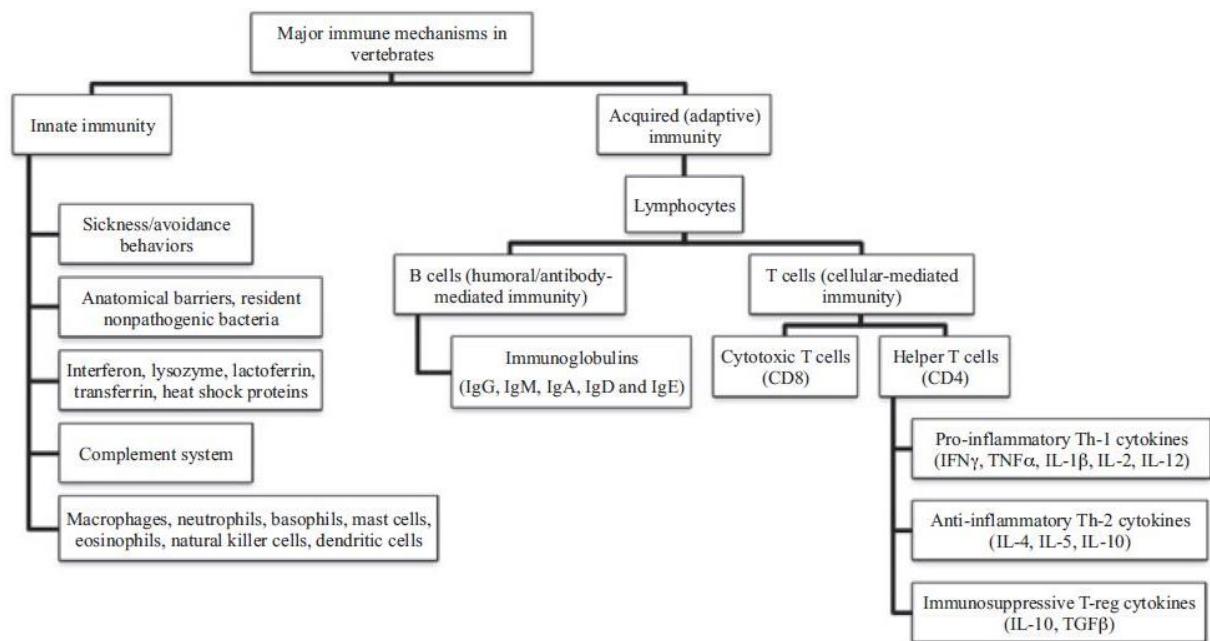
immunity would be more energetically costly to develop (due to the diversification process of specific cells, such as lymphocytes) but would have a low cost of use (Lee, 2006).

#### **Box 4 - Into the immunity of wildlife...**

The immune system of vertebrates is composed of the innate and the adaptive arms, which are complementary, but have each specific functions and effectors (Figure 5). Overall, the immune system is composed of specialised cells whose function is to differentiate “self” from “non-self” and to eliminate any foreign “non-self” substance than could damage the host.

**Innate immunity:** This component of the immune system represent the first line of defence of an organism and is relatively rapid to set up (hours), but not specific to pathogens. Such defences include anatomical barriers (e.g., mucus membranes or skin), humoral factors such as lysozyme, complement and acute phase proteins, but also cellular responses such as phagocytic cells (e.g. neutrophils, monocytes and macrophages), inflammatory mediators produced by basophils and eosinophils, and natural killer cells. The complement system includes enzymes that promote inflammatory response. In addition, complement is important for stimulating the adaptive immune responses (Dempsey et al., 1996). Developmental costs of innate immunity are thought to be relatively low in terms of energy, while activation costs are particularly high (McDade et al., 2016).

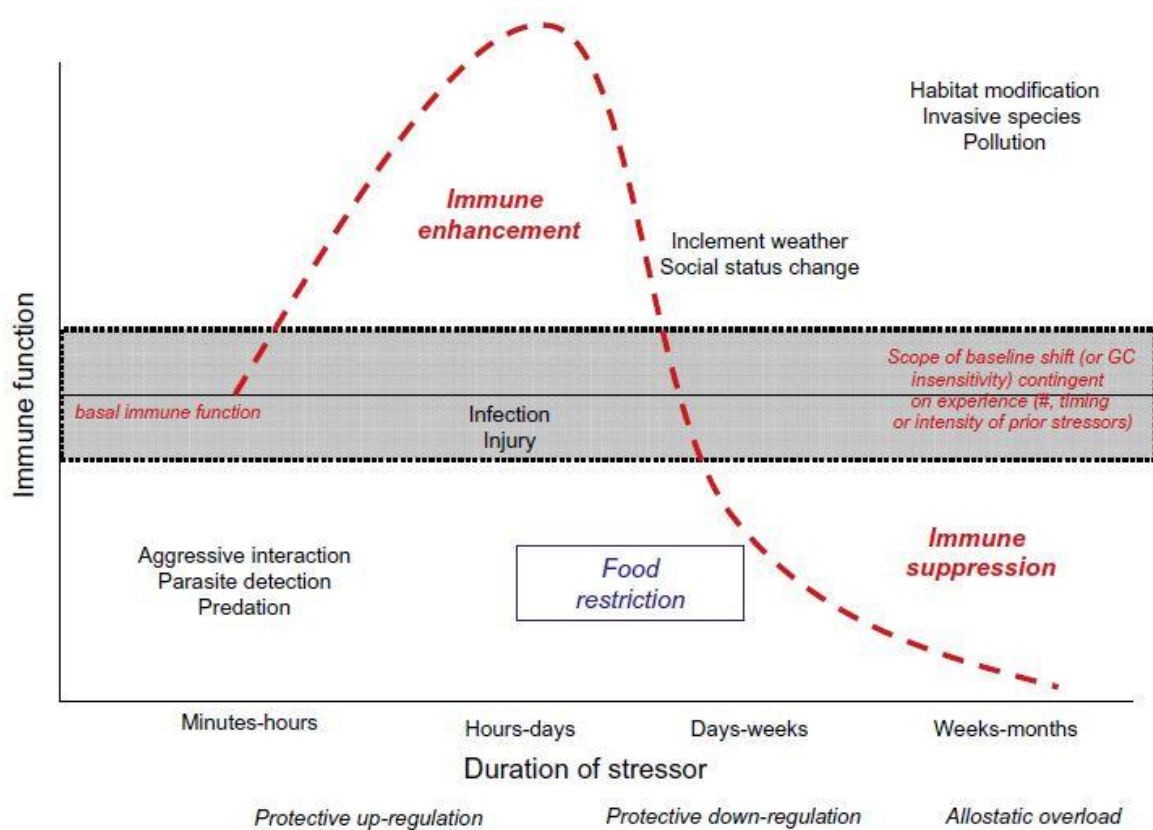
**Adaptive immunity:** This component of the immune system comes as an additional line of defence. It is typically slower than the innate immunity (days) at first exposure to a given pathogen, but is pathogen-specific with longer lasting effects and a higher efficiency and rapidity upon secondary exposure. Such defences are mediated by lymphocyte cells that subsequently develop into B cells, T cells or NK cells. B cells produce antibodies that neutralise pathogens and their products, block binding of parasites to host cells, stimulate the complement system, promote cell migration to infection sites and enhance phagocytosis, among other actions (Demas et al., 2011). T cells do not produce antibodies but require direct contact with an infected cell to eliminate it. T cells can be further divided into different subtypes, including cytotoxic T cells, which recognize and destroy cells with intra-cellular pathogens, helper T cells which coordinate immune response to infection, and regulatory T cells which play a key role in maintaining peripheral immunological tolerance by actively suppressing the immune response (Demas et al., 2011). Compared to innate immunity, this arm is known to have particularly high developmental costs, but relatively low energy costs of activation (McDade et al., 2016).



**Figure 5** - Major immune mechanisms in vertebrates (extracted from Demas et al., 2011).

A central assumption in ecology is that trade-offs should exist between immune defences and any other functions that share a common source of energy and that contribute to an individual's fitness. This assumption has been the basis for many hypotheses and researches on the potential link between glucocorticoid levels and efficiency of immune responses (Lee, 2006 ; Martin, 2009). However, most of our knowledge on this topic comes from the biomedical literature. The commonly accepted findings is that chronically elevated glucocorticoid levels are immunosuppressive (Dhabhar and McEwen, 1997; Sapolsky et al., 2000; Dhabhar, 2009), while transient response levels can enhance immune functions (Dhabhar, 2014), in both innate and adaptive arms (Dhabhar, 2002). In response to an acute stressor, glucocorticoids elevation initiates a cascade of events and induces the trafficking of immune cells (e.g., neutrophils and lymphocytes) out of compartments such as the spleen, bone marrow, and lymph nodes into the blood, to ultimately reach target organs where an individual is most likely to be injured (e.g. skin, gastrointestinal track, or lungs) (Dhabhar et al., 2012). Conversely, in response to a chronic stressor, the maintenance of elevated

glucocorticoid levels has been linked to impaired immune response to vaccination, and delayed healing after standardised wound inductions (Glaser et al., 1998; Glaser et al., 1999). Based on these results from the biomedical field, it has been generalised that, in wild conditions, chronic stress would be detrimental to the immune system of individuals while acute stress would improve it (Figure 6 extracted from Martin, 2009). However, natural conditions differ from laboratory, and domesticated animals do not react to the same stimuli than wild animals. In free ranging conditions, short term elevations of glucocorticoid levels can suppress, enhance, or have no measurable effects on immune functions of wild animals (Delehanty and Boonstra, 2009; Matson et al., 2006; Merrill et al., 2012). Regarding the effects of long term elevations and maintenance of elevated baseline glucocorticoid levels, researches on wild avian species showed that corticosterone levels may differentially affect different components of the immune response (Bourgeon and Raclot, 2006). For instance, Kim Silvana Stier and her colleagues (2009) experimentally demonstrated in barn owl (*Tyto alba*) nestlings that elevated corticosterone levels affected the adaptive response through reduced production of antibodies, but did not significantly affect agglutination scores, a component of the constitutive innate immunity. However, patterns are not consistent among studies, for example a group of Belding's ground squirrels (*Urocitellus beldingi*) that underwent experimentally elevated cortisol levels exhibited a lower bacteria killing ability, another component of the constitutive innate immune response, compared to a control group (Brooks and Mateo, 2013). To date, the understanding of the relationship between stress and immunity in the wild remains a large field of investigations to explore.



**Figure 6** - Juxtaposition of stressors that wild animals experience and known effects of similar duration stressors in domesticated rodents. In response to stressors lasting minutes to hours, such as failed predation events, a concomitant enhancement in most immune processes, particularly inflammatory ones has been noticed. Longer duration stressors such as adverse weather conditions (days to weeks) and habitat changes (weeks to months) have been correlated with the reduction of most immune processes. Here, the down regulation of immune functions may either represent an adaptive energy saving mechanism or may be the consequence of allostatic overload (McEwen and Wingfield, 2003). Red dashed line depicts changes in immune function as a consequence of stressors of various durations. Shaded area depicts potential long-term increase or decrease in sensitivity to stressors driven by experience. Plain text indicates variety of possible stressors. Italicized text below x-axis hypothesises evolutionary relevance of changes in immunity as a function of stressor duration.

## 5-2 Reproduction and survival

The consequences of short- and long-term glucocorticoids elevation on survival and reproductive success have been the focus of numerous investigations because of their consequences on population dynamics. Stress response is adaptive, and seeks to enhance immediate survival in the face of stressful situations (Romero and Wingfield, 2001). However, as pointed out in a recent review (Breuner et al., 2008), few studies have examined the link between short-term elevation of glucocorticoids and fitness measures. In this review, only 11 studies over the past 20 years have evaluated the effects of short-term elevation of glucocorticoids and direct measures of fitness. It is also worth mentioning that over the 62 studies considered in this review, only one was conducted on amphibians, three on mammals, three on fishes, and 14 on reptiles, while 41 have been conducted on birds. Overall, results of the studies suggest that short-term elevation of glucocorticoid levels is positively linked to survival, at the expense of current reproduction. However, the only two studies examining a link between direct survival measures and short-term elevation of glucocorticoid levels found a negative relationship. A recent study on North American red squirrels (*Tamiasciurus hudsonicus*) also found different results. In this study, a short-term experimental increase in glucocorticoids impaired adrenal gland function but had no effect on body mass or survival of adults and offspring (Van Kesteren et al., 2019).

Due to their role in the metabolism and maintenance of the energy balance, it has been hypothesised that high baseline glucocorticoid levels (as a result of encountering a challenging conditions) would be linked to low reproductive success and survival rates (Bonier et al., 2009). A comprehensive review from Frances Bonier and her colleagues (2009) focused on this topic and showed that the empirical data did not strongly support this hypothesis. Of the 53 studies considered in this review, almost half of them (41%) did not found any significant

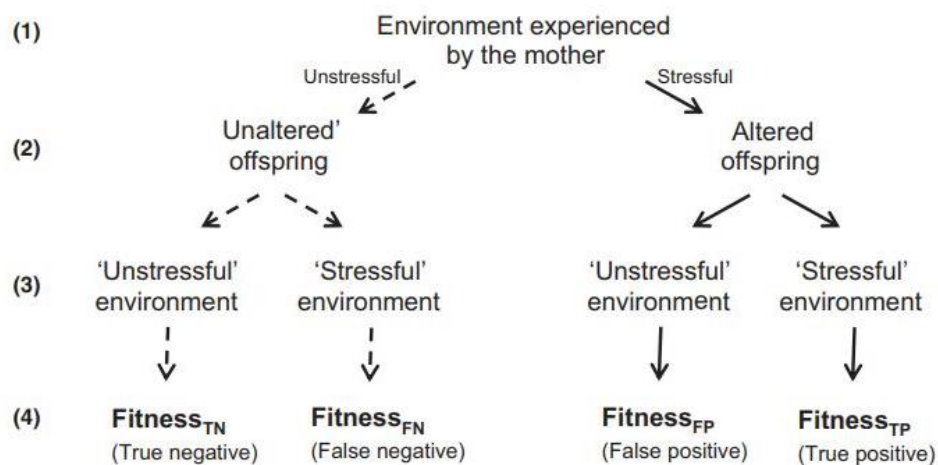
relationship between glucocorticoid levels and reproductive success or survival. For the studies showing a significant relationship, most results were not consistent, with negative as well as positive relationships. As for the review on the effect of response levels of glucocorticoids, it is worth to note that most of the studies considered were carried on birds (70%). This discrepancy between studies can be explained by differences between environmental contexts, but also a lack of information regarding some elements such as the life history stage. For instance, a recent study on tree swallows (*Tachycineta bicolor*) showed that females with low corticosterone levels during the incubation period had a higher reproductive success. However, during the provisioning period, there was a trade-off between baseline and response levels of corticosterone. Indeed, reproductive success was higher among females that maintained low baseline corticosterone levels while having strong response levels, but also among females with high baseline corticosterone levels while having weak response levels (Vitousek et al., 2018).

This brief overview of the literature highlights the complexity of the relationships between glucocorticoid levels and fitness, and the importance of considering additional parameters when studying this relationship, such as life history stages or environmental conditions. Taking into account these potential mediators of the glucocorticoid levels-fitness relationships, a recent phylogenetic meta-analysis across vertebrates revealed that response as well as baseline levels of glucocorticoids were consistently linked to lower reproductive success. However the relationships were much more complex with respect to survival. These relationships were highly context-dependent and differed according to life history strategies, with negative relationships occurring more often in long-lived species (Schoenle et al., 2019).

### 5-3 Inter-generational effects

Maternal stress effects occur when maternal stress hormones alter offspring phenotype (Love et al. 2013). Exposure to maternal glucocorticoids can originate from the placenta or the milk in mammals and the yolk in non-mammalian vertebrates (Seckl and Meaney, 2004; Sullivan et al., 2011; Almasi et al., 2012). Detrimental effects of elevated maternal glucocorticoid levels on the offspring phenotypes (body mass, growth, behaviour, physiology) have been hypothesised. Again, these predictions were largely based on the biomedical literature (Barbazanges et al., 1996; Seckl and Meaney, 2004; Meaney et al., 2007; Weinstock, 2008), but several studies conducted on wild animals also found decreased birth body size, body mass and growth rate of offspring from mothers exhibiting high glucocorticoid levels (Saino et al., 2005; Meylan and Clobert, 2005; Sheriff et al., 2009; Meylan et al., 2012). In addition to direct effects of maternally-derived glucocorticoids on the offspring phenotype, indirect effects can originate from a reduction of maternal care due to high glucocorticoid levels (Love et al., 2013). Usually, the observed changes in offspring phenotype occur through epigenetic changes in the brain and result in a change in neural development and the alteration of the HPA axis of the offspring, resulting for instance in offspring with elevated glucocorticoid levels (Hayward and Wingfield, 2004; Seckl and Meaney, 2004). However, elevated maternal glucocorticoid levels do not always lead to poorer quality offspring. For instance, Michael Sheriff and his colleagues (2017a) showed that although glucocorticoid levels were higher in offspring of females arctic-ground squirrels coming from a site where they exhibited higher glucocorticoid levels, maternal glucocorticoid levels had no effect on offspring's size at either of the two sites. Another result that contradicts this theory is the famous experimentation conducted by Ben Dantzer and his colleagues (2013) on red squirrels which showed that females subjected to experimental or natural elevation of maternal glucocorticoids produced

offspring with a higher growth rate than controls. As for the fitness consequences of elevated glucocorticoid levels, these mixed results could be explained by the environmental context, timing of measures (i.e. how young are the offspring and how independent of their mother they are). For instance, hatchlings from corticosterone injected eggs of house wrens (*Troglodytes aedon*) were lighter than controls, but were heavier at fledging due to a compensatory growth mechanisms (Strange et al., 2016). In addition, stating that a reduced size, or growth rate, of offspring is maladaptive (i.e. reduced fitness) is not necessarily true without taking into account the long-term consequences of this phenotype, for the mother as for the offspring. This more integrative view have been proposed by Michael Sheriff and his colleagues (2017b) and suggests that modulation of the offspring phenotypes by mother's glucocorticoids could prepare them for their post-natal environment, and therefore be beneficial to offspring. In other words, when the environment of the mother and the future environment of the offspring match, maternal stress can be adaptive (Sheriff and Love, 2013). This new framework therefore emphasises the importance of considering past, present and future environment of the mothers and offspring to improve our understanding of the potential adaptive value of maternally derived glucocorticoids (Figure 7).





**Figure 7** - The environment experience by mothers during reproduction can either be unstressful (leading to the dashed arrow pathway) or stressful (leading to the pathway represented by solid arrows), with the latter occurring when stress hormone levels of mothers increase beyond some threshold of normal baseline (1). This dichotomy leads to “unaltered” offspring phenotypes or “altered” offspring phenotypes in response to elevated maternal stress (2). These offspring then have the potential to also encounter two different environments; an “unstressful” environment, or, alternatively, a “stressful” environment (3), and their fitness value will depend upon the interaction between their phenotype and the environment they experience (4). Additionally, the adaptive potential of maternal stress to maternal (inclusive) fitness can also be evaluated within this framework if the end fitness outcomes (4) are that of the mother (i.e., do mothers survive better and have greater future reproduction if they raise altered offspring in a stressful environment as opposed to attempting to raise unaltered offspring?). (Extracted from Sheriff et al. 2018).

## **6- Background synthesis and aims of this thesis**

In the wild, animals adapt to unpredictable events through physiological and behavioural adjustments, most of which are mediated by glucocorticoid hormones. Wide among-individual variability in glucocorticoid levels occurs and different consequences on fitness are expected. However, to which extent these variations are consistent over time and what are the drivers of these observed variations and their consequences on fitness remains poorly understood. Most studies investigating changes in glucocorticoid level have considered environmental and individual characteristics separately. Studies aiming to understand the influence of environmental context often include variables such as sex or age in their analyses, however they rarely include information about among-individual differences in behavioural

response to stressors. Conversely, studies of the relationship between behavioural response to stressors and glucocorticoid levels rarely consider the environmental context. The general aim of this thesis is to use a comprehensive approach to identify the main determinants of glucocorticoid levels and to investigate their consequences in a long-lived mammal, the roe deer.

The first aim of this thesis (**chapter I**) was to investigate simultaneously individual and environmental drivers of stress levels in wild animals. First, I investigated whether there were consistent among-individual differences in baseline stress levels, and what are the environmental and individual drivers of these among-individual variations. I use a comprehensive approach including environmental (spatial and temporal variations in resources quantity and quality) and individual (sex, age, body mass, behavioural response to a stressor) characteristics in three wild populations of roe deer. Second I specifically investigated the interaction between spatial behaviour and physiological stress response in one population benefiting from an intensive monitoring of spatial behaviour. I tested whether spatial and temporal behavioural adjustments allowed individuals to mitigate the elevation of baseline glucocorticoid levels observed in a human dominated landscape.

While the first chapter of this thesis focuses on among-individual variations in adults, the second chapter aims to investigate some of the fitness outcomes of elevated glucocorticoid levels, with a focus on mother-offspring relationships (**chapter II**). Indeed, while the factors underlying among-individual variations of glucocorticoids in the adult stage are well described, the literature on this subject is almost non-existent in the offspring. First, I aimed to understand among-individual variations of glucocorticoid levels in offspring. Second, I investigated the consequences of elevated maternal glucocorticoid levels on maternal reproductive success and offspring phenotype.

In a third time (**chapter III**), I addressed other potential consequences of elevated glucocorticoid levels, focussing on their interaction with immune functions. While a framework with strong and several testable hypotheses have been proposed (Martin, 2009), few studies have examined this relationship in wild populations. In particular, most studies have focused on one arm of the immune system, the innate or the adaptive one, but have rarely considered the effects of glucocorticoids on both. Therefore, the goal of this last chapter was twofold. First, I investigated the relationship between baseline glucocorticoid levels and baseline levels of eleven immune parameters reflective of both the innate and adaptive arms as well as inflammatory markers in three different populations of wild roe deer. Second, I used an experimental approach to understand how variations in glucocorticoid levels could induce variations in immune parameters, while considering among-individual differences in the behavioural response to a stressful situation.

## **6-1 Outline**

The work presented in my thesis can therefore be summarised with the following questions:

**Chapter I:** What are the drivers of baseline stress levels in wild populations?

- a) Do consistent among-individual differences in baseline stress levels exist?
- b) To what extent behavioural and physiological stress responses are linked and may form a coping-style?
- c) How spatial behaviour can mitigate the elevation of glucocorticoids in the face of human disturbance?

**Chapter II:** What are the drivers of baseline stress levels in offspring in wild populations? And what are the consequences of higher maternal baseline glucocorticoid levels?

- a) To which extent behavioural and physiological responses to stressors are linked in offspring?

b) Are glucocorticoid levels of offspring linked to maternal glucocorticoid levels?

c) Does maternal stress influence offspring's phenotype?

**Chapter III:** What are the consequences of baseline glucocorticoid levels on immunity, both the innate and adaptive arms?

a) Are baseline glucocorticoid levels negatively linked to immune parameters in wild populations? (Observational approach)

b) Are variations of baseline glucocorticoid levels related to variations in immune parameters of both arms? (Experimental approach)

## **6-2 Model species**

In order to investigate these questions, I used the European roe deer (*Capreolus capreolus*) (Box 5) as a study model focusing on four different populations (Box 6). The European roe deer has largely increased its distribution in the recent years and is now widespread across the European continent, with the exception of Ireland, Cyprus, Corsica, Sardinia, Sicily, and most of the smaller islands (Figure 8). Primarily considered a forest species, the roe deer exhibits a high behavioural plasticity and has now colonised a wide variety of habitats (Andersen et al., 1998). From forest, roe deer expended to agricultural, mountain, Mediterranean garrigue and moorland environments (Andersen et al., 1998). This diversity of new habitats increases the variety of potential stressors for this species. In particular, humans-related stressors are increasingly prevalent since the roe deer can occupy large agricultural plains without refuges such as woodland patches (Cibien et al., 1989), with meadows and cultivated crops providing particularly abundant and rich food resources, but also leading to an exposition to anthropogenic disturbance (Hewison et al., 2001). In addition to its exposition to a wide range of stressors, roe deer also appears as an interesting species since it tends to be an income breeder (Andersen et al., 1998), which means that it does not accumulate fat reserves in the

body and can only rely on the current food resources to achieve its life cycle. This characteristic makes this species particularly sensitive to availability and quality of resources, the scarcity of which can rapidly become a source of stress, revealing trade-off between glucocorticoid elevation and other functions. Another interesting feature regarding roe deer is that this species is particularly prone to fluctuation in environmental conditions such that under favourable conditions, females may give birth at one year old (Hewison, 1996) whereas under poor conditions first reproduction is often delayed to the age of 3 years old (Gaillard et al., 1992; Hewison, 1996). Reproductive success can therefore depend on environmental conditions, and glucocorticoid levels appear to be a possible underlying cause of these among-individual differences. Finally, spatial proximity of roe deer with domestic fauna makes this model interesting. Being exposed to both wild and domestic fauna, roe deer are exposed to a wide range of pathogens and therefore rely heavily on their immune system to persist (Jolles et al., 2015).



**Figure 8** - Area of distribution of the European roe deer. Map from the IUCN (International Union for Conservation of Nature) Red List of Threatened Species. Version 2019-2. In orange: current area of distribution, in red: area of distribution where the species is extinct.

## Box 5 - Biology of the European roe deer (*Capreolus capreolus*) (1/2)

### Basic information

**Class:** *Mammalia*

**Order:** *Artiodactyla*

**Family:** *Cervidae*

**Genus:** *Capreolus*

**Withers height:** 60 to 80 cm

**Total length:** 1 to 1.30 m

**Weight:** 20 to 30 kg with males weighting 2 to 3 kg more than females

Presence of antler in males that can reach about 30 cm



### Biology of the species

Roe deer can be considered as a crepuscular species, with activity peaks at the sunrise and sunset, even though it can exhibit lower activity peaks during the rest of the day. It is a solitary species most of the year, but form small groups during winter. While the size of the groups can reach about 200 individuals in large plains, in more forested environments the group is often composed of one or a few adult females with their offspring and yearling daughters, and some time one or two adult males. It is an herbivore which feeds on a large number of plant species depending on the habitat where it lives. Diet is mainly composed of shrubs, trees and forbs in forest, but also crop plants in cultivated habitats, and various plant parts such as buds, leaves, flowers, fruits and seeds (R. Andersen et al., 1998; Tixier and Duncan, 1996; Cornelis et al., 1999). Cultivated leaves and seeds, as well as acorn constitute a particularly nutritive food for roe deer (Abbas et al., 2011).

In roe deer natal dispersal occurs at approximately 8 months of age for approximately 33% of the animals, both males and females. Adult roe deer may exhibit migratory behaviour with seasonal non-overlapping home range (Cagnacci et al., 2011) or multi-range space use throughout the year (Couriot et al., 2018), but with a high degree of spatial fidelity all their life long (Hewison et al., 1998).

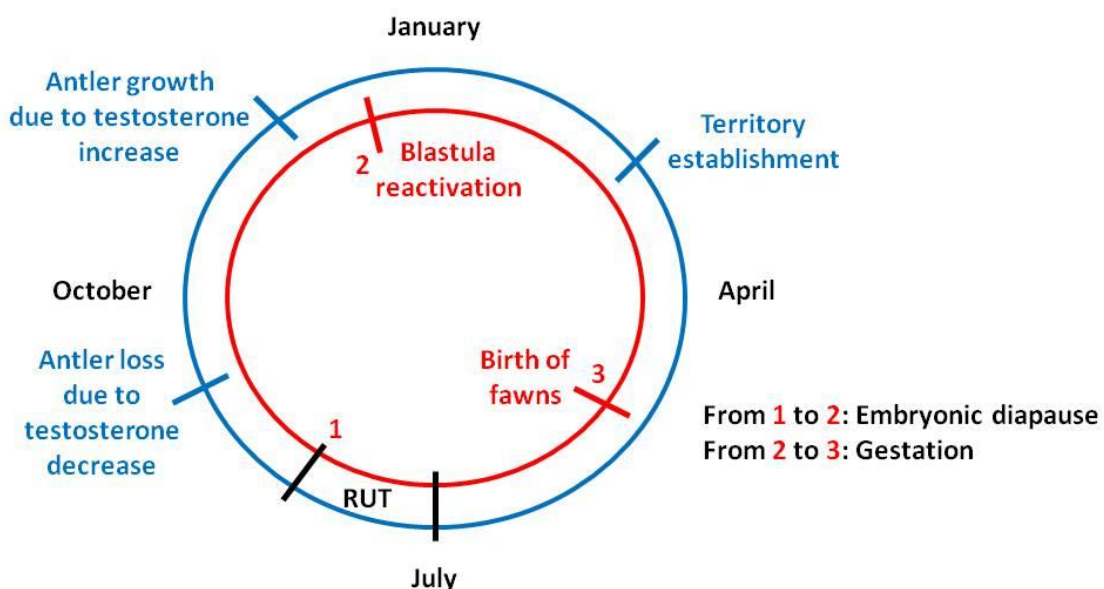


**Figure 9** - A roe deer named Babine feeding in a field of lucerne (*Medicago sativa*) near the village of Fabas.



### Box 5 - Biology of the European roe deer (*Capreolus capreolus*) (2/2)

The rut, or breeding season, occurs between mid-July to mid-August. Males become aggressive and maintain exclusive territories against the other males from March to August. During the rest of the year, males remain solitary or join small groups. Although mating occurs in summer, the fertilised egg does not implant and grow until December or January. This phenomenon is called embryonic diapause and is thought to be an adaptation to avoid giving birth during harsh northern winters (Aitken, 1974). The gestation period is nine months (four months of no embryonic growth, followed by five months of foetal growth) and fawns (between one and three, usually two) are born between the end of April and the month of June. Females usually stay with their fawns during the first summer and until the autumn. Roe deer is a relatively low polygyny species and females usually mate with only one male (Andersen et al., 1998).



**Figure 10** - Biological cycle of roe deer; in blue for males, red for females, black for both.

**Did you know this? – Male or female...**



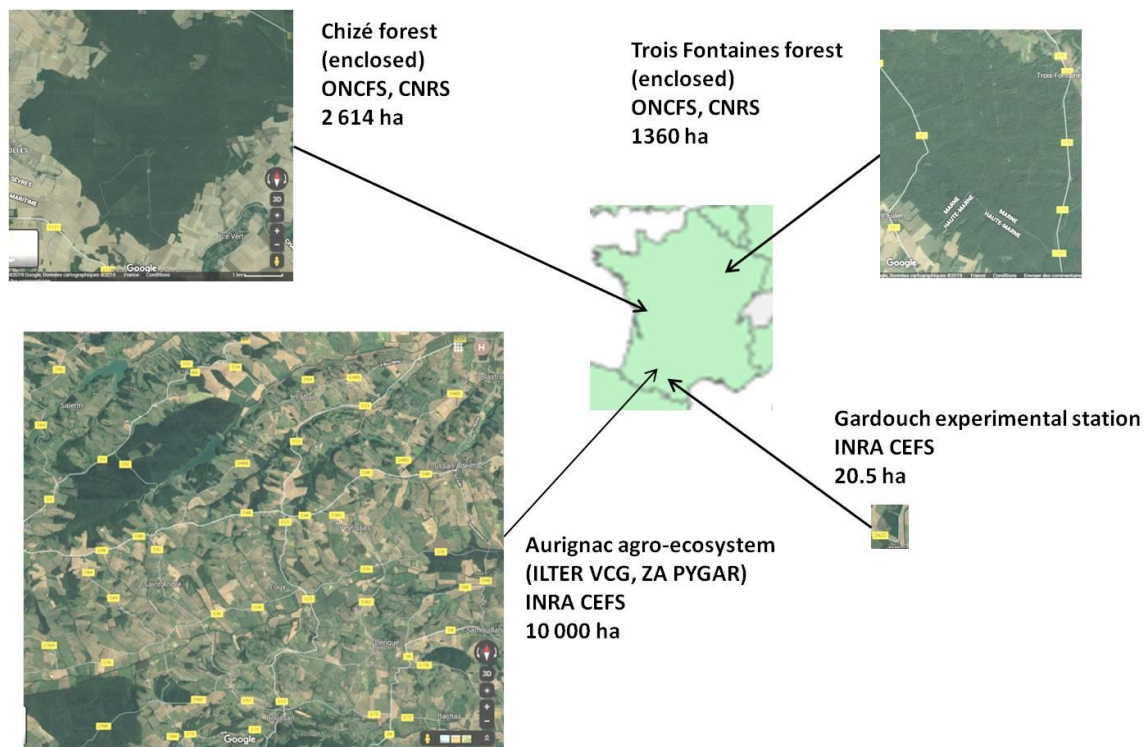
Males (bucks) and females (does) can be distinguished by the presence of antler. In addition, both sexes have a prominent white rump with no tail. But females have a small tuft of hair similar to a tail at the base of the rump patch that makes it look like a heart, while males do not have it, which makes the rump patch looks like a bean.



**Figure 11** - Female (left) and male (right) roe deer, with the typical shape of their rump patch

### Box 6 – Getting to know three wild and one captive population of roe deer... (1/2)

**Aurignac:** The Aurignac population located in the south-west of France (43°13'N, 0°52'E, 10 000 ha) is exposed to an oceanic climate with summer droughts. This area is a designated LTSE (long-term socio-economic and ecological research) and belongs to the Zone Atelier PyGar (Pyrénées Garonne). The area is characterised by a fragmented agricultural landscape mostly covered by open habitats, meadows and cultivated fields (respectively 31% and 36% of the study site), interspersed with small woodland patches (19%) and two large forests (14%; see Martin et al., 2018 for further details). Human presence occurs throughout the site with small villages and farms distributed along an extensive road network of 270 km. Therefore, while meadows and cultivated fields offer abundant and high-quality resources for roe deer (Abbas et al., 2011), they also represent a potential source of stress due to more intense human activities compared to forest habitat (Bonnot et al., 2013). This roe deer population does not face any natural predator, with the exception of the red fox (*Vulpes vulpes*) which exerts predation on fawns. Roe deer may occasionally be preyed by farm dogs. However, this population is hunted on a regular basis by stalking (bucks only) during summer (June-August) and by drive hunts with dogs during autumn-winter (September - February). Roe deer density is about 8 individuals per 100ha in open habitats while it is 8 to 10 times higher in forest.



**Figure 12** - Location and scaled representation of the four populations studied throughout this thesis.



#### **Box 6 – Getting to know three wild and one captive population of roe deer... (2/2)**

**Chizé:** The Chizé population is located in western France (46°05'N, 0°25'W, 2 614 ha), with a temperate oceanic climate and frequent summer droughts. Created in 1970, this integral biological reserve is enclosed and there is no hunting on this roe deer population. However, wild boars are hunted during winter, and roe deer are caught to monitor the population which causes disturbance for roe deer. Forest productivity is low and constitutes an overall poor-quality habitat in terms of food resources (Gaillard et al., 1993). Furthermore, spatial differences exist in vegetation's composition that results in spatial heterogeneity in resources quality (Pettorelli et al., 2003). The study site can therefore be divided into three sectors as follow: sector 1 is of high quality, with oak (*Quercus sp.*) and hornbeam (*Carpinus betulus*) associated with herbaceous species; sector 2 is of intermediate quality, dominated by oak and maple (*Acer sp.*) and sector 3 is of poor quality, mainly composed of beech (*Fagus sylvatica*). This heterogeneity in the vegetation results in a spatial variability in body mass and annual survival rate (Pettorelli et al. 2003; Douhard et al. 2013). The population density is around 14 individuals per 100 ha.

**Trois-Fontaines:** As for the Chizé population, roe deer from this population occur in an enclosed forest. The Trois-Fontaines population is located in north-east of France (48°43'N, 4°55'E, 1 360 ha), with a continental climate characterised by cold winters and warm rainy summers. This population is hunted some part of the year (less than 30 roe deer per year) and capture operations occurs in winter to monitor the population. The forest offers a homogenous habitat principally composed of oak and beech and provides high quality of resources for roe deer (Pettorelli et al., 2006) with high annual survival rate (Douhard et al., 2013). The population density is around 20 individuals per 100 ha.

**Gardouch:** This study site is a research station located in south-west of France, approximately 30 km south-east of Toulouse. The station is located on the slopes of a hill, around 230 meters above sea level. The climate is oceanic with summer droughts, as for Aurignac. The station is owned and managed by the French Institute for Agricultural Research (Institut de la Recherche Agronomique - INRA) and composed of 11 enclosures of 0.5 ha with captive and habituated roe deer of both sexes. In each enclosure, roe deer are supplemented with pellets. Roe deer from these enclosures are mainly used for research on behaviour. In addition, a small population of about 7 roe deer freely roams in a larger enclosure of 15 ha.



# Chapter 1

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## Among-individual differences in baseline stress levels





# **The Neutrophil to Lymphocyte ratio indexes individual variation in the behavioural stress response of wild roe deer across fluctuating environmental conditions**

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## Abstract

Understanding how wild animals adapt to perturbations and their consequences for life history traits and population dynamics is a current focus of attention in ecology and conservation biology. Here, we analysed variation in the neutrophil to lymphocyte ratio (N:L ratio), a proxy of stress level, in wild roe deer *Capreolus capreolus* from three populations experiencing markedly different environmental conditions. We first assessed whether among-individual differences in the N:L ratio were consistent over time and across environmental contexts. We then investigated how both individual characteristics (behaviour at capture, age, sex, body mass), and environmental context (habitat and year quality) were linked to this indicator of stress level. We found moderate, but consistent, repeatability of the N:L ratio in all three populations, indicating stable among-individual differences in the way individuals cope physiologically with varying environmental conditions. In addition, we found a weak, but consistent, association between the N:L ratio and behaviour at capture in two of the three populations. Finally, the N:L ratio also varied in relation to temporal changes in environmental conditions. In particular, individuals had, on average, higher levels of stress in poor quality years in two of the three populations. We discuss our results in relation to the coping styles framework.

## Significance statement

Due to global change, natural populations are increasingly faced with unpredictable fluctuations of their environment. The stress response, via the release of glucocorticoids, is a key mechanism that enables individuals to cope with these variations. However, all individuals do not necessarily cope with life threatening and/or stressful situations in the same way, but

as yet, the major drivers underlying variation in stress level remain unclear. We showed that the N:L ratio, reflecting baseline stress level, was repeatable and influenced by both individual and environmental factors. In particular, variation in the N:L ratio was linked to the quality of the year in terms of resource availability and, to a lesser extent, to the individual's behaviour at capture. Our study demonstrates that both environmental context and individual characteristics drive variation in the N:L ratio in a wild vertebrate population.

## Keywords

stress - *Capreolus capreolus* - N:L ratio - environmental conditions - behaviour

## Introduction

Natural populations are living in a rapidly changing world (Wingfield 2015). Although some sources of environmental variation are regular and predictable (e.g. circadian or seasonal cycles), the majority are more erratic and unpredictable (e.g. extreme climatic events, human disturbance) (Romero & Wingfield 2015). Unpredictable environmental fluctuations have increased markedly due to climate change and the intensification of human activities (Sih et al. 2011; Hooke et al. 2012), and have become major selective forces in natural populations (Wingfield & Romero 2001). Therefore, understanding how wild populations respond to these perturbations and their consequences for life history traits and population dynamics is a focus of increasing attention in ecology and conservation biology.

One of the major axes of adaptation to environmental perturbations is the stress response that involves the stimulation of the Hypothalamic-Pituitary-Adrenal (HPA) axis, leading to the secretion of glucocorticoids into the blood stream (Sapolsky et al. 2000). Consistent among-individual differences in the behavioural and physiological stress response are likely

widespread and can be characterised in terms of a coping style continuum, ranging from reactive to proactive individuals (Koolhaas et al. 1999; Réale et al. 2007). Reactive individuals are typically shy, not aggressive, thorough explorers, and are particularly attentive to modifications of their environment. They are flexible in their behavioural repertoire and exhibit a pronounced stress-induced hormonal response (high reactivity of the HPA axis). Conversely, proactive individuals are bold, aggressive, actively explore their environment and follow routines, exhibiting a weak hormonal response (low reactivity of the HPA axis) to stressful situations (Koolhaas et al. 1999; Sih et al. 2004). These differences in coping styles are often associated with differences in fitness outcomes (Dingemanse & de Goede 2004; Groothuis et al. 2008) which may depend on environmental conditions (Dingemanse et al. 2004; Monestier et al. 2015). The persistence of different coping styles in wild populations could, therefore, be linked to a variable and unpredictable environment. Considering among-individual differences in both physiology and behaviour is therefore important to understand the response of wild animals to environmental perturbations. By combining data on individual characteristics and environmental drivers across a number of years and populations, we explored their potential effects in shaping the physiological stress response of wild animals to environmental perturbations.

Measuring stress in natural populations remains a methodological challenge. Circulating levels of corticosteroids in the blood provide information on the level of stress an individual is exposed to. However, blood sampling procedures often involve stressful events (e.g. capture, restraint, manipulation) that induce a rapid increase in corticosteroids. Unless sampled almost immediately (< 3 minutes, Romero & Reed 2005), levels of glucocorticoids in the blood thus reflect the stress induced by the sampling procedure, but not baseline levels. To overcome this limitation, proxies of stress level that are less rapidly affected by sampling procedures are

required. The leukocyte profile, and particularly the neutrophil to lymphocyte ratio (N:L ratio), or heterophil to lymphocyte ratio (H:L ratio) in birds, has been investigated (Maxwell 1993; Vleck et al. 2000), but few studies have used this approach in free-ranging mammals (but see Monestier et al. 2016; Cohas et al. 2018). Specifically, the use of the leukocyte profile has been shown to be a reliable indicator of the stress response because an increase in glucocorticoids is followed by an increase in the N:L ratio (Davis et al. 2008), provoked by a simultaneous rise in circulating neutrophils and a decline in circulating lymphocytes (Dhabhar 2006). The N:L ratio is considered as a proxy of long-term (chronic) rather than short-term (acute) stress (Davis et al. 2008; Monestier et al. 2016; Cohas et al. 2018), mainly because the N:L ratio is not rapidly impacted following exposure to a stressor (Burguez et al. 1983; Bennett & Harbottle 1968). Typically, the response takes from hours to days, although this time scale is species-dependent. For instance, in the Pyrenean chamois *Rupicapra pyrenaica*, the N:L ratio increases within 1 hour after exposure to a stressor (Lopez-Olvera et al. 2006), whereas it takes around 2 hours in roe deer *Capreolus capreolus* (Montané et al. 2007), 4 hours in horses *Equus ferus caballus* (Burguez et al. 1983) and up to 6 days in the American bull frog *Rana catesbeiana* (Bennett & Harbottle 1968).

Here, we analysed variation in the N:L ratio in roe deer living in three wild populations subjected to markedly different environmental conditions. We first looked for consistent among-individual differences over time and across environmental contexts in baseline stress level. To this end, we measured across year individual repeatability in the N:L ratio in each of the three populations. We then assessed how stress level was associated with individual characteristics (i.e. sex, age, body mass and behaviour at capture) and environmental context within these free-ranging populations. Behaviour at capture was characterised by the degree of struggle at capture and during manipulation, as well as escape speed at release. Moreover,

this behavioural score at capture has previously been validated as an index of the proactive-reactive gradient in roe deer, with the behavioural stress response being linked to physiological parameters such as temperature, microhematocrit levels and the N:L ratio (Monestier et al. 2016). As previous studies have suggested that stress-induced and baseline levels of glucocorticoids are negatively related to the reactive-proactive gradient (Cockrem 2007; Montiglio et al. 2012), we predicted that individuals exhibiting a less pronounced behavioural response to capture would have a higher N:L ratio than individuals exhibiting a more pronounced behavioural response. Finally, because poor habitat quality is a source of stress (Marra & Holberton 1998; Fodikis et al. 2012), we predicted that roe deer living in poorer and /or more unpredictable quality habitats would exhibit a higher N:L ratio.

## **Material and methods**

### **Study sites**

This study was carried out on three wild populations of roe deer living in markedly different habitats. The Aurignac population located in the south-west of France (43°13'N, 0°52'E, 7500 ha) is exposed to an oceanic climate with summer droughts. It is a fragmented agricultural landscape mostly covered by open habitats interspersed with small woodland patches and two large forests. Meadows and cultivated fields occupy a high proportion of the study site (see Hewison et al. 2009 for further details) and offer abundant high quality food for roe deer (Abbas et al. 2011), but may be a potential source of stress due to more intense human activities compared to forest habitat (Bonnot et al. 2013). This roe deer population is hunted on a regular basis by stalking (bucks only) during summer (June-August) and by drive hunts with dogs during autumn-winter (September - January). The two other populations occur in enclosed forests. The Trois-Fontaines population is located in north-eastern France (48°43'N,



4°55'E, 1.360 ha), with a continental climate characterized by cold winters and warm rainy summers and constitutes a productive forest habitat. Roe deer are occasionally hunted in this population (less than 30 individuals per year). The Chizé population is located in western France (46°50'N, 0°25'W, 2.614 ha), with a temperate oceanic climate, frequent summer droughts and constitutes a poor quality habitat in terms of food resources for roe deer (Gaillard et al. 1993). Roe deer are not hunted in this population. In all sites, wild boars are hunted during winter, which could induce disturbance for roe deer.

In Aurignac, the six capture sites can be assigned to three sectors according to landscape openness (see Morellet et al. 2009 for more details) which vary in terms of habitat quality and potential exposure to stressors (sector 1: highly fragmented woodland with cultivated crops that provide high quality food resources compared to the more wooded sectors, sector 2: partially wooded area, sector 3: woodland). This variation in habitat quality translates into differences in juvenile body mass: juveniles in sector 3 weighed on average, 2.0 kg (SE=0.59) less than in sector 2 and 3.1 kg (SE=0.43) less than in sector 1 (Hewison et al. 2009). In Chizé, the forest also comprises three sectors that differ in terms of habitat quality (Pettorelli et al. 2001): sector 1 is of high quality, with oak and hornbeam associated with herbaceous species; sector 2 is of intermediate quality, dominated by oak and maple and sector 3 is of poor quality, mainly composed of beech. Juveniles of both sexes were 2 kg heavier in sector 1 compared to sector 3 (Pettorelli et al. 2003). Trois-Fontaines is a spatially homogenous forest in terms of habitat quality and/or exposure to stressors. The quality of the year (thereafter year quality) in terms of resource availability for a given population was estimated using mean body mass of fawns born in a given year and captured during the following winter (Pettorelli et al. 2003, Douhard et al. 2013).

## Field data collection

As part of long-term Capture-Mark-Recapture programs initiated in 1996 in Aurignac, in 1977 at Chizé, and in 1975 at Trois-Fontaines, 6 to 12 days of capture are organised between December and March each year. We used large-scale drives with 30 to 100 beaters (and dogs in Chizé and Trois-Fontaines) and up to 4 km of long-nets. Once a deer was captured, it was removed from the net and transferred to a wooden retention box providing darkness and ventilation until the marking procedure. In Aurignac, roe deer were tranquilized immediately following capture by intramuscular injection of acepromazine (Calmivet, Vetoquinol, France; targeted dose of 0.075 mg/kg).

Marking procedures lasted for approximately 15 minutes and were performed by experienced handlers. Each individual was weighed (to the nearest 10 g at Aurignac and 50 g at Chizé and Trois-Fontaines) and sex and age were recorded. In Aurignac, individuals were assigned to one of two age classes (juveniles for 8-10 month-old individuals vs. adults for >18 month-old individuals) based on tooth eruption patterns (Hewison et al. 1999). In Chizé and Trois-Fontaines, age in years was known for most individuals because they were first caught within their first year of life.

Blood samples have been collected since 2009 at Aurignac (9 years of collection) and 2010 at Chizé and Trois-Fontaines (8 years of collection) from the jugular vein (up to 20 mL for a 20 kg roe deer). Whole blood was EDTA-preserved at 4°C. Concentrations of total leukocytes (white blood cell [WBC] count of complete blood count) were determined at the Clinical Pathology laboratory (Biovelys) of VetAgro-Sup for Chizé and Trois-Fontaines, and at Ecole Nationale Vétérinaire de Toulouse (ENVT) for Aurignac. The two laboratories used the same type of automat (Sysmex 2000iV, Sysmex). To obtain percentages of each leukocyte type, a

differential cell count was performed on the first 100 WBCs on Wright-Giemsa-stained blood smears by trained technicians (Houwen 2001). A possible drawback of using the N:L ratio to measure stress level is that the leukocyte profile may be affected by immune activation (Davis et al. 2008; Romero & Wingfield 2015). Hence, we examined other haematological parameters (e.g. concentrations of monocytes and total WBC) to distinguish between unhealthy individuals undergoing an immune response and healthy individuals expressing a stress response (Davis et al. 2008). No individual presented abnormally elevated values of neutrophils, lymphocytes, monocytes or total WBC, therefore, no individual was removed from our dataset.

### **Behavioural assessment**

Behaviour at capture, during handling and at release has been recorded since 2009 in Aurignac (9 years of collection) and since 2015 in Chizé and Trois-Fontaines (3 years of collection). In Aurignac, we attributed a score of 1 (presence) or 0 (absence) for the following items: 1) the roe deer turned upside down in the box, 2) the roe deer attempted to remove its collar at release, 3) the roe deer struggled in the net (here we attributed a score of 1 for a high level of struggle, 0.5 for medium struggle, and 0 for low struggle), 4) the roe deer struggled and panted on the table during handling (1 for both, 0.5 for struggling only, 0 otherwise), and 5) the roe deer ran at release (1 for high speed running, 0.5 for moderate speed, 0 for trotting). An index of proactivity was then calculated as the mean score for these five behavioural items. The resulting value ranged from 0 to 1, with 1 indicating highly proactive individuals (Monestier et al. 2016). This index has been shown to be highly repeatable ( $r = 0.47$ , 95% confidence interval = [0.24, 0.66]) (Bonnot et al. 2018) and has previously been used to index among-individual behavioural differences in the Aurignac roe deer population (Bonnot et al. 2015; Debeffe et

al. 2014; Monestier et al. 2015). In Chizé and Trois-Fontaines, behaviour at release was recorded as follows: a score of 0 for slow escape with several stops, 1 for runs away, but stops after a short distance, 2 for runs away without stopping until out of sight, 3 for fell, jumped and attempted to remove its collar at release and 4 for unable to stay upright. Due to the small number of individuals with scores of 3 or 4 in these two populations, we pooled them in order to have three representative categories for further analyses (i.e. 0, 1 and 2 or more). This trait was also similarly repeatable in Chizé:  $r = 0.40$ , 95% confidence interval = [0.12, 0.37],  $P$  value  $< 0.05$  and Trois-Fontaines:  $r = 0.32$ , 95% confidence interval = [0.13, 0.34],  $P$  value  $< 0.05$ .

The time elapsed between capture and blood sampling depended on when the deer were caught (morning vs. afternoon), the number of roe deer to be processed, and the total duration of capture operations. Given that the N:L ratio may be affected by capture after a certain amount of time, estimated at 2 hours in roe deer (Montané et al. 2007), we expected the delay between capture and blood sampling to influence the observed level of the stress response. More precisely, we expected N:L levels to be higher in individuals where the delay between capture and blood sampling was close or longer than 2 hours. Therefore, time at capture and at handling were recorded for each individual in Aurignac and Chizé so that we could account for this delay. In Trois-Fontaines, the exact time at capture and handling was not reported, but was estimated using the median capture time (10:30 am for animals caught in the morning or 2:30 pm for those caught in the afternoon), and the estimated time of manipulation (starting at 4:00 pm and incremented by 15 min using the order of handling for all roe deer).

## **Statistical analyses**

### **Repeatability of the N:L ratio**

Individual repeatability of the N:L ratio was estimated using mixed models (Nakagawa & Schielzeth 2010). Repeatability was estimated as the ratio of among-individual variance to total variance with linear mixed-effects models (with individual identity as a random factor) using the restricted maximum likelihood (REML) method, using the “rptR” package (Stoerckel et al. 2017) for Gaussian distributions. For each population, we calculated “agreement repeatability” and “adjusted repeatability”, the latter included the fixed effect of the delay between capture and blood sampling in the linear mixed models. To avoid including the among-year variance in the among-individual variance component, we included the year of capture as a random effect on the intercept. Individuals sampled only once were included in these analyses as this procedure may improve power to estimate among-individual variance, so avoiding biased results (Martin et al. 2011).

### **Variability of the N:L ratio**

To assess variation in the N:L ratio, analyses were performed using linear mixed-effect models (LMMs). The N:L ratio was analysed as the response variable and transformed as  $\log(x+1)$  to achieve normality of model residuals. We built a reference model which included all pertinent variables to test our predictions on the effect of individual characteristics (age, sex, body mass, behaviour at capture) and environmental quality (sector and year quality). The reference model also included the time delay between capture and blood sampling to account for this effect on the N:L ratio, plus the two-way interactions between age and time delay and between age and sex. These interactions were included to account for i) a possible effect of age on the influence of the time delay between capture and blood sampling as the efficiency of the stress response can be impaired with age (Romero et al. 2009), and ii) to account for a possible between-sex differences in age-dependence of the N:L ratio that may occur with

physiological traits such as the neutrophil concentration (Cheynel et al. 2017). Our set of candidate models included the reference model and all simpler sub-models. Body mass was standardised by age and sex. Individual identity and year of capture were included as random effects to avoid pseudo-replication issues (Hurlbert 1984) and to control for unexplained variance due to among-individual differences and inter-annual variation. Date of capture was also included as a random effect to take into account the possible effect of particular uncontrolled events that could have occurred on the day of capture (e.g. weather events, number of persons during animal manipulation).

To select the best model describing variation in the N:L ratio, we used a model selection procedure based on the Akaike Information Criterion (AIC, Burnham & Anderson 2002). We considered all the most parsimonious competing models within 2 AIC units of the model with the lowest AIC (Arnold 2010; Burnham and Anderson 2002:87-88), averaged parameter estimates and calculated importance score (w+) for each variable. Importance scores were calculated by summing the Akaike weights (AICw) across all the models in the set in which a given variable features (Burnham & Anderson 2002:167-169). The normality of the residuals was tested (Shapiro-Wilk test) and visually assessed with histograms. Goodness-of-fit was assessed through calculating conditional (i.e. total variance explained by the best supported model) and marginal (i.e. variance explained by the fixed effects alone)  $R^2$  values (Table 1) and standard residual plot techniques (Nakagawa & Schielzieth 2013). All analyses were carried out in R version 3.2.5 (R Development Core Team 2016) using the function lmer in the lme4 package (Bates et al. 2014) and model.avg function in the MuMIn package (Barton 2016).

## **Data Availability**

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

## Results

### Repeatability of the N:L ratio

Analyses were conducted on 395 (Aurignac, 9 years), 470 (Chizé, 8 years) and 506 (Trois-Fontaines, 8 years) observations and concerned 321, 275 and 259 individuals for Aurignac, Chizé and Trois-Fontaines, with a mean of 1.23, 1.88 and 1.95 repetitions per individual, respectively. In each of the three studied populations, the N:L ratio was moderately repeatable (Aurignac:  $r = 0.299$ , 95% confidence interval = [0.093, 0.503],  $P$  value = 0.004 ; Chizé:  $r = 0.244$ , 95% confidence interval = [0.123, 0.367],  $P$  value < 0.001 ; Trois-Fontaines:  $r = 0.228$ , 95% confidence interval = [0.131, 0.339],  $P$  value < 0.001). When corrected for the delay between capture and blood sampling, the N:L ratio was still similarly repeatable (Aurignac:  $r = 0.275$ , 95% confidence interval = [0.055, 0.475],  $P$  value < 0.05 ; Chizé:  $r = 0.263$ , 95% confidence interval = [0.141, 0.388],  $P$  value < 0.001 ; Trois-Fontaines:  $r = 0.278$ , 95% confidence interval = [0.176, 0.4],  $P$  value < 0.001).

### Variability of the N:L ratio

Analyses were conducted on 361 (Aurignac, 9 years), 164 (Chizé, 3 years) and 178 (Trois-Fontaines, 3 years) observations. Based on cumulative AICc weights, age and the time elapsed between capture and blood sampling (delay) were the most important parameters influencing the N:L ratio ( $w_+ = 1.00$ ) in the three studied populations. The N:L ratio increased linearly with the time delay between capture and blood sampling (Table 1 ; Figure 1) and with age, although this effect was less marked in Aurignac and Trois-Fontaines than in Chizé (Figure 2). Males also

exhibited a more rapid increase in N:L ratio with age than females in Chizé and a similar trend was observed in Aurignac (Table 1). In addition, in both Trois-Fontaines and Aurignac, the N:L ratio decreased as the quality of the year increased (Table 1 ; Figure 3), with a similar trend in Chizé, although the importance score was smaller ( $w_+ = 0.43$ ). The N:L ratio was affected by spatial variation in habitat quality in the Chizé population only such that roe deer living in the poorest part of the forest (sector 3) had a lower N:L ratio than those in the other two sectors (Table 1). Behaviour at capture also featured in the selected models in both Aurignac and Chizé ( $w_+ = 1.00$ ) such that the N:L ratio decreased with increasing values of the behavioural score. However, this relationship was not linear in Chizé, with a slight increase in N:L ratio between behavioural scores of 0 to 1, and a decrease between 1 and higher values of behavioural score (Table 1 ; Figure 4). Finally, there was some indication that body mass was positively correlated with the N:L ratio in Trois-Fontaines ( $w_+ = 0.62$ , Table 1).

## Discussion

### Stable among-individual differences in the stress level

Our first aim was to assess among-individual differences over time and across varying environmental contexts in the stress level of wild, free ranging roe deer using the neutrophil to lymphocyte ratio. Compared to the meta-analysis by Bell and colleagues (2009) on behavioural traits, we found that the N:L ratio was moderately repeatable across years, with an average value around 0.26 against 0.37 in Bell and colleagues' analysis, indicating a certain degree of consistency in among-individual differences in this species. This result is coherent with two recent meta-analyses that estimated an overall repeatability of  $R \sim 0.29$  for glucocorticoid levels (Taff et al. 2018), with a somewhat lower repeatability for baseline



( $R=0.23$ ) than stress-induced ( $R=0.39$ ) glucocorticoid levels across studies (Schoenemann & Bonier 2018). In the only other roe deer study performed in that context, Monestier and colleagues (2016) reported a quite high repeatability in the N:L ratio ( $R = 0.46$ ) of captive animals sampled every two months over a single year. Repeatability in the N:L ratio is likely to be higher when samples are collected over shorter time intervals, as previously reported for glucocorticoid levels (Schoenemann & Bonier 2018). Hence, the longer sampling interval in our study may explain the lower repeatability we found. In addition, captivity provides a more controlled and predictable environment which may limit fluctuations in stress levels compared to natural populations.

The moderate level of repeatability in the N:L ratio of roe deer suggests that a single measurement per year may be insufficient to predict the stress response of an individual from one year to another, or over a longer time period (Niemelä & Dingemanse 2018). However, the finding that the repeatability in the N:L ratio was consistent among the three studied populations suggests that among-individual differences for coping with environmental constraints have either been conserved, or generated independently, to approximately the same degree in the three populations. Roe deer are sedentary and exhibit high fidelity to their home range (Hewison et al. 1998). Hence, they are likely to be exposed to local sources of stress that are stable from year to year, such as hunting and human disturbance, and to have similarly stable access to refuge, which may contribute to the repeatability of the stress level indexed by the N:L ratio. Repeatability in stress level could also imply that these among-individual differences have a genetic basis (Groothuis & Carere 2005; Stedman et al. 2017), so that the stress response could be under selection. Although preliminary results tend to suggest a genetic basis for individual differences in the stress response (Jenkins et al. 2014;

Stedman et al. 2017), studies on the heritability of the glucocorticoid metabolism are in their infancy and more work needs to be conducted in this area.

### **Phenotypic and environmental drivers of the stress level**

Our second aim was to obtain a better understanding of the drivers of variation in the N:L ratio as an index of stress levels in natural populations. Our results demonstrate that a combination of methodological, environmental, and individual factors may all affect this index. From previous work on several species, we can assume that the N:L ratio reflects baseline, rather than stress-induced, levels of glucocorticoids (Davis et al. 2008; Vleck et al. 2000). However, as it is the case for glucocorticoids, it was important to account for the time delay between the initiation of capture-induced stress and blood sampling. In line with previous work on roe deer (Montané et al. 2007), we found a linear increase in the N:L ratio with this time delay in all three populations. Together, these results strongly suggest that the N:L ratio not only provides information regarding the baseline stress level of an individual, but may also be an informative indicator of its stress response to capture. We emphasise that ignoring the time elapsed between capture and blood sampling in analyses of the N:L ratio may lead to an erroneous interpretation of lower stress levels for animals which are sampled immediately after capture compared to those sampled later.

To identify how environmental conditions impact the level of stress indexed by the N:L ratio, we exploited both spatial (i.e. sectors) and temporal (i.e. year quality) variations in habitat quality within the study populations. As expected, we found that the N:L ratio was higher in poor years, characterized by low fawn body mass, in both the Aurignac and Trois-Fontaines populations. This effect of the environment in terms of year quality contrasts with the absence of a clear effect of spatial variation in habitat quality, despite marked within-site

heterogeneity, especially in Aurignac where juveniles in the best habitat weighed up to 3 kg more than those in the poorest habitat (Hewison et al. 2009). Indeed, juvenile body mass is a reliable proxy of conditions experienced by a given cohort (Gaillard et al. 1996), and has been shown to affect the subsequent performance of individuals (Pettorelli et al. 2002, Douhard et al. 2013). Surprisingly, the N:L ratio was lowest in the poorest quality habitat (sector 3) in the Chizé population. As suggested by Escribano-Avila and colleagues (2013), animals living in poor habitat may favour allocation to immune function rather than reproduction to maximize fitness. This hypothesis implies that the stress level should be attenuated in poor habitats to avoid impairing the immune function (Davis & Maney 2018). Overall, in accordance with our predictions and previous studies, our results suggest that roe deer are able to adjust to their local spatial environment over the long term, but are less easily able to do so to temporal and unpredictable variations in habitat quality. Indeed, the quantity and quality of resources in the environment are known to act as ecological stressors that influence the level of stress in vertebrates (Fodikis et al. 2012). Numerous studies have reported elevated stress levels in individuals exposed to poor food availability in some years (Hellgren et al. 1993), adverse weather conditions (Huber et al. 2003), or poor overall habitat quality (Marra & Holberton 1998).

Once we had accounted for methodological and environmental factors, we found that the N:L ratio was also related to individual phenotype. First, compared to females, males exhibited an increase in the N:L ratio with age in Chizé, with a similar trend in Aurignac, suggesting that males have higher stress levels in these populations. The degree of sex-specific variation in stress levels differs across species (Touma & Palme 2005). Studies on Steller sea lion *Eumetopias jubatus* or domestic chicken *Gallus gallus* showed higher faecal glucocorticoids in males compared to females (Hunt et al. 2004; Rettenbacher et al. 2004), while other studies

on red deer *Cervus elaphus*, caribou *Rangifer tarandus granti* and reindeer *Rangifer tarandus tarandus*, found no difference between the sexes in faecal and hair glucocorticoids (Huber et al. 2003; Ashley et al. 2011). However, sex-differences in N:L levels in roe deer, may also be due to differential immune allocation between males and females, with a higher concentration of neutrophils in males compared to females but similar concentrations of lymphocytes (Cheynel et al. 2017). In all three populations, there was a trend for the N:L ratio to increase with age. While several studies have demonstrated that age can influence the level of glucocorticoids, there is no consensus about the nature of this relationship. Our findings are in accordance with the hypothesis of an increase in the baseline stress level with age, as observed in humans (Bauer 2005). However, one may again expect that neutrophil and lymphocyte concentrations vary with age differently as a result of immunosenescence. Indeed, in two of the same roe deer populations (Chizé and Trois-Fontaines), Cheynel and colleagues (2017) found an increase in neutrophils with age in females but not males, while the pattern was less clear for lymphocytes. Further studies of baseline levels in stress hormones are needed to clarify the role of immune function in generating age- and sex-specific variation in the physiological stress level.

Finally, we found a slight increase in the N:L ratio as the behavioural score decreased in two of the three study populations (Aurignac & Chizé), although the relationship was less marked in Chizé. A possible explanation for the small effect size in Chizé and for the lack of relationship between behaviour at capture and the N:L ratio in Trois-Fontaines could be due to differences in the ecological context faced by roe deer among the three study populations. In particular, pathogen exposure markedly differed among populations. In the fragmented landscape of Aurignac roe deer are sympatric with domestic stock, leading them to be exposed to a more diverse and abundant pathogen reservoir (Candela et al. 2014; Sevilla et al. 2014; Chastagner

et al. 2017) than in the enclosed forests of Chizé and Trois-Fontaines, where parasite diversity and abundance is much lower (Gilot et al. 2012, Gotteland et al. 2014). Because the N:L ratio is influenced by pathogen exposure (Minias 2019), different exposure among populations might have influenced the link between the N:L ratio and the behavioural response to stressful situations. However, specific mechanisms underlying the link between pathogen exposure, immune levels and behaviour are currently unknown and further investigation is required to test this hypothesis. Our relationship between a physiological and a behavioural trait could suggest a gradient of coping styles in these wild populations, in line with previous laboratory (Koolhaas et al. 1999) and field (Montiglio et al. 2012; Montiglio et al. 2014) studies. However, an alternative explanation for the relatively moderate effects we report could be a degree of independence between the physiological (HPA axis) and behavioural responses to a stressful event. This hypothesis was proposed by Koolhaas and collaborators in their more recent two-tier model of coping styles (Koolhaas et al. 2010). They suggested that the behavioural response that is primarily linked to the sympathetic stress response (release of catecholamines) is independent from the HPA axis activity (release of glucocorticoids) which represents “stress reactivity”. Although recent studies seem to support this alternative view of coping styles in wild populations (Qu et al. 2018; Westrick et al. 2018), further work is needed to assess the importance of these two models in the wild and the individual-specific link between physiological and behavioural traits in response to stressful events. Stress influences numerous individual traits, such as immunity and parasitism for instance. Further work is now needed to investigate the fitness consequences of different individual strategies for coping with environmental perturbations in various environments.

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## **Compliance with ethical standards**

### **Conflict of interest**

The authors declare that they have no conflict of interest.

### **Ethical approval**

All applicable institutional and/or national guidelines for the care and use of animals were followed. For Trois-Fontaines and Chizé populations, the protocol of capture and blood

sampling of roe deer under the authority of the Office National de la Chasse et de la Faune Sauvage (ONCFS) was approved by the Director of Food, Agriculture and Forest (Prefectoral order 2009-14 from Paris). All procedures were approved by the Ethical Committee of Lyon 1 University (project DR2014-09, June 5, 2014). For the Aurignac population, the study was permitted by the land manager (hunting groups and farmers) and the prefecture of the Haute Garonne. All procedures were approved by the Ethical Committee 115 of Toulouse and were authorized by the French government (APAFIS#7880-2016120209523619\_v5).

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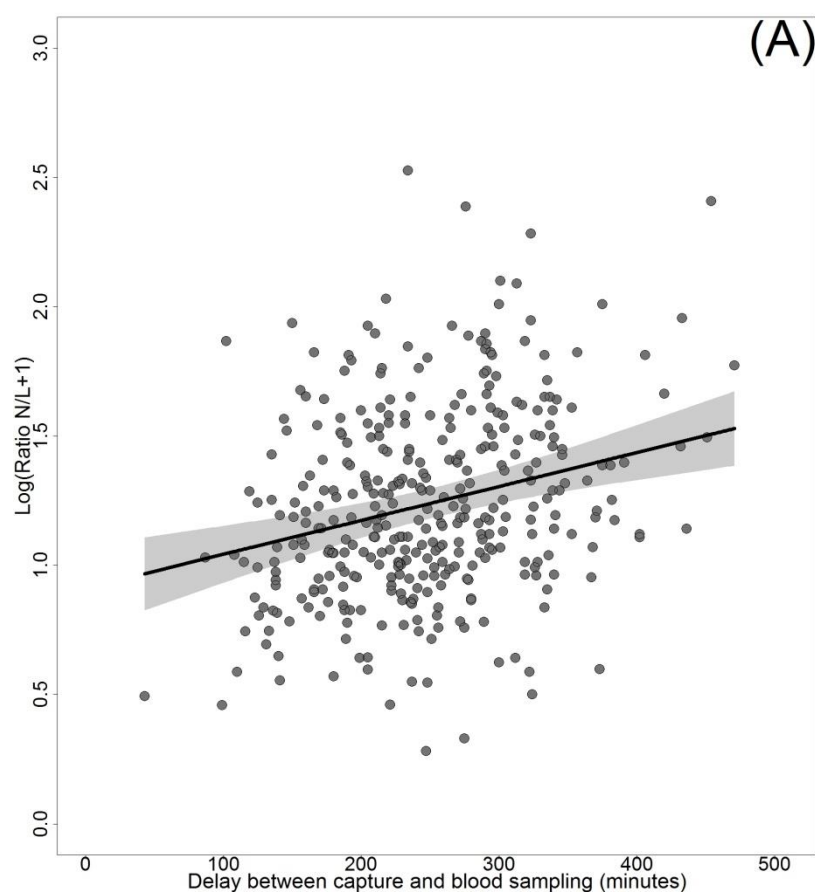
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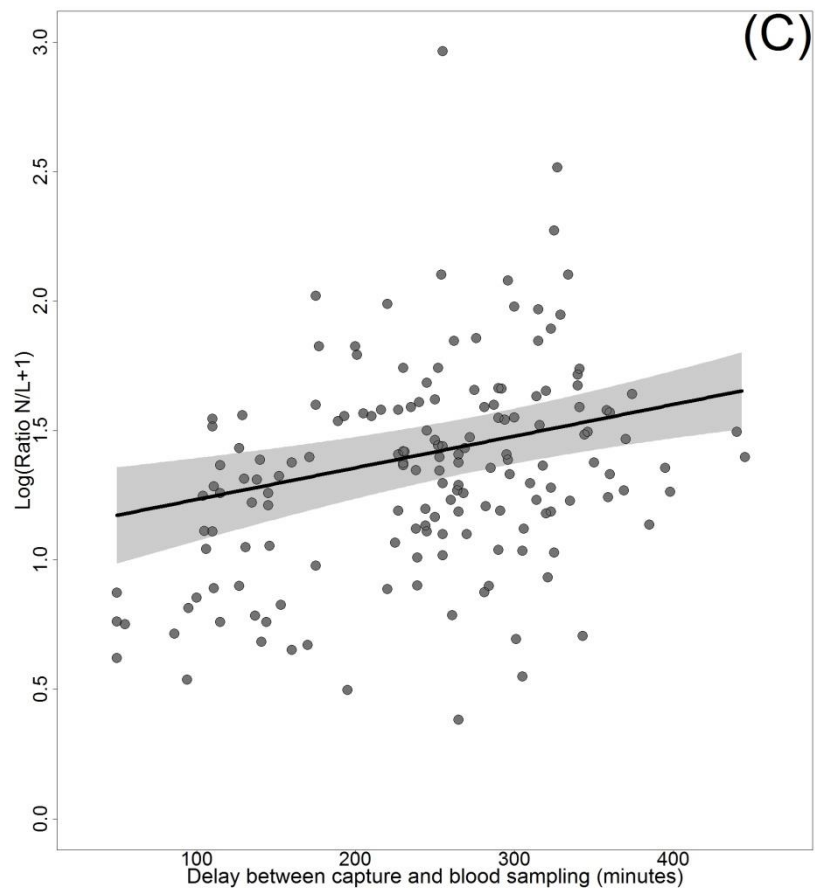
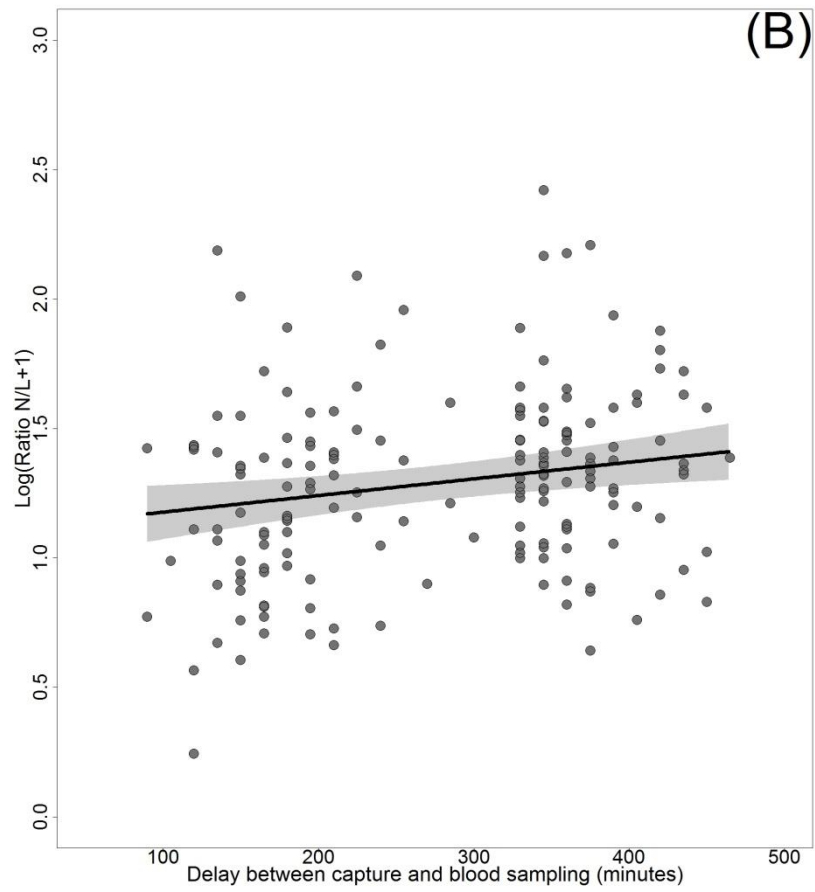
| Parameter   | w <sub>+</sub> | Estimate   | CI                 |
|---|----------------|------------|--------------------|
| <b>Aurignac</b><br>(R <sup>2m</sup> :0.17 ; R <sup>2c</sup> :0.40)        |                |            |                    |
| Intercept   |                | 2.161      | 1.1877 to 3.1343   |
| Age (juveniles)   | 1.00           | -0.286     | -0.6170 to 0.0459  |
| Delay   | 1.00           | 0.001      | 0.0007 to 0.0019   |
| Behaviour   | 1.00           | -0.225     | -0.4314 to -0.0189 |
| Year quality  | 1.00           | -0.068     | -0.1228 to -0.0140 |
| Sex (Males)   | 1.00           | 0.121      | 0.0261 to 0.2162   |
| Age (juveniles)*Delay   | 0.67           | 0.001      | -0.00005 to 0.0022 |
| Age (juveniles)*Sex (males)   | 0.54           | -0.125     | -0.2796 to 0.0298  |
| <b>Chizé</b><br>(R <sup>2m</sup> :0.36 ; R <sup>2c</sup> :0.40)           |                |            |                    |
| Intercept   |                | 1.535      | -0.5167 to 3.5872  |
| Age   | 1.00           | 0.025      | 0.0057 to 0.0447   |
| Delay   | 1.00           | 0.001      | 0.0006 to 0.0019   |
| Behaviour 1   | 1.00           | 0.107      | -0.0253 to 0.2400  |
| Behaviour 2   |                | -0.108     | -0.2449 to 0.0284  |
| Year quality  | 0.43           | -0.114     | -0.2750 to 0.0469  |
| Sex (Males)   | 1.00           | -0.030     | -0.2097 to 0.1488  |
| Sector 2  | 1.00           | 0.025      | -0.1105 to 0.1608  |
| Sector 3  |                | -0.220     | -0.3864 to -0.0547 |
| Age*Sex (males)   | 1.00           | 0.042      | 0.0061 to 0.0774   |
| <b>Trois-Fontaines</b><br>(R <sup>2m</sup> :0.16 ; R <sup>2c</sup> :0.61) |                |            |                    |
| Intercept   |                | 2.663      | 1.1018 to 4.2233   |
| Age   | 1.00           | 0.036      | -0.0039 to 0.0762  |
| Delay   | 1.00           | 0.0009     | 0.0001 to 0.0016   |
| Year quality  | 1.00           | -0.104     | -0.1956 to -0.0117 |
| Body mass   | 0.62           | 0.023      | -0.0016 to 0.0468  |
| Age*Delay   | 0.49           | -9.620e-05 | -0.0002 to 0.00003 |

**Table 1** Linear mixed effect models resulting from the model averaging procedure of models within a  $\Delta AIC_c < 2$  of the best model for explaining variation in the Neutrophil:Lymphocyte ratio in the three roe deer populations (Aurignac, Chizé, Trois-Fontaines). The effects of age, time delay between capture and blood sampling (Delay), behaviour at capture (Behaviour), year quality, body mass, sex, sector (only for Aurignac and Chizé), and the two-way interactions between age and sex, and age and time delay were included in the full model. All models included individual identity, year of capture, and day of capture as random effects. R<sup>2m</sup> and R<sup>2c</sup> are the marginal and conditional estimates for explained variance of the model, respectively. W<sub>+</sub> stands for importance scores of variables and is calculated by summing the

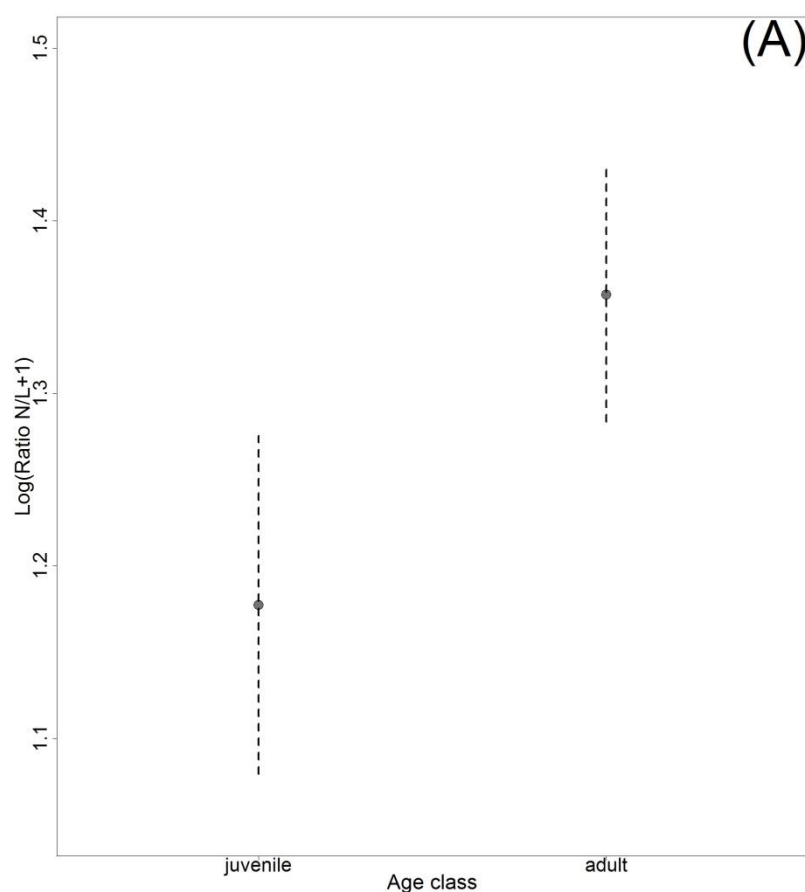
Akaike weights across all the models including the target variable. CI stands for Confidence Interval. CI values that do not overlap 0 are presented in *italics*.

**Fig. 1** Relationship between the Neutrophil:Lymphocyte ratio and the time delay between capture and blood sampling in the roe deer populations of Aurignac (A), Trois-Fontaines (B) and Chizé (C). Points represent observed values. Lines represent model predictions. Shaded areas represent the 95% confidence intervals

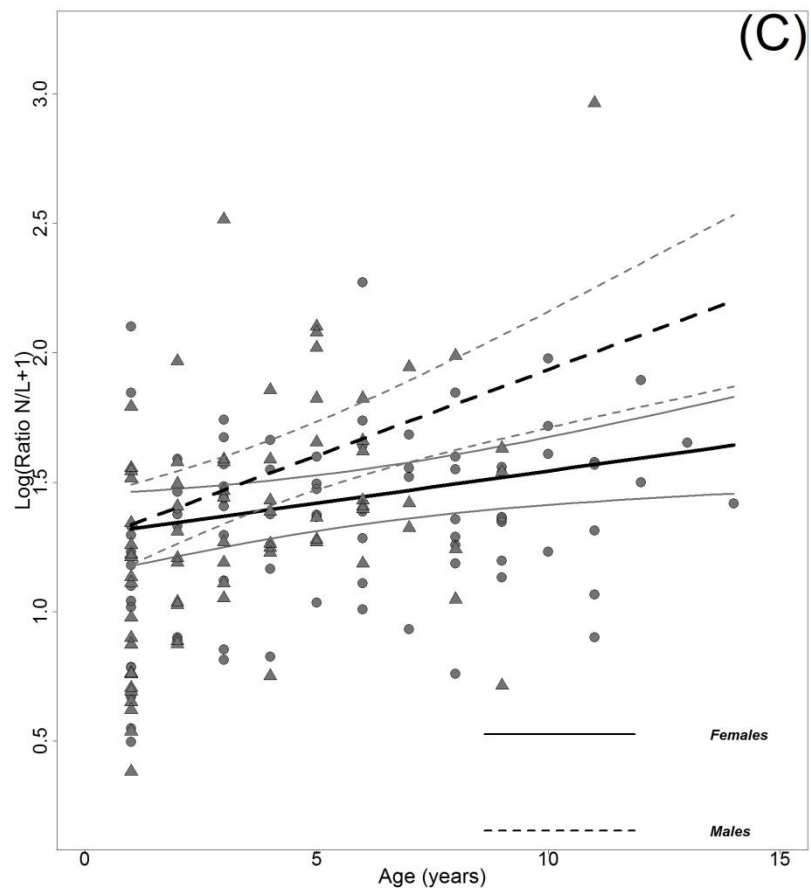
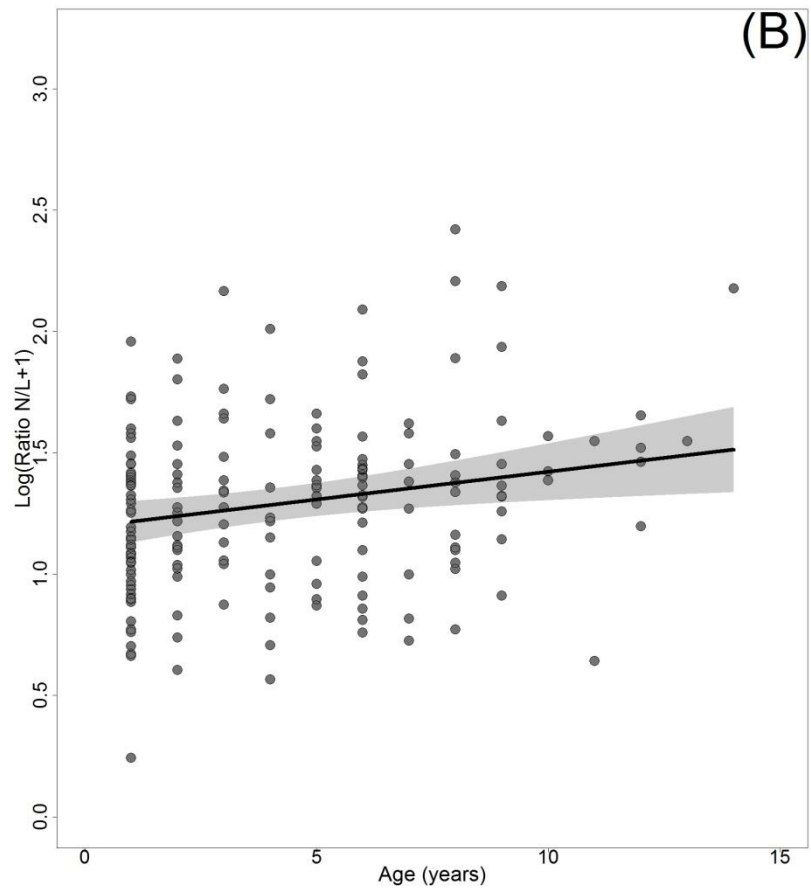




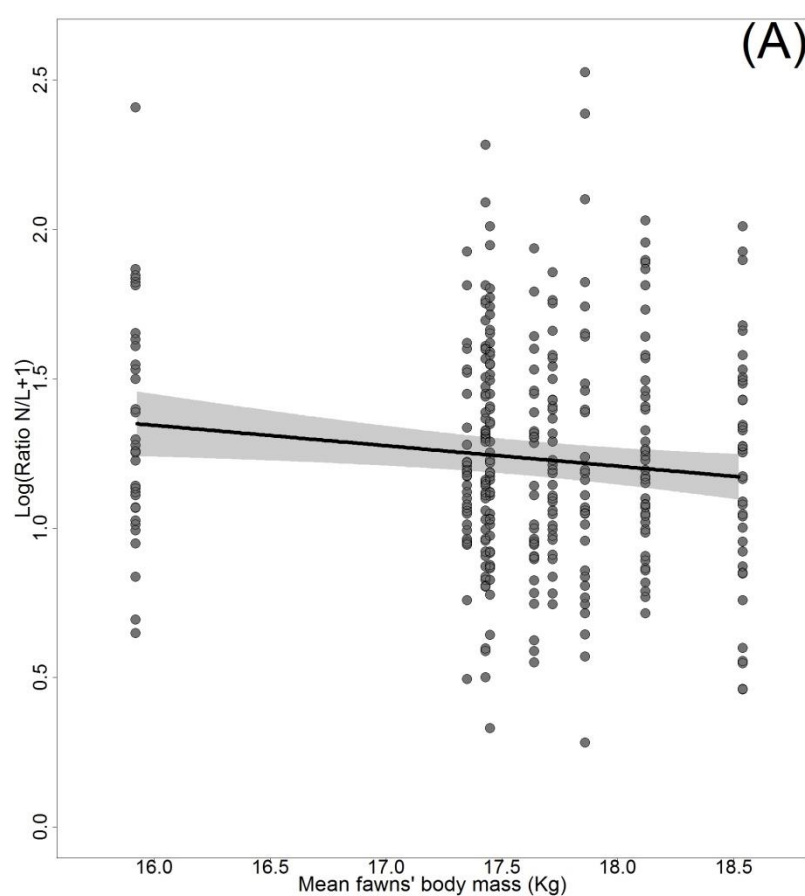
**Fig. 2** Relationship between the Neutrophil:Lymphocyte ratio and age in the roe deer populations of Aurignac (A), Trois-Fontaines (B) and Chizé (C). Age is expressed as two classes in Aurignac with juveniles (8-10 month old) and adults (more than 18 month old). Points represent observed values. In Figure 2B, males are indicated with triangles and females with circles. Lines represent model predictions and shaded areas (figures A & B) or dashed lines (figure C) represent the 95% confidence intervals for Chizé and Trois-Fontaines. The points represent model predictions and the bars represent the 95% confidence intervals for Aurignac

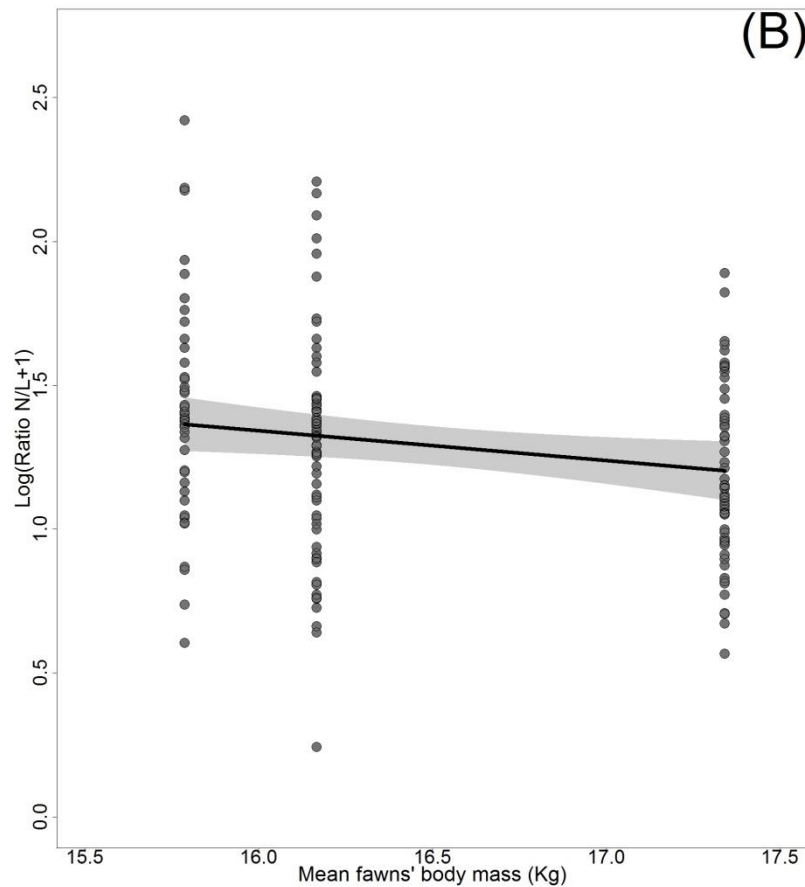




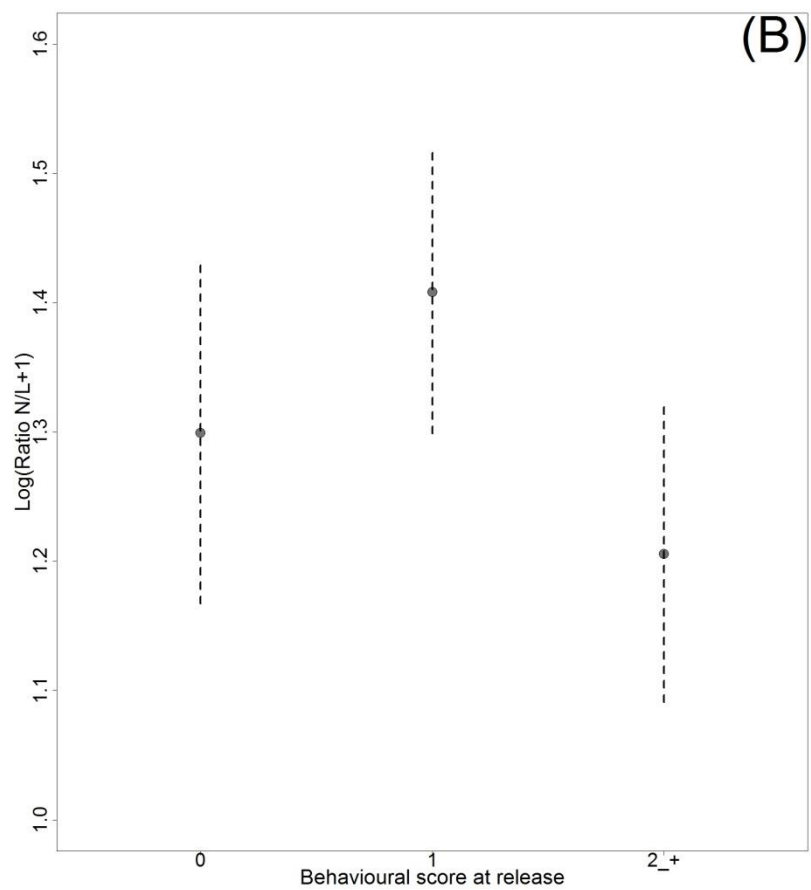
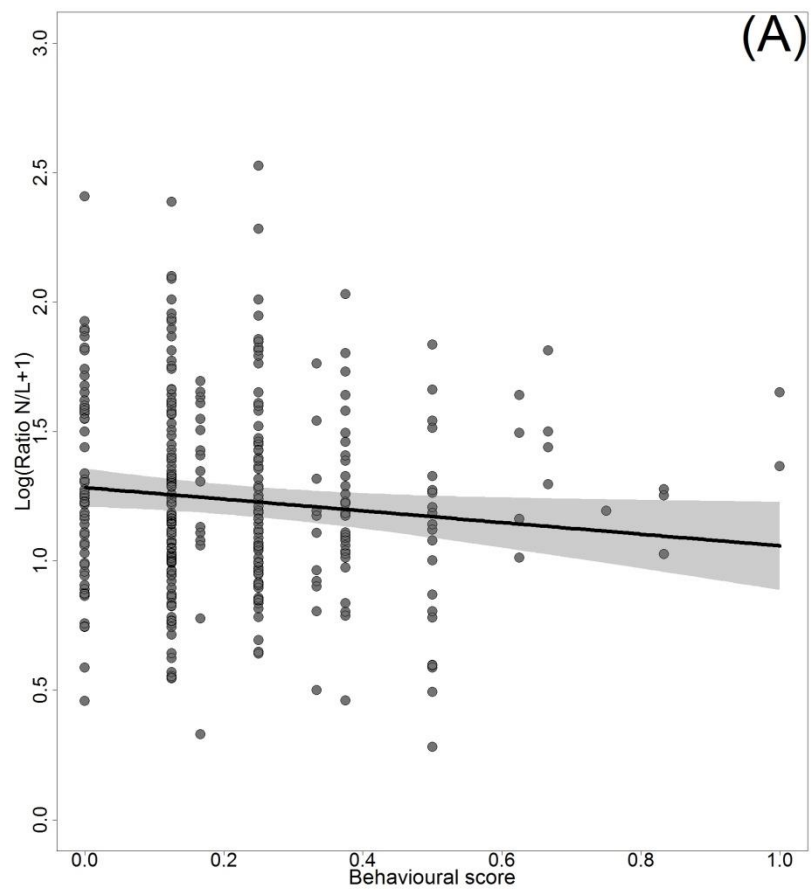


**Fig. 3** Relationship between the Neutrophil:Lymphocyte ratio and year quality in the roe deer populations of Aurignac (A) and Trois-Fontaines (B). Year quality was estimated using average body mass of fawns captured during the following winter. Points represent observed values. Lines represent model predictions and the shaded areas represent the 95% confidence intervals





**Fig. 4** Relationship between the Neutrophil:Lymphocyte ratio and the behavioural score at capture and release in the roe deer populations of Aurignac (A) and Chizé (B) respectively. The behavioural score ranged from 0 to 1 at Aurignac and is expressed in three classes in Chizé, with a score of 0 (slow escape with several stops), 1 (runs away, but stops after a short distance), 2 or higher (runs away without stopping until out of sight, or fell, jumped and attempted to remove its collar, or unable to stay upright). High values indicate proactive individuals, *i.e.*, with a marked behavioural response to capture. Points indicate observed values. Lines represent model predictions and the shaded areas represent the 95% confidence interval



# **Under cover of the night: context-dependency of anthropogenic disturbance on stress levels of wild roe deer *Capreolus capreolus***

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## **Lay summary**

We tested whether proximity to sources of anthropogenic disturbance influenced baseline stress levels of a wild ungulate in relation to their space use behaviour. We found that faecal cortisol metabolites increased as distance to human infrastructure decreased, but this relationship was buffered by the use of refuge habitats during daytime.

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## Abstract

Wild populations, in particular large-mammals, are increasingly exposed to human-induced modifications of their habitats. To cope with anthropogenic stressors, large mammals can adjust their behaviour, for example, shifting their activity to more sheltered habitats, or becoming more nocturnal. However, whether spatial and temporal adjustments in behaviour allow animals to buffer the endocrine response to human disturbance is poorly documented. Here, we analysed faecal cortisol metabolites (FCMs) of wild roe deer *Capreolus capreolus* living in a human dominated agro-ecosystem. Using Global Positioning System monitoring of 116 individuals, we assessed their spatial behaviour and tested whether proximity to anthropogenic structures (linear distance to built-up areas) and the use of refuge habitats (woods, groves and hedges) influenced FCM levels. In accordance with our predictions, individuals which ranged closer to anthropogenic structures during daytime had higher FCM levels, but this relationship was buffered as use of refuge habitats increased. In addition, this link between proximity to anthropogenic structures and FCM levels disappeared when we analysed spatial behaviour at night. Finally, FCM levels were higher when ambient temperature was lower, and during years of low resource availability. Our results demonstrate that the stress levels of large mammals may be strongly influenced by their proximity to anthropogenic activities, but that these effects may be buffered by behavioural adjustments in terms of space use and circadian activity rhythm. Whereas most studies have focused on the influence of environmental heterogeneity, our analysis highlights the need to also consider the fine-scale spatial response of individuals when studying the hormonal response of wild animals to human disturbance. We emphasise the potential to mitigate this hormonal stress response, and its potential negative consequences on population dynamics, through

the preservation or restoration of patches of refuge habitats in close proximity to human infrastructure.

## Keywords

Stress hormones - *Capreolus capreolus* - faecal glucocorticoid metabolites - human disturbance - space use

## Introduction

The intensification of anthropogenic activities over the last three decades brought Crutzen and Stoermer (2000) to name the modern era the “Anthropocene”. Changes in land use, particularly within agricultural areas, has resulted in landscape modifications and habitat fragmentation over more than 50% of the earth’s land surface (Hooke et al., 2012). Wild animals are, therefore, increasingly exposed to human-induced modifications of their habitats which can have consequences on ecosystem processes through the modification and restriction of their movements and space use (Tucker et al., 2018). For example, large mammals, and particularly large herbivores, usually occupy a large home range and are widely hunted, so that they are particularly exposed to the consequences of human activities, including landscape fragmentation. These habitat modifications may act as chronic stressors since large mammals usually do not easily adapt to erratic and unpredictable environmental variations, such as those associated with human activities (Romero and Wingfield, 2015).

The stress response is one of the main mechanisms by which individuals cope with environmental perturbations or life-threatening situations (Romero and Wingfield, 2015). In addition to the autonomic nervous system, the stress response includes the activation of the endocrine system and, more specifically, the hypothalamic-pituitary-adrenal (HPA) axis (Möstl



and Palme, 2002). This response is primarily an adaptive mechanism by which immediate survival is prioritised over other functions (Wingfield et al., 1998; Breuner et al., 2008). Activation of the HPA axis results in the secretion and release of glucocorticoids (cortisol and corticosterone) by the adrenal gland cortex into the blood stream (Sapolsky et al., 2000; Reeder and Kramer, 2005). As levels of glucocorticoids increase, the hormone supports the “emergency life history stage” (sensu Wingfield et al., 1998), for example, making stored energy available and increasing foraging and locomotor activity (Hau et al., 2016; Palme 2019). When stressors are persistent or regularly repeated, glucocorticoid regulation becomes less effective and glucocorticoid secretion is maintained at a high level for longer. This physiological state, referred to as chronic stress, is often associated with negative consequences (McEwen and Wingfield, 2010), including a decreased immune response and increased susceptibility to disease (Dhabhar 2014), reduced growth (Busch et al., 2008) and a decrease in reproductive performance (Sapolsky et al., 2000). These adverse effects can impact individual fitness (Romero et al., 2009) and, ultimately, population dynamics (Wingfield and Sapolsky, 2003).

Over recent years, studies investigating the effects of human disturbance on the stress levels of wild animals have increased (Dantzer et al., 2014; Rehnus et al., 2014; Jachowski et al., 2015; Formenti et al., 2018), generally indicating that individuals living in human-disturbed areas exhibit higher glucocorticoid levels than those living in undisturbed areas. In addition to elevated glucocorticoid levels, fear induced by human disturbance may also drive animals to modify their space use in order to reduce exposure to such stressors (Jachowski et al., 2012 on African elephants *Loxodonta africana*; Martin et al., 2010 on brown bear *Ursus arctos*; Thiel et al., 2008 on capercaillie *Tetra urogallus*). In the landscape of fear framework (Laundré et al., 2001), animals are therefore predicted to adjust their spatial behaviour to spatio-temporal

variations in the perceived risk of predation or disturbance. For instance, Martin and colleagues (2018) showed that roe deer *Capreolus capreolus* confined their movements to safe habitats during daytime and during the hunting season, when human activity is high. To minimise exposure to anthropogenic stressors, wild animals also tend to shift their activity patterns to become more nocturnal (Gaynor et al., 2018). These behavioural tactics may attenuate the physiological stress response and, hence, reduce the risk of the chronic adverse effects of high glucocorticoid levels. However, to our knowledge, no study has yet investigated the relative importance of spatial and temporal modifications of behaviour to mitigate the stress response of wildlife to anthropogenic disturbance.

Here, we analysed variation in faecal cortisol metabolites (FCMs) of roe deer from a wild population living in a heterogeneous landscape composed of agricultural fields providing rich food resources with woodlands and hedgerows that can be considered as refuge habitats. Arguably, measuring FCMs is the best way of assessing the baseline or cumulative levels of stress to which an individual has been exposed (Dantzer et al., 2014; Palme 2019). This measure is non-invasive and reflects the overall concentration of plasma glucocorticoids that an individual has experienced over a variable time-window, the length of which varies between species (Palme et al., 2005; Sheriff et al., 2011). Our aim was to test how baseline stress levels were associated with potential anthropogenic stressors (such as roads, houses and built-up areas), and to what extent this relationship might be modulated by behavioural adjustments in terms of use of refuge habitats over the day-night cycle. Because human activities and infrastructure are often perceived as sources of risk for wildlife (Frid and Dill, 2002), we expected that roe deer living closer to anthropogenic structures would exhibit higher baseline stress levels. However, we also predicted that this relationship would be buffered as the use of refuge habitats increased. Finally, previous studies have shown that roe

deer avoid using human disturbed habitats during the day due to the perceived increased level of risk (Bonnot et al., 2013). We thus expected stress levels to be more strongly related to proximity with anthropogenic structures during the day rather than at night.

## **Material and methods**

### **Study sites**

This study was carried out on a population of wild roe deer living in south-western France, near Aurignac (43°13'N, 0°52'E, 10 000 ha). This population experiences an oceanic climate with summer droughts and lives in a fragmented agricultural landscape mostly composed of open habitats, such as meadows and cultivated fields (respectively 31% and 36% of the study site), inter-mixed with small woodland patches (19%) and two main forests (14%; see Martin et al., 2018 for further details). The open habitats provide abundant and high-quality food resources for roe deer most of the year (Abbas et al., 2011), but they may be associated with potential sources of stress due to higher intensity of human activities compared to forest habitat (Bonnot et al., 2013). This roe deer population is hunted on a regular basis by stalking (bucks only) during summer (June-August) and by drive hunts with dogs during autumn-winter (September - February).

### **Field data collection**

As part of a long-term capture-mark-recapture program initiated in 1996, 6 days of capture occur each year, between the beginning of January and the beginning of March. We used large-scale drives with 30 to 100 beaters and up to 4 km of long-nets. Upon capture, deer were transferred to a wooden retention box providing darkness and ventilation until the marking

procedure. Roe deer were tranquilized just after capture by intramuscular injection of acepromazine (Calmivet, Vetoquinol, France; targeted dose of 0.075 mg/kg).

Marking procedures lasted approximately 15 minutes and were performed by trained handlers. Each individual was weighed (to the nearest 10 g) and sex and age were recorded. Individuals were divided in two age classes (juveniles for 8-10 month-old individuals or adults for >18 month-old individuals) based on tooth eruption patterns (Hewison et al., 1999). At the end of the procedure, each individual was marked with 2 ear tags and most were equipped with a GPS collar (Lotek 3300 GPS, Lotek Small WildCell GSM, Vectronic GPS PLUS-1C Store On Board, Vectronic GPS PLUS Mini-1C) programmed to obtain a GPS location every six hours (at 00:00, 06:00, 12:00 and 18:00). All capture and marking procedures were done in accordance with French and European laws for animal welfare (prefectural order from the Toulouse Administrative Authority to capture and monitor wild roe deer, agreement no. A31113001 delivered by the Departmental Authority of Population Protection, ethical authorisation by French Government).

### **Collection and extraction of FCMs**

Faecal samples were collected directly from the rectum during the marking procedure between 2012 and 2017 and held at -4°C for a maximum of 3h before being stored at -20°C until analyses.

FCM were extracted following a methanol-based procedure and assayed using a group-specific 11-oxo-aetiocholanolone enzyme immunoassay (EIA), as previously described (Möstl et al., 2002) and validated for roe deer (Zbyryt et al., 2017). Briefly, each faecal sample was homogenised and  $0.5 \pm 0.005$  g of homogenate was transferred to a glass tube containing 5 ml of a 80% methanol solution. The suspended samples were vortexed at 1500 rpm for 30 min

and centrifuged at 2500 g for 15 min (Palme et al., 2013). An aliquot of the supernatant was further diluted (1:10) with assay buffer prior to EIA analysis. Measurements were carried out in duplicate (intra- and inter-assay coefficients of all samples were less than 10% and 15%, respectively) and the results expressed as nanograms per gram of wet faeces (ng/g). Following the suggestion of Taff and colleagues (2018), we also calculated repeatability estimates of FCMs in order to evaluate the extent to which there is consistent among-individual variation in FCM levels.

### **Home range determination and landscape variables**

Home ranges were calculated using GPS data after removing the first 10 days following capture and release, since capture and handling are known to induce a transient modification of space use (Morellet et al., 2009). For each individual, we calculated a home range based on the 15 following days using the 90% fixed kernel method excluding locations during excursions (Worton 1989; Börger et al., 2006) with the “adehabitatHR” R package (Calenge 2006). We hypothesised that the home range used during this particular time period would be representative of the home range used just prior to capture, since roe deer exhibit high spatial fidelity (Hewison et al., 1998). Based on aerial photographs of the study site (from the IGN's BD Ortho, <http://professionnels.ign.fr/bdortho-50cm>), we manually digitized homogeneous habitat polygons (in ArcView GIS 3.3, Esri, Redlands, CA, U.S.A.). Each polygon was then assigned to a habitat type (e.g. woodland, hedgerow, meadow or crop) from field observations each summer.

For each home range, we calculated the proportion of refuge habitat (woodland, hedgerows, and scrubland). Here, we considered that the proportion of available refuge habitat within the home range (comprised between 0.07 and 0.99, with an average value of 0.35) reflected

habitat selection when that individual established its home range within the study area (second order habitat selection sensu Johnson 1980). We then calculated the probability that an individual used refuge habitat during the day (comprised between 0.11 and 1.00, with an average value of 0.72) and at night (comprised between 0.00 and 1.00, with an average value of 0.32) as the ratio of the number of locations within a refuge habitat over the total number of locations during daytime (at 12:00) and night-time (at 00:00) for that individual (see Bonnot et al., 2015 for a similar approach). This describes the selection of refuge habitat within the home range (third-order habitat selection sensu Johnson 1980). We considered these two different scales because, so far, most studies (e.g. Jachowski et al., 2012) have only considered what we refer to here as available refuge habitat, whereas animals likely respond to environmental stressors at multiple spatial and temporal scales. Finally, for each individual we calculated the average distances in meters between the GPS location and the nearest anthropogenic structure (roads, houses, and other buildings) considering their locations during daytime (at 12:00), and night-time (at 00:00) separately. This distance varied from 122 to 734 m during daytime, with an average of 280 m; and from 67 to 549 m during night-time, with an average of 211 m.

### **Statistical analyses**

Individual repeatability of FCM was estimated using 261 observations of 221 individuals and mixed models using the restricted maximum likelihood (REML) method, with the “rptR” package (Stoeffel et al., 2017) for Gaussian distributions.

To analyse variation in FCM levels, we performed linear mixed-effects models (LMMs) on 125 observations of 116 individuals. Fewer animals were used for these analyses compared to the repeatability analyses because not all animals were equipped with GPS collars. Three

individuals had extreme values for FCM and so were removed from our dataset ( $< 100$  or  $> 4500$  ng/g). The lowest value (96 ng/g) was removed because we suspected some problems with sample quality and possible contamination (Lexen et al., 2008), whereas the two highest values (4692 and 4914 ng/g) were removed because they were more likely indicative of an acute stress response during the hours preceding capture, rather than baseline levels which were the target of our study. FCM level was analysed as the response variable in 4 sets of models, each testing a specific hypothesis:

Set 1 and 2: To test the effect of proximity to anthropogenic structures on stress level and to evaluate the extent to which use of refuge habitats during daytime (set 1) and night-time (set 2) could influence this relationship, we included mean distance to the nearest anthropogenic structure during daytime (or night-time), probability of using refuge habitat during daytime (or night-time), and their two-way interaction.

Set 3 and 4: To test the effect of proximity to anthropogenic structures on stress level and to evaluate the extent to which availability of refuge habitats could influence this relationship, we included mean distance to the nearest anthropogenic structure during daytime (set 3) or night-time (set 4), proportion of woodland patches in the home range, and their two-way interaction.

We ran these 4 sets of models separately because of the strong collinearity between some explanatory variables for daytime and night-time (e.g. mean distance to the nearest anthropogenic structure  $r = 0.73$  ;  $p < 0.001$ ) and for some landscape variables (e.g. correlation between the probability of using a refuge habitat during daytime and proportion of woodland patches in the home range  $r = 0.74$  ;  $p < 0.001$ ). In addition to the variables mentioned above, candidate models included age (two modalities, juveniles versus adults), sex, body mass,



temperature, year quality, and the Julian date of capture. Indeed, FCM levels can be affected by ambient temperature (Huber et al., 2003). Temperature was taken as the maximal ambient temperature the day prior to capture (comprised between -0.3 and 19.2 °C, with an average value of 9.7 °C) because FCM indexes plasma cortisol 8 to 23 hours before faeces sampling (Dehnhard et al., 2001). In addition, we included the population average body mass of juveniles captured the following winter (comprised between 16.4 and 17.4 kg, with an average value of 16.9 kg) to control for annual variation in resource availability and quality (see Hamel et al., 2009). In each set of models, body mass was standardised (using residuals from a model including the additive effects of sex and age). Individual identity was included as random effects to avoid pseudo-replication issues (Hurlbert 1984). FCM was log transformed to achieve normality of model residuals. To select the best models of variation in FCM level, we used a model selection procedure based on the second-order Akaike Information Criterion (AICc, Burnham and Anderson, 2002). Models with a difference in AICc ( $\Delta AICc$ ) > 2 units from the best model were considered to have less support, following Burnham and Anderson (2002). In addition, we removed models within two AICc units of the top model that differed from a higher-ranking model by the addition of one or more parameters. These were rejected as uninformative, as recommended by Arnold (2010) and Richards (2008). We then applied a conditional model averaging procedure to estimate parameters. In addition, we calculated AICc weights (AICcw) to measure the relative likelihood that a given model was the best among the set of fitted models. Normality of the residuals for the selected models was tested (Shapiro-Wilk test) and visually assessed with histograms. Goodness-of-fit was assessed by calculating conditional (i.e. total variance explained by the best supported model) and marginal (i.e. variance explained by fixed effects alone)  $R^2$  values (Table 2) and standard residual plot techniques (Nakagawa and Schielzieth, 2013). In order to interpret the results

from models using space use behaviour during daytime and night-time, we also compared the average distance to the nearest anthropogenic structure between day and night locations using a paired t-test. All analyses were carried out with R version 3.6.0 (R Development Core Team 2016), using the lmer function from the lme4 package (Bates et al., 2014) and model.avg function from the MuMIn package (Barton 2016).

## Results

In our sample ( $n = 125$ ), FCM levels ranged from 167 to 3591 ng/g, with an average value of 872 ng/g. FCM levels were also moderately repeatable:  $R = 0.28$ , 95% confidence interval = [0.01, 0.52],  $P$  value < 0.01. The model selection procedure to explain FCM variations in relation to roe deer spatial behaviour during daytime (Supplementary data 1 & 2 - daytime models) led to consistent results and included year quality, mean distance to the nearest anthropogenic structure, probability of using refuge habitats (in set 1) or the proportion of available refuge habitats in the home range (in set 3), as well as the two-way interaction between refuge and distance to anthropogenic structures (Table 1 & Table 2, daytime). Specifically, roe deer that were closer to anthropogenic structures during daytime also exhibited higher FCM levels, but this relationship disappeared as the probability of using refuge habitat during daytime increased (Table 1; Figure 1) or as the proportion of refuge habitat in the home range increased (Table 2; Figure 2). For example, for individuals that did not use refuge habitat very frequently (probability lower than 0.65), FCM levels increased, on average, by 72% for every 100 m nearer to an anthropogenic structure, while they remained stable for individuals that made intensive use of refuge habitat (probability higher than 0.65). On the contrary, when there was only a low availability of refuge habitat in an individual's home range (proportion of available refuge habitat less than 0.18), FCM levels increased by

only 24% for every 100 m nearer an anthropogenic structure, while they remained stable when the availability of refuge habitat was higher (proportion higher than 0.18). In addition, FCM levels increased as the quality of the year decreased such that FCM levels in the poorest year of our study were, on average, 36% higher than those in the best year (Figure 3). Maximal ambient temperature the day before capture also explained some variation of FCM levels, with the highest FCM levels being observed when maximal ambient temperature was lower (Figure 4).

Both models selected to explain FCM variations in relation to night-time spatial behaviour (Supplementary data 1 & 2 - night-time models) included the effects of year quality and maximum ambient temperature the day prior to capture (Table 1 & Table 2 night-time). However, in contrast with daytime, neither the main effects of mean distance to the nearest anthropogenic structure and the probability of using refuge habitats nor their two-way interaction (set 2) or the proportion of refuge habitat in the home range (set 4) were retained in the best models.

Finally, our results showed that, on average, individuals ranged closer to anthropogenic structures during night-time compared to daytime ( $t = 9.1019$  ;  $df = 124$ ;  $P < 0.001$ ).

## Discussion

The aims of this study were twofold. First, we explored the influence of sources of anthropogenic disturbance on baseline stress levels in wild roe deer, while taking into account potential effects of ambient temperature, age, sex, body mass and resource availability. Second, we determined the extent to which spatio-temporal adjustments of behaviour could contribute to buffer the effects of anthropogenic disturbance on baseline stress levels. Our

results demonstrate, for the first time in a wild mammal, that exposure to anthropogenic disturbance is not always associated with higher stress levels. In particular, proximity to anthropogenic structures at night was unrelated to FCM levels, while the availability and use of refuge habitat during the day attenuated the negative impact of these sources of stress on FCM levels.

The average level of FCM observed is similar to the levels reported by Zbyryt and colleagues (2017) in two roe deer populations exposed to human disturbance, but free from predators (mean FCM levels of 874 ng/g). The repeatability is also consistent with a recent meta-analysis that estimated an overall repeatability of  $R \sim 0.29$  for glucocorticoid levels (Taff et al., 2018). Consistent with previous studies on other species (Arlettaz et al., 2007 on black grouse *Tetrao tetrix*; Bourbonnais et al., 2013 on brown bear *Ursus arctos*; Rehnus et al., 2014 on mountain hare *Lepus timidus*), our results showed that FCM levels increased as roe deer ranged closer to anthropogenic structures. However, this relationship was observed only under certain conditions. In particular, an individual roe deer was more likely to be stressed by proximity to anthropogenic structures when it used open habitats during the day, or when it did not make extensive use of refuge habitat, or when refuges were not widely available. Previous studies on the same roe deer population have shown that roe deer modified their spatial behaviour when in proximity with anthropogenic structures (Coulon et al., 2008), restricted their routine movement to safe habitats during daytime and the hunting season (Martin et al., 2018), but also decreased their use of risky habitat and reduced their distance to cover when risk increased (Padié et al., 2015). We were able to demonstrate that the protective influence of wooded refuge habitat resulted in an attenuated stress response, indicating that these behavioural adjustments do actually mitigate stress levels from a physiological point of view. This suggests that the physiological effects of human disturbance on wild mammals may be

more complex than previously assumed (Dantzer et al., 2014), and depends not only on the spatial pattern of human disturbance, but also on the individual's ability to access refuge habitat when disturbance is high.

The observation that proximity to anthropogenic structures at night was unrelated to an individual's stress level suggests that darkness alters the perception of risk in roe deer. This suggests that night also acts as a form of refuge from perceived sources of stress for wild animals, as suggested by the recent global study of Gaynor and colleagues (2018). Again, this result is coherent with previous work on the same roe deer population, which showed that roe deer adjusted their spatial behaviour, using more woodland habitat during daytime when proximity with human was high, but that there was no difference between daytime and night-time use of woodland habitat when far from human infrastructures (Bonnot et al., 2013). Indeed, large herbivores face a trade-off between acquisition of high quality food resources and exposure to anthropogenic stressors (Bonnot et al., 2015). In our study site, although they are associated with high levels of disturbance, open habitats have been shown to provide higher quality food resources for roe deer (Abbas et al., 2011, Hewison et al., 2009), such that juveniles weigh up to 3 kg more than those in the more forested part of the study site (Hewison et al., 2009), with consequences for individual performance (Pettorelli et al., 2002, Douhard et al., 2013). While it is widely reported that many wild animals have increased their level of nocturnal activity in the face of human disturbance (Gaynor et al., 2018), we are not aware of any study to date that has investigated the relationship between FCM levels and space use behaviour in relation to the degree of nocturnality. Our results suggest that the cost of exploiting open habitats in terms of exposure to stress is lower during night-time compared to daytime. Animals may therefore mitigate the costs of living in close proximity to humans by adjusting their temporal use of open habitat towards a more nocturnal mode.

Our study also identified the effects of environmental conditions, in particular, of temporal variation in habitat quality (year quality), on baseline stress levels. We found that during years of low resource availability and quality (indexed by mean juvenile body mass), roe deer had, on average, higher levels of FCM. This result was expected as the quantity and quality of available food resources are both known to act as ecological stressors in vertebrates (Fodikis et al., 2012) and a large body of literature already reported elevated stress levels in individuals exposed to poor food availability in some years (Hellgren et al., 1993) or poor overall habitat quality (Marra and Holberton, 1998). Furthermore, year to year variations in resource abundance and quality are not easily predictable for wild animals and our results suggest that roe deer do not easily cope with these unpredictable temporal variations in their environment. We also found that FCM levels were negatively correlated with ambient temperature the day before capture, suggesting that colder temperatures are perceived as more stressful for roe deer at this time of the year (winter - between the 5<sup>th</sup> of January and the 5<sup>th</sup> of March). These results are consistent with other studies on ungulates that found much higher cortisol levels during the winter months compared to the rest of the year (e.g. Bubenik et al., 1983 on white-tailed deer *Odocoileus virginianus*). Huber and colleagues (2003) also investigated effects of climatic conditions on FCM levels in red deer *Cervus elaphus* during a complete year and suggested that “high winter glucocorticoid levels may act via catabolic function during adaptation of deer to cold winter month when resources are limited”.

Overall, our results suggest that the stress levels of wild ungulates are strongly influenced by proximity to human activities, but that these effects are buffered by both spatial and temporal behavioural adjustments. From an evolutionary point of view, we can therefore expect wild animals to exhibit temporal shifts in their spatial behaviour, with an increase in the use of risky habitats during the night. In terms of locally applicable management and conservation

strategies for free-ranging animals, preservation or restoration of patches of refuge habitat in proximity to human infrastructure could help to mitigate stress levels and the potential negative consequences on health, fitness and, ultimately, population dynamics of living in the Anthropocene.

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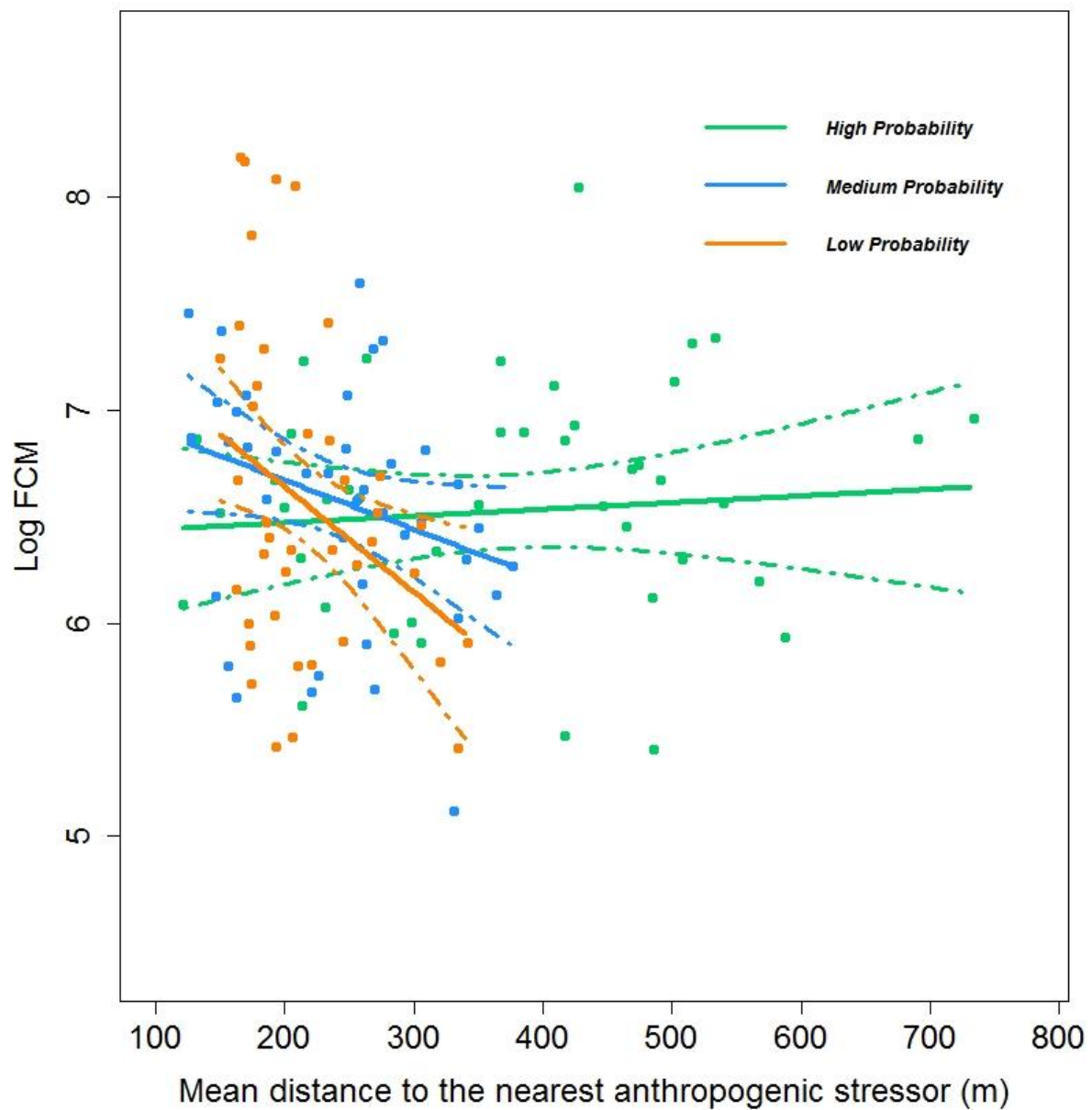


| Parameter  | Estimate | CI               |
|--|----------|------------------|
| <b>Daytime set 1</b><br>( $R^{2m}$ :0.18 ; $R^{2c}$ :0.43)             |          |                  |
| Intercept  | 15.36    | 9.826 to 20.923  |
| Distance to human infrastructure                                       | -0.010   | -0.015 to -0.005 |
| Probability of using refuge habitat                                    | -2.470   | -3.822 to -1.117 |
| Temperature  | -0.029   | -0.056 to -0.002 |
| Year quality   | -0.369   | -0.697 to -0.043 |
| Distance to human infrastructure * Probability of using refuge habitat | 0.011    | 0.005 to 0.016   |
| <b>Night-time set 2</b><br>( $R^{2m}$ :0.08 ; $R^{2c}$ :0.41)          |          |                  |
| Intercept  | 13.98    | 8.258 to 19.803  |
| Temperature  | -0.032   | -0.059 to -0.005 |
| Year quality   | -0.419   | -0.763 to -0.082 |

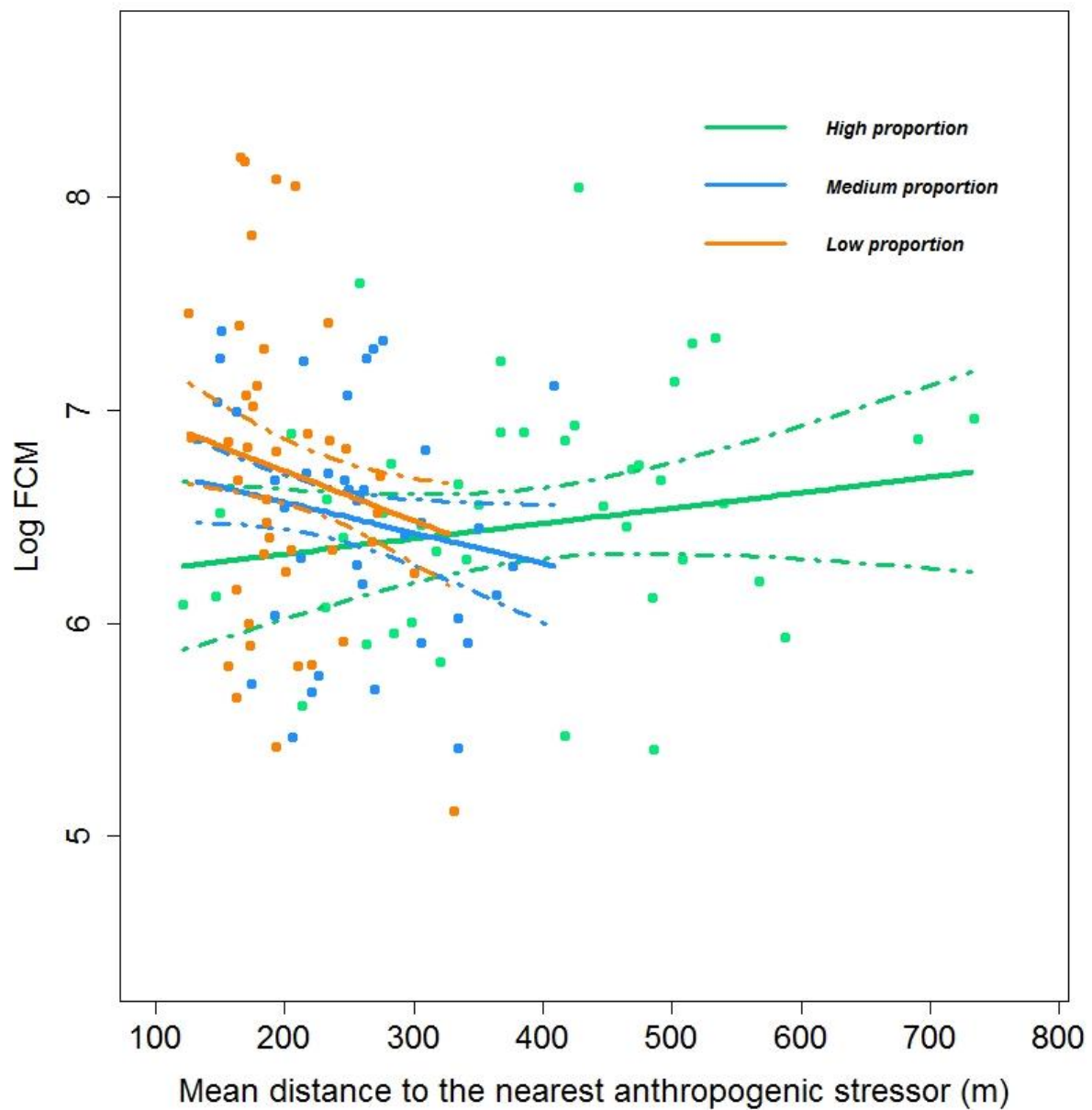
**Table 1** Characteristics of the selected linear mixed-effect models for explaining variation in FCM levels in the roe deer population of Aurignac in relation to use of refuge habitat and proximity to anthropogenic infrastructure during daytime and night-time. The effect of mean distance to the nearest anthropogenic structure during daytime (Distance to human infrastructure), probability of using refuge habitat during daytime, maximal temperature the day before capture (Temperature), year quality (indexed by the population average body mass of juveniles captured during the following winter), and the two-way interaction between mean distance to the nearest anthropogenic structure and probability of using refuge habitats during daytime were fitted. Models included individual identity and year of capture as random effects.  $R^{2m}$  and  $R^{2c}$  are the marginal and conditional explained variance of the models, respectively. CI stands for Confidence Interval. See text for definition of model sets.

| Parameter   | Estimate | CI               |
|---|----------|------------------|
| <b>Daytime set 3</b><br>( $R^{2m}$ :0.14 ; $R^{2c}$ :0.41)        |          |                  |
| Intercept   | 14.794   | 9.033 to 20.555  |
| Distance to human infrastructure                                  | -0.003   | -0.006 to -0.001 |
| proportion of woodland patches                                    | -2.071   | -3.500 to -0.642 |
| Temperature   | -0.029   | -0.056 to -0.002 |
| Year quality  | -0.433   | -0.770 to -0.096 |
| Distance to human infrastructure * proportion of woodland patches | 0.006    | 0.002 to 0.011   |
| <b>Night-time set 4</b><br>( $R^{2m}$ :0.08 ; $R^{2c}$ :0.41)     |          |                  |
| Intercept   | 13.98    | 8.258 to 19.803  |
| Temperature   | -0.032   | -0.059 to -0.005 |
| Year quality  | -0.419   | -0.763 to -0.082 |

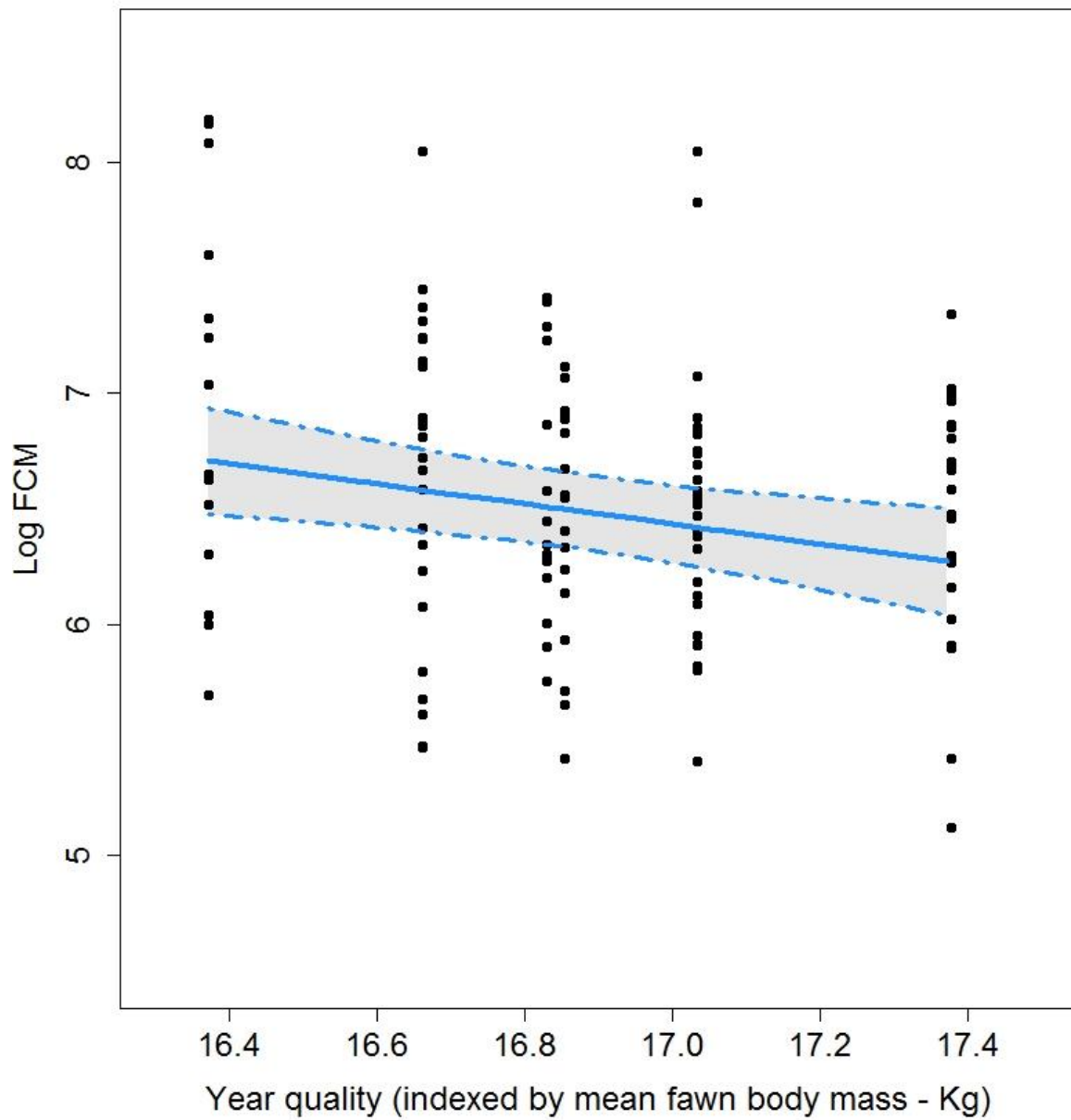
**Table 2** Characteristics of the selected linear mixed-effect models for explaining variation in FCM levels in the roe deer population of Aurignac in relation to available refuge habitat and proximity to anthropogenic infrastructure during daytime and night-time. The effect of mean distance to the nearest anthropogenic structure during daytime (Distance to human infrastructure), proportion of woodland patches in the home range, maximal temperature the day before capture (Temperature), year quality (indexed by the population average body mass of juveniles captured during the following winter), and the two-way interaction between mean distance to the nearest anthropogenic structure during daytime and proportion of woodland patches in the home range were fitted. Models included individual identity and year of capture as random effects.  $R^{2m}$  and  $R^{2c}$  are the marginal and conditional explained variance of the model, respectively. CI stands for Confidence Interval. See text for definition of model sets.



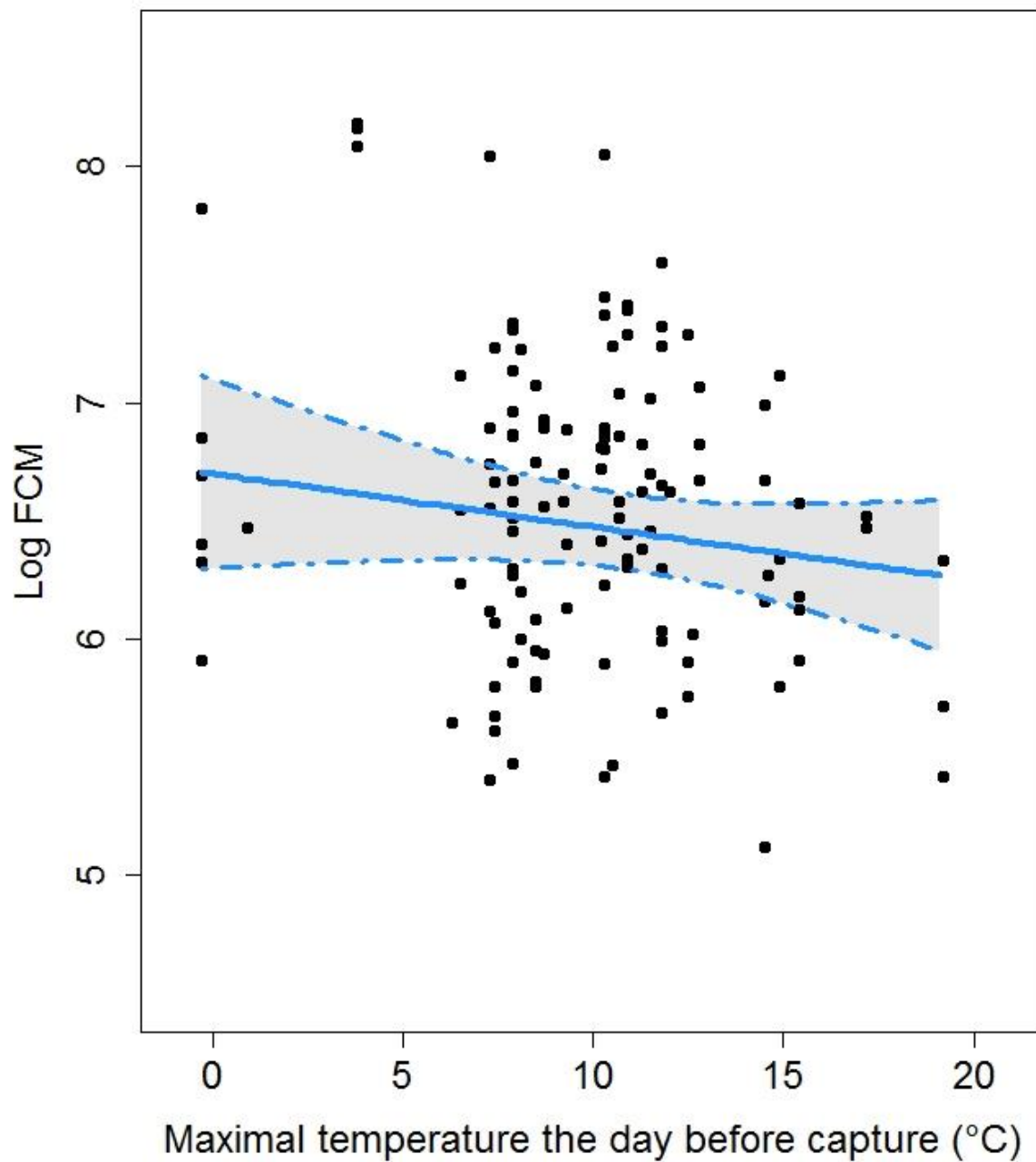
**Fig. 1** Relationship between FCM level and mean distance to the nearest anthropogenic structure during daytime in relation to the probability of using refuge habitat during daytime in the roe deer population of Aurignac. Points represent observed values, lines represent model predictions and dashed lines represent the 95% confidence interval. High probability corresponds to a probability of using refuge habitat  $> 0.77$ ; Medium probability is comprised between 0.65 and 0.77; and Low probability is  $< 0.65$



**Fig. 2** Relationship between FCM level and mean distance to the nearest anthropogenic structure during daytime in relation to proportion of woodland in the home range in the roe deer population of Aurignac. Points represent observed values, lines represent model predictions and dashed lines represent the 95% confidence interval. High proportion corresponds to a proportion of woodland in the home range  $> 0.35$ ; Medium probability is comprised between 0.18 and 0.35; and Low probability is  $< 0.18$



**Fig. 3** Relationship between FCM level and year quality in the roe deer population of Aurignac. Year quality was indexed using the population average body mass of juveniles captured during the following winter. Points represent observed values, lines represent model predictions and dashed lines represent the 95% confidence interval



**Fig. 4** Relationship between FCM level and maximal temperature the day before capture in the roe deer population of Aurignac. Points represent observed values, lines represent model predictions and dashed lines represent the 95% confidence interval

**Supplementary data 1:** Performance of the subset of candidate linear mixed-effect models within a  $\Delta AICc < 2$  fitted to investigate variation in faecal glucocorticoids metabolite levels in the roe deer population of Aurignac according to daytime (A) and night-time (B) use of refuge habitats. Model(s) in bold was/were used for estimation of parameters, and averaged when more than one model was considered after removing models that differed from a higher-ranking model by the addition of one or more parameters. These were rejected as uninformative, as recommended by Arnold (2010) and Richards (2008). Our set of candidate models was composed of all simpler models that included sex, age, year quality, body mass, Julian date of capture (Date), mean distance to the nearest anthropogenic structure (Anthropogenic distance) during daytime (A) and night-time (B), probability to use refuge habitats during daytime (PRHD) or night-time (PRHN), maximal temperature the day before capture (Temperature), and the two-way interaction between mean distance to the nearest anthropogenic structure and probability to use refuge habitats during daytime (A) and nighttime (B). Individual identity was included as a random effect. AICc is the value of the corrected Akaike's Information Criterion and K is the number of estimated parameters for each model. The ranking of the models is based on the differences in the values for  $\Delta AICc$  and on the Akaike weights (AICw).

### A) Daytime model

| Models   | K        | AICc         | $\Delta AICc$ | AICw         |
|--|----------|--------------|---------------|--------------|
| <b>Anthropogenic distance+Year quality+PRHD+Temperature+<br/>Anthropogenic distance*PRHD</b>   | <b>8</b> | <b>226.4</b> | <b>0.00</b>   | <b>0.441</b> |
| Anthropogenic distance+Year quality+PRHD+Temperature+<br>Anthropogenic distance*PRHD+Age       | 9        | 227.9        | 1.55          | 0.203        |
| Anthropogenic distance+Year quality+PRHD+Temperature+<br>Anthropogenic distance*PRHD+Body mass | 9        | 228.0        | 1.67          | 0.191        |
| Anthropogenic distance+Year quality+PRHD+Temperature+<br>Anthropogenic distance*PRHD+Sex       | 9        | 228.3        | 1.96          | 0.165        |

### B) Night-time model

| Models                             | K        | AICc         | $\Delta AICc$ | AICw        |
|------------------------------------|----------|--------------|---------------|-------------|
| <b>Year quality+Temperature</b>    | <b>5</b> | <b>233.3</b> | <b>0.00</b>   | <b>0.36</b> |
| Year quality+Temperature+Body mass | 6        | 234.7        | 1.42          | 0.18        |
| Year quality+Temperature+Age       | 6        | 234.7        | 1.43          | 0.18        |
| Year quality+Temperature+Sex       | 6        | 235.2        | 1.86          | 0.14        |
| Year quality+Temperature+Date      | 6        | 235.2        | 1.92          | 0.14        |



**Supplementary data 2:** Performance of the subset of candidate linear mixed-effect models within a  $\Delta AICc < 2$  fitted to investigate variation in faecal glucocorticoids metabolite levels in the roe deer population of Aurignac according to daytime (A) and nighttime (B) space use behaviour and available refuge habitats in the home range. Model(s) in bold was/were used for estimation of parameters, and averaged when more than one model was considered after removing models that differed from a higher-ranking model by the addition of one or more parameters. These were rejected as uninformative, as recommended by Arnold (2010) and Richards (2008). Our set of candidate models was composed of all simpler models that included sex, age, year quality, body mass, Julian date of capture (Date), mean distance to the nearest anthropogenic structure (Anthropogenic distance) during daytime (A) and night-time (B), proportion of woodland patches in the home range (Woodland), maximal temperature the day before capture (Temperature), and the two-way interaction between mean distance to the nearest anthropogenic structure and proportion of woodland patches in the home range. Individual identity was included as a random effect. AICc is the value of the corrected Akaike's Information Criterion and K is the number of estimated parameters for each model. The ranking of the models is based on the differences in the values for  $\Delta AICc$  and on the Akaike weights (AICw).

## A) Daytime model

| Models   | K        | AICc         | $\Delta AICc$ | AICw        |
|--|----------|--------------|---------------|-------------|
| <b>Anthropogenic distance+Year quality+Woodland+Temperature+<br/>Anthropogenic distance*Woodland</b>   | <b>8</b> | <b>233.6</b> | <b>0.00</b>   | <b>0.37</b> |
| Anthropogenic distance+Year quality+Woodland+Temperature+<br>Anthropogenic distance*Woodland+Age       | 9        | 234.9        | 1.32          | 0.19        |
| Anthropogenic distance+Year quality+Woodland+Temperature+<br>Anthropogenic distance*Woodland+Body mass | 9        | 235.4        | 1.82          | 0.15        |
| <b>Anthropogenic distance+Year quality+Woodland +<br/>Anthropogenic distance*Woodland</b>              | <b>7</b> | <b>235.4</b> | <b>1.85</b>   | <b>0.15</b> |
| Anthropogenic distance+Year quality+Woodland+Temperature+<br>Anthropogenic distance*Woodland+Sex       | 9        | 235.5        | 1.94          | 0.14        |

## B) Night-time model

| Models  | K        | AICc         | $\Delta AICc$ | AICw        |
|---|----------|--------------|---------------|-------------|
| <b>Year quality+Temperature</b>   | <b>5</b> | <b>233.3</b> | <b>0.00</b>   | <b>0.23</b> |
| Year quality+Temperature+Anthropogenic<br>distance+Woodland+Anthropogenic distance*Woodland     | 8        | 233.7        | 0.43          | 0.19        |
| Year quality+Temperature+Body mass  | 6        | 234.7        | 1.42          | 0.11        |
| Year quality+Temperature+Age  | 6        | 234.7        | 1.43          | 0.11        |
| Year quality+Temperature+Woodland   | 6        | 235.2        | 1.85          | 0.09        |
| Year quality+Temperature+Sex  | 6        | 235.2        | 1.86          | 0.09        |
| Year quality+Temperature+Date   | 6        | 235.2        | 1.92          | 0.09        |
| Year quality+Temperature+Anthropogenic<br>distance+Woodland+Anthropogenic distance*Woodland+Age | 9        | 235.3        | 1.96          | 0.09        |





## **Chapter 2**

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# **Early-life baseline stress levels and maternal effects**





# **Human activities and behaviour influence salivary cortisol concentrations during early life of roe deer fawns**

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## Abstract

Early-life conditions exert a profound and long lasting influence on life history trajectories and overall individual fitness. Although the underlying mechanisms are not yet fully understood, stress hormones, and in particular glucocorticoids, seem to play a major role in orchestrating this long-term programming. Describing the environmental and individual characteristics that may affect stress hormone levels in newborns is thus a necessary step to better understand how early life experiences may influence life-history trajectories later on. Here, we analysed the salivary cortisol concentrations in wild roe deer fawns *Capreolus capreolus* (<16 days old) living in an agro-ecosystem of various degree of human activities, and whose quality and availability of resources varies over time. We tested whether environmental (proximity to anthropogenic structures) and individual (age, sex, body mass and behaviour) characteristics influenced baseline cortisol concentrations. We found that newborn fawns located near anthropogenic structures had higher cortisol concentrations than those living far from human disturbance, but this relationship was modulated by age of fawns, and appeared only after few days of life. Finally, we found that baseline cortisol concentrations were higher in individuals exhibiting proactive behaviour during handling. Our results demonstrate that, from the first stage of life, the stress levels of wild mammals may be affected not only by human disturbance, but may also be related to the among-individual variability of the behavioural response to stressful situations. The application of a comprehensive approach that takes into account human impact and individual characteristics may appear necessary in order to depict the impact of early-life stress hormones on adult performance and population dynamics.

## Introduction

Glucocorticoid hormones (i.e. cortisol and corticosterone) are metabolic hormones that play a major role in the regulation of an individual's energy through acquisition, storage and mobilisation of resources (Landys et al., 2006; Busch and Hayward, 2009). In addition, these hormones are also important mediators of the physiological and behavioural responses of organisms to changes in their environment (Wingfield et al., 1998). While solid knowledge has been collected over the years on the factors influencing glucocorticoid hormones in adults, the topic remains poorly documented in newborns, and almost only in birds. Yet, understanding these early variations could be valuable in order to better understand the early-life history trajectories of individuals and their long-term consequences over the all life on an individual, and ultimately, on population dynamics. Indeed, early developmental trajectories may significantly shape the subsequent morphology, behaviour and physiology of adults (Lindström, 1999; Metcalfe and Monaghan, 2001). In particular, newborns with elevated glucocorticoids during development may suffer from immediate effects, such as inhibition of growth and immune function (Wada and Breuner, 2008; Stier et al., 2009). They can also become hypersensitive to stressors as adults, and might transmit this alteration on their HPA axis to their future offspring (Meaney, 2001; Hayward and Wingfield, 2004).

Usually, newborns are mostly confined to nesting sites during early development and have limited ability to move, making them particularly sensitive to environmental perturbations, (Starck, 1993). Consequently, anthropogenic activities and structures, such as roads, may be particularly stressful for newborns, as it has been shown in nestling birds for instance (Walker et al., 2005). Anthropogenic structures can affect newborns directly, through traffic noise or increased human presence (see Fahrig and Rytwinski, 2009 for a review in adults), but also

indirectly by modifying parental care for instance (Morgan et al., 2010). The effect of anthropogenic disturbance on glucocorticoid hormones in adults has been a major focus of research these past years (Thiel et al., 2008; Rehnus et al., 2014; Jachowski et al., 2015; Formenti et al., 2018). Specifically, results of these studies generally indicate that individuals living in human-disturbed areas exhibit higher glucocorticoid concentrations than those living in undisturbed areas (Dantzer et al., 2014).

In addition to the influence of the environment, individual characteristics have also brought a large body of literature showing that they may underlie among-individual variations in glucocorticoid hormones (Hau et al., 2016; Romero and Wingfield, 2015). In particular, behaviour during stressful situations has been linked to stress-induced and baseline concentrations of glucocorticoids, both in laboratory (Koolhaas et al., 1999) and wild (Montiglio et al., 2012) animals. Consistent among-individual differences in the behavioural and physiological stress response can be characterised in terms of a coping style continuum, ranging from reactive to proactive individuals (Koolhaas et al. 1999; Réale et al. 2007). While reactive individuals are typically shy, thorough explorers, and exhibit higher glucocorticoid levels (high reactivity and activity of the HPA axis), the opposite occurs for proactive individuals (Koolhaas et al. 1999; Sih et al. 2004). However, how this link between the physiological and behavioural responses to stressful situations also occur during early-life remains poorly know.

Here, we used data on roe deer fawns from a free-ranging population to analyse, for the first time in a wild mammal, the variation of cortisol in newborns according to environmental and behavioural characteristics. We assessed baseline stress levels in roe deer fawns by measuring salivary cortisol concentrations, and investigated the relationship with anthropogenic



pressure and individual characteristics (age, sex, body mass and behaviour). Because human activities and infrastructures are often perceived as sources of risk for wildlife (Frid and Dill, 2002), we expected that fawns nested closer to anthropogenic structures would exhibit higher baseline stress levels than those nested further away, as it occurs in adult roe deer (Carbillet et al., unpublished data). In addition, we expected baseline cortisol concentrations of newborn fawns to be related to their behaviour at capture, with higher levels in shier individuals compared to bolder, as predicted by the coping styles framework (Koolhaas et al., 1999).

## **Material and methods**

### **Study sites**

This study was carried out on a population of wild roe deer living in south west of France, near the Aurignac canton (43°13'N, 0°52'E, 10 000 ha). This population experiences an oceanic climate with summer droughts and lives in a fragmented agricultural landscape mostly composed of meadows and cultivated fields (respectively 31% and 36% of the study site), inter-mixed with small woodland patches (19%) and two main forests (14%; see Martin et al., 2018 for further details). This roe deer population is hunted on a regular basis by stalking (bucks only) during summer (June-August) and by drive hunts with dogs during autumn-winter (September - February).

### **Field data collection**

Initiated in 2004, the capture-mark-recapture program of fawns occurs each year between the end of April and the middle of June. In 2017 and 2018, 47 newborn fawns (on average 4.6 days old at first capture) were caught by hand. The marking procedure occurred once the

mothers moved away from the fawns. Fawns were sexed, aged, weighed to the nearest 50g, and behaviour during handling was recorded with two modalities (0: the fawn called; 1: the fawn did not call). Several behaviours during handling, including calling, have been shown to be highly repeatable among individuals adult roe deer ( $R = 0.47$ , 95% confidence interval = [0.24, 0.66]) (Bonnot et al. 2018a), and have previously been used to index among-individual behavioural differences in the Aurignac roe deer population (Debeffe et al. 2014; Bonnot et al. 2015; Monestier et al. 2015). Consequently, individuals that responds actively to capture (e.g. by calling or struggling) can be considered as bolder compared to individuals that respond passively to the capture event (e.g. no call and no struggle). The age of fawns was established in relation to daily monitoring of the mother's appearance (belly size, presence/absence of udders) and behaviour (reaction to a whistle mimicking the call of a fawn), and in relation to the fawn's behaviour (Jullien et al., 1992). Since 2017, a salivary sample was taken using a cotton swab (SalivaBio Children's Swab; Salimetrics) and maintained à 4°C in the field before being conserved at -20°C within 2 hours until further analyses. Salivary samples were collected in order to further assess baseline cortisol levels. Their collection occurred within 10 minutes following the fawn's capture as salivary cortisol is expected to reflect baseline cortisol within a window of 30 minutes following disturbance (Sheriff et al., 2011; Dantzer et al., 2014). More than half (30 / 47) of the fawns were recaptured (on average 7.1 days later), were weighed again and a second salivary sample was taken. In addition, at both captures, we recorded the GPS locations of the fawn.

### **Salivary cortisol analyses**

Cotton swabs that were used for the salivary sampling during handling of fawns were centrifuged at 1500 g for 15 min and an aliquot of the supernatant was diluted (1:2) with assay

buffer prior to EIA analysis with a commercial kit (Item No. 1-3002; Salimetrics). Measurements were carried out in duplicate (intra- and inter-assay coefficients of all samples were 6.03% and 7.61%, respectively) and results expressed as picograms per millilitre (pg/mL).

### **Habitat composition and landscape variables**

The habitat of each fawn was characterised over the surface of circles centred on GPS data recorded at captures. For the first capture, we used circles with a radius of 50 m because newborn fawns are almost motionless during the first days of life. At the second capture, the habitat was characterised on a circle of 100 m radius since fawns were more likely to explore their environment (Andersen et al. 1998). We manually digitised homogeneous habitat polygons (in ArcView GIS 3.3, Esri, Redlands, CA, U.S.A.) based on aerial photographs of the study site (from the IGN's BD Ortho, <http://professionnels.ign.fr/bdortho-50cm>). Each polygon was then assigned to a habitat type (e.g. woodland, hedgerow, meadow or crop) from field observations each summer. For each home range, we calculated the proportion of refuge habitat (woodland, hedgerows, and scrubland), which was comprised between 0 and 1, with an average value of 0.21. In addition, for each individual we calculated the distance in meters between the GPS location of capture and the nearest anthropogenic structure (roads, houses, and other buildings). Distance of the capture to the nearest anthropogenic structure varied from 29 to 528 m, with an average of 208 m.

### **Statistical analyses**

To analyse variation in salivary cortisol concentration, we performed linear mixed-effects models (LMMs) on 77 observations of 47 individuals corresponding to two years of capture. Salivary cortisol concentration was analysed as the response variable and the data was log transformed to achieve normality of model residuals. We built a reference model which

included all pertinent variables to test our predictions on the effect of landscape structure (proportion of refuge habitat and distance to the nearest anthropogenic structure) and individual characteristics (age in days, sex, body mass, behaviour at capture). The reference model also included the two-way interactions between distance to the nearest anthropogenic structure and age; and between distance to the nearest anthropogenic structure and proportion of refuge habitats. These interactions were included to test for the potential differential effect of risk perception with age; and to account for the potential buffering effect of refuge habitats on the effect of anthropogenic structures on cortisol concentrations, as shown in adult roe deer (Carbillet et al., unpublished data). Our set of candidate models included the reference model and all simpler sub-models. Body mass was standardised (using residuals from a model including the additive effects of sex and age). Individual identity and year of capture were included as random effects to avoid pseudo-replication issues (Hurlbert, 1984) and to control for unexplained variance due to among-individual differences and inter-annual variation. To select the best models of variation in salivary cortisol levels, we used a model selection procedure based on the second-order Akaike Information Criterion (AICc, Burnham and Anderson, 2002). Models with a difference in AICc ( $\Delta\text{AICc}$ ) > 2 units from the best model were considered to have less support, following Burnham and Anderson (2002). In addition, we removed models within two AICc units of the top model that differed from a higher-ranking model by the addition of one or more parameters. These were rejected as uninformative, as recommended by Richards (2008) and Arnold (2010). We then applied a conditional model averaging procedure to estimate parameters. In addition, we calculated AICc weights (AICcw) to measure the relative likelihood that a given model was the best among the set of fitted models. Normality of the residuals for the selected models was tested (Shapiro-Wilk test) and visually assessed with histograms. Goodness-of-fit was assessed by

calculating conditional (i.e. total variance explained by the best supported model) and marginal (i.e. variance explained by fixed effects alone) and standard residual plot techniques (Nakagawa and Schielzieth, 2013). All analyses were carried out with R version 3.6.0 (R Development Core Team, 2016), using the lmer function from the lme4 package (Bates et al., 2014) and model.avg function from the MuMIn package (Barton, 2016).

## Results

Model selection procedure for explaining salivary cortisol concentration variation retained the effects of landscape structure, such that fawns that were closer to anthropogenic structures exhibited higher baseline cortisol concentrations, but this relationship was only significant when considering the age of the fawn (Table 1; Fig. 1). For example, for a 10 days old fawn, baseline cortisol concentrations increased, on average, by 33% for every 200 m nearer to an anthropogenic structure, while they remained nearly stable for fawns that were younger than 5 days. In addition, we found that individual characteristics such as age at capture influenced baseline cortisol concentrations, with nearly three times higher concentrations in fawns at age 15 days compared to age 1 day (Table 1; Fig. 2). We also found an effect of behaviour during handling (Table 1), such that fawns that called had baseline cortisol concentrations that were 32% higher than in fawns that did not call (Fig. 3). Model selection procedure did not provide any support for an effect of sex or body mass on baseline cortisol concentrations.

## Discussion

The main goal of this study was to gain more insight into the environmental and individual characteristics that may influence baseline cortisol concentrations during early life in a wild mammal. Our results supported the prediction that both environmental characteristics such as proximity with anthropogenic structures and individual characteristics such as those revealed by behavioural response at capture and age have the potential to influence early baseline cortisol concentrations. To our knowledge, this is the first study that successfully used salivary samples in a free-ranging population, opening opportunities for future studies on wild young individuals. In addition, this study was the first that investigated the drivers of baseline cortisol concentrations in newborns of a wild population.

First, our study highlighted that environment may exert a strong influence on the baseline cortisol concentrations of fawns. Baseline cortisol concentrations increased as fawns were located closer to anthropogenic structures. However, this relationship was observed only under certain conditions. In particular, an individual roe deer was more likely to be stressed by proximity to anthropogenic structures when it got older. This modulation of the effect with age could be due to the fact that perception of the risk may not be fully developed within the first week of life. Another non-exclusive explanation could be the increase in locomotor activity that occurs with age, which could lead fawns to increase their proximity with anthropogenic structures. The finding of an effect of human activities on stress level is in line with one of the rare study on newborns, who showed that nestling birds located closer to roads exhibited higher corticosterone concentrations at both stress-induced and baseline levels (Crino et al., 2011). This result is also coherent with previous work on adults of the same population, which showed that roe deer ranging closer to anthropogenic structures had higher

faecal cortisol levels, even if this relationship was mediated by the use of refuge habitats and period of the day (Carbillet et al., unpublished data). Our result suggests that the physiological effects of human disturbance on wild mammals may occur early in the life of an individual, which could have persistent consequences on adult life and ultimately on population dynamics. Indeed, inhibition of growth and immune function may occur in response to elevated cortisol concentrations during development (Wada and Breuner, 2008; Stier et al., 2009), which can influence survival, but also reproductive success. In addition, individuals with elevated stress hormones might modify the phenotype of their future offspring through maternal effects (Meaney, 2001; Hayward and Wingfield, 2004). Accordingly, it might be possible that the ability of fawns to express elevated cortisol concentrations may have been transmitted from their mothers (Love et al., 2008; Haussman et al., 2012). Especially, considering the fact that fawns may experience a similar environment, and consequently similar exposure to stressors, than their mothers, this transmission might be adaptive for fawns' survival (Sheriff et al. 2018). However, an analysis on a subset of our dataset did not reveal a link between maternal and offspring cortisol concentrations (Carbillet et al., unpublished data), suggesting that early-life cortisol concentrations may not only result from maternal transmission, but may already be modulated by early-life environment and human activities, as suggested by our data.

Second, we found that individual characteristics may influence early baseline cortisol concentrations. In particular, a non-negligible amount (on average 1235 pg/mL; compared to 2707 pg/mL for captive adults, unpublished data) of cortisol was present in saliva of newborn fawns. In addition, we found that within the first 15 days of life, cortisol concentrations increased linearly with age. This result may seem surprising in light of the literature which generally recognises the existence of a hypo-responsiveness period (De Kloet et al., 1988;

Romero et al., 2004) which minimises the circulating cortisol and favours neuronal development in mammalian newborns. This hypo-responsive period seems closely linked to the altricial-precocial gradient, with newborns who are quickly independent of their parents showing a short hypo-responsive period (Wada, 2008). Fawns of roe deer highly depend on the care provided by their mother during the first weeks of life (Andersen et al. 1998) and may therefore be considered as a rather altricial species compared to several bird species where chicks can move and collect their own food within one or two weeks (Starck and Ricklefs, 1998). Nonetheless, another study on wild roe deer found that newborn fawns can elicit an increase in cortisol concentrations comparable to that exhibited by adults in the face of a stressor (Bonnot et al., 2018b). These results may suggest that our results were not impacted by hypo-responsiveness, and that the relationship between the hypo-responsive period and dependence of offspring requires more investigations.

Finally, individuals who exhibited an active behaviour during handling (as evidenced by calling during handling) had higher cortisol concentrations than quiet individuals. A link between cortisol secretion and behaviour during stressful situations has been proposed within the coping-style framework (Koolhaas et al., 1999). This relationship was supported by several studies in adult laboratory (Koolhaas et al., 1999) and wild (Montiglio et al., 2012; Montiglio et al., 2015) animals and showed that individuals reacting passively to stressful situations exhibited a higher cortisol secretion compared to proactive ones. Our results on newborns are therefore not in line with those found on adults. Such discrepancy may be explained, at least in part, by differences in developmental stages (newborns vs. adults). At this stage, behavioural and physiological responses to stressful situations might not be fixed, but still both under development, and possibly at a different degree. The observed pattern may also be explained by the fact that fawns that exhibited a calling behaviour were simply fawns that



were more advanced in their behavioural and physiological development. However, we would need to test this hypothesis by investigating the same relationship on the same individuals later in life, which we were not able to do with our current data.

To our knowledge, this study was the first to investigate the individual and environmental drivers of cortisol concentrations during early life in a wild mammal. Our results highlighted the possibility for a rapid development of the HPA axis in roe deer, but also the potential for an effect of maternal stress and human disturbance early in the life of wild animals. Further investigations in other species would be needed to evaluate the potential for human activities to shape stress hormone profiles of newborns and their long-lasting consequences for individual fitness.

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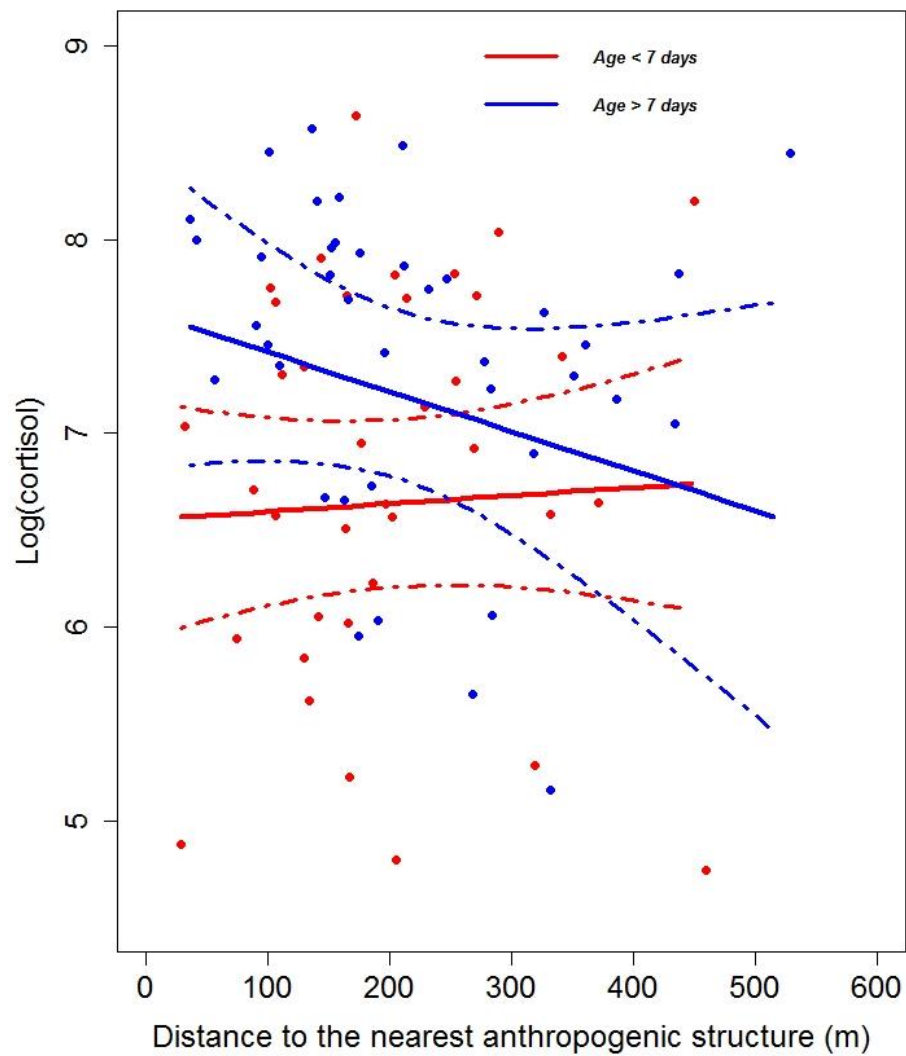
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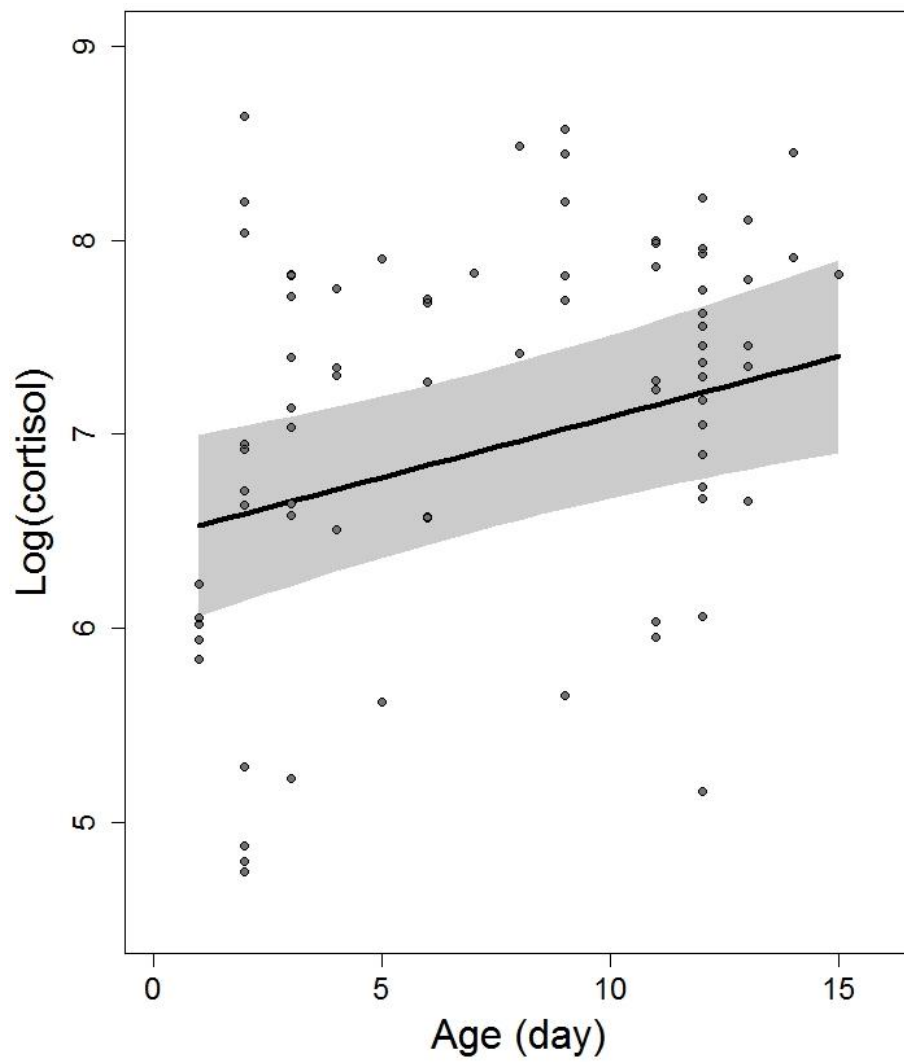


| Parameter                      | Estimate | Lower CI       | Upper CI       |
|--------------------------------|----------|----------------|----------------|
| Intercept                      | 6.14     | <i>5.34</i>    | <i>6.94</i>    |
| Age (days)                     | 0.12     | <i>0.03</i>    | <i>0.22</i>    |
| Calling behaviour (yes)        | 0.65     | <i>0.26</i>    | <i>1.04</i>    |
| Anthropogenic distance (m)     | 0.002    | -0.001         | 0.005          |
| Proportion of woodland patches | 0.72     | -0.08          | 1.52           |
| Age*Anthropogenic distance     | -0.0004  | <i>-0.0007</i> | <i>-0.0001</i> |

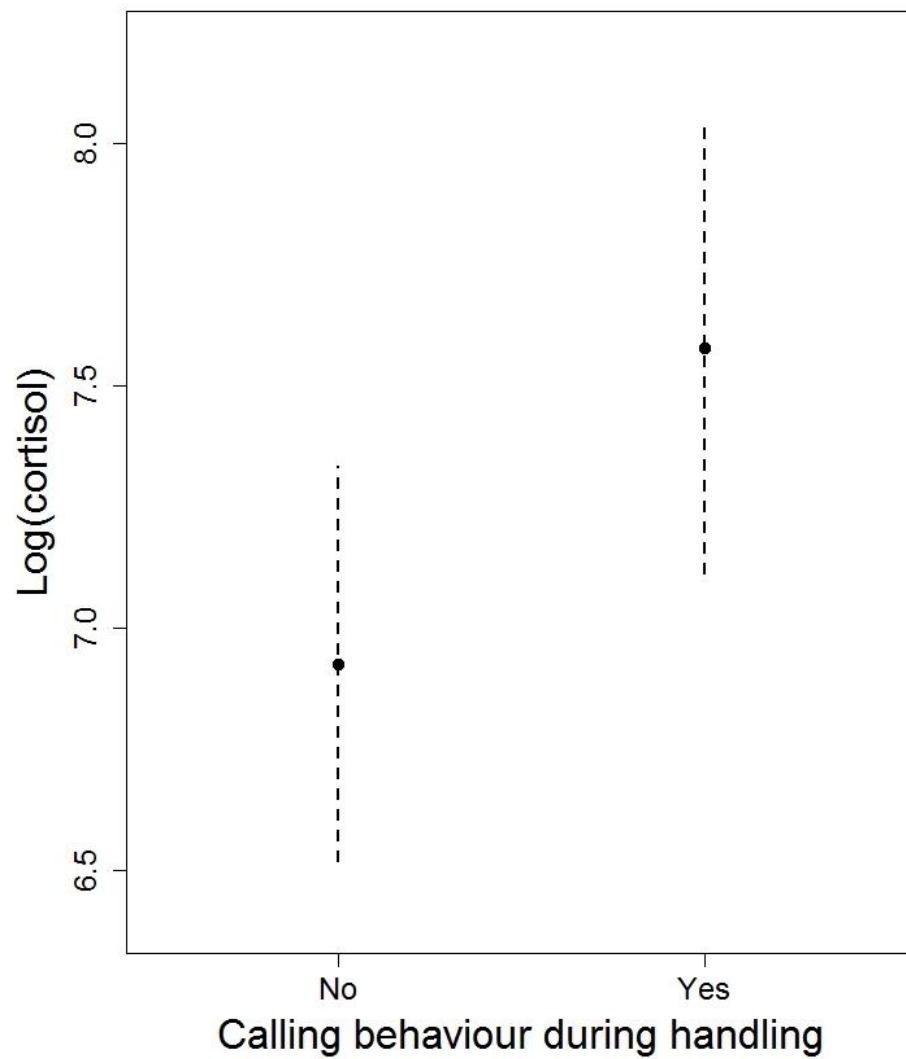
**Table 1.** Characteristics of the selected mixed linear model for explaining variation in salivary cortisol concentrations of roe deer fawns within the 15 first days of their life. Calling behaviour stands for the calling behaviour during handling of the fawn (2 modalities, yes or no); Anthropogenic distance stands for the distance to the nearest anthropogenic structure; Marginal variance ( $R^2_m$ ) of the model was 0.26; conditional variance ( $R^2_c$ ) was 0.64. CI stands for 95% Confidence Interval. See text for definition of model set. CI values that do not overlap 0 are presented in italics.



**Fig. 1** Relationship between salivary cortisol concentrations and distance to the nearest anthropogenic structure in relation to age of roe deer fawn at capture. Points represent observed values, lines represent model predictions and dashed lines represent the 95% confidence interval.



**Fig. 2** Relationship between salivary cortisol concentration variation and age of the fawn at capture. Points represent observed values, lines represent model predictions and shaded area represents the 95% confidence interval.



**Fig. 3** Relationship between offspring salivary cortisol concentrations and calling behaviour of the fawn during handling. Behaviour during handling was expressed in two classes such that the fawn was calling (Yes) or it was not calling (No). The points represent model predictions and the bars represent the 95% confidence intervals.

**Supplementary data 1:** Performance of the subset (within a  $\Delta AICc < 2$  units from the best model) of candidate mixed effect linear models fitted to investigate variation in salivary cortisol concentrations. Model(s) in bold were used for estimation of parameters, and averaged when more than one model was considered after removing models that differed from a higher-ranking model by the addition of one or more parameters. These were rejected as uninformative, as recommended by Richards (2008) and Arnold (2010). Our set of candidate models was composed of all simpler models that included sex, age, body mass, calling behaviour during handling (Calling behaviour), distance to the nearest anthropogenic structure (Anthropogenic distance), proportion of woodland patches (Woodland), and the two-way interactions between distance to the nearest anthropogenic structure and age; and between distance to the nearest anthropogenic structure and probability to use refuge habitats. Individual identity and year of capture were included as random effects to avoid pseudo-replication issues and to control for unexplained variance due to among-individual differences and inter-annual variation. AICc is the value of the corrected Akaike's Information Criterion and K is the number of estimated parameters for each model. The ranking of the models is based on the differences in the values for  $\Delta AICc$  and on the Akaike weights (AICw).

| <b>Models</b>   | <b>K</b> | <b>AICc</b>  | <b><math>\Delta AICc</math></b> | <b>AICw</b> |
|---|----------|--------------|---------------------------------|-------------|
| <b>Age + Anthropogenic distance + Calling behaviour + Anthropogenic distance*Age + Woodland</b> | <b>9</b> | <b>200.5</b> | <b>0.00</b>                     | <b>0.32</b> |
| <b>Age + Anthropogenic distance + Calling behaviour + Anthropogenic distance*Age</b>            | <b>8</b> | <b>200.6</b> | <b>0.01</b>                     | <b>0.31</b> |
| <b>Age + Calling behaviour</b>  | <b>6</b> | <b>201.1</b> | <b>0.57</b>                     | <b>0.24</b> |
| Age + Anthropogenic distance + Calling behaviour  | 7        | 202.4        | 1.86                            | 0.13        |

# **Maternal stress during early gestation does not influence offspring phenotype in fawns of roe deer**

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## Abstract

Maternal stress exposure refers to the ability for maternal hormones such as glucocorticoids to modify the offspring phenotype. While maternal stress has been considered to have unavoidable negative consequences on reproductive success and phenotype of the offspring, a more recent framework proposed it to be viewed as adaptive. Results from the literature remains contradictory and focus mainly on birds, reptiles and small mammals in the wild. Here we used a free-ranging population of a large herbivore, the roe deer *Capreolus capreolus*, to assess whether maternal levels of glucocorticoids during early gestation had negative or positive consequences the early offspring phenotype. Our results did not show any influence of maternal stress on birth weight, growth rate, behaviour during handling and baseline cortisol level of offspring within their twenty first days of life. Our results add to a growing body of literature investigating the effect of maternal stress in the wild. The absence of effect in our study, combined with the contradictory findings within the literature, suggest that further investigations on the mechanisms underlying the effects of maternal glucocorticoids on the phenotype of offspring are needed to better understand if maternal stress may be adaptive or not.

## Introduction

Glucocorticoids are steroid hormones secreted by the hypothalamic-pituitary-adrenal (HPA) axis and that play an important role in the stress response in order to enhance immediate survival (Wingfield and Romero, 2001). However, when individuals are exposed for long period of time to high circulating concentration of glucocorticoids, functions such as growth (Sapolsky et al., 2000) or reproductive success (Schoenle et al., 2019) may be impaired. In addition to these effects during the lifespan of an individual, inter-generational negative consequences of elevated glucocorticoids have been hypothesised through maternal stress exposure (Love et al., 2013). Maternal stress may alter the offspring phenotype through maternal stress hormones, such as glucocorticoids (Love et al., 2013). Negative consequences of maternal stress on body mass, growth, behaviour and physiology of the offspring have first been highlighted by the biomedical literature (Barbazanges et al., 1996; Seckl and Meaney, 2004; Meaney et al., 2007; Weinstock, 2008), and then in wild animals (Meylan and Clobert, 2005; Sheriff et al., 2009; Meylan et al., 2012; Love et al., 2013). However, other empirical studies showed positive consequences of maternal stress on the phenotype of the offspring (Sheriff and Love, 2013; Dantzer et al., 2013; Strange et al., 2016; Rivers et al., 2017). These results led Sheriff and colleagues (2017) to propose a more recent framework examining the adaptive potential of maternal stress. This framework suggests that maternal stress effects adaptively prepare the offspring to their future environment (match between maternal and offspring environment), a view that is supported by empirical studies. Negative and positive consequences of maternal stress are therefore co-existing in the literature, suggesting that more knowledge of the underlying mechanisms are need in order to better understand these contradictory results.



In the wild, most of our knowledge on the long-term consequences of maternal stress relies on studies of birds, reptiles, and relatively small mammals. In the present study, we used a wild population of a large herbivore, the roe deer (*Capreolus capreolus*), living in a fragmented landscape to investigate the potential consequences of maternal stress on the phenotype of fawns. Consequently, Our main aim was to test whether maternal stress could explain fawn's phenotype, while considering other potential determinants of fawn performances, namely other maternal traits (boldness and body condition), and environmental conditions (quality of the available resources during the year of birth). Regarding the phenotype of the fawn, we focused on fawn's growth (birth weight, growth rate) and stress reactivity (indexed by both the baseline glucocorticoid levels and behaviour during stressful situation). According to the adaptive hypothesis of maternal stress (Sheriff et al., 2017) we expected mothers with high glucocorticoid levels and/or shy ones to prepare their offspring to have high performance in a challenging environment. This should include the production of fawns with a high birth weight and growth rate, but also relatively high baseline glucocorticoid levels to support the associated energetic needs, and finally, a tendency to be shy in order to improve hiding behaviour to reduce the risk of predation.

## Material and methods

### Study site

This study was carried out on a population of wild roe deer living in south west of France, near the Aurignac canton (43°13'N, 0°52'E, 10 000 ha). This population experiences an oceanic climate with summer droughts and lives in a fragmented agricultural landscape mostly composed of meadows and cultivated fields (respectively 31% and 36% of the study site), inter-mixed with small woodland patches (19%) and two main forests (14%; see Martin et al.,

2018 for further details). The open habitats provide abundant and high-quality food resources for roe deer most of the year (Abbas et al., 2011), but they may be associated with potential sources of stress due to higher intensity of human activities compared to forest habitat (Bonnot et al., 2013). This roe deer population is hunted on a regular basis by stalking (bucks only) during summer (June-August) and by drive hunts with dogs during autumn-winter (September - February). The quality of the available resources in the environment may vary among years. To take into account this temporal variation, quality of the year was assessed using the population average body mass of juveniles (approximately 8 months old) captured the following winter to control for annual variation in resource availability and quality (see Hamel et al., 2009).

### **Data collection on mothers**

As part of a long-term capture-mark-recapture program initiated in 1996, 6 days of capture occur each year, between the beginning of January and the beginning of March. We used large-scale drives with 30 to 100 beaters and up to 4 km of long-nets. Upon capture, deer were transferred to a wooden retention box providing darkness and ventilation until the marking procedure during which faecal samples were collected directly from the rectum during the marking procedure and held at  $-4^{\circ}\text{C}$  for a maximum of 3h before being stored at  $-20^{\circ}\text{C}$  until analyses. In addition, each individual was weighed (to the nearest 10 g), and sex and age (expressed as two classes: juveniles for 8-10 months-old individuals vs. adults for >18 months-old individuals) were recorded. A behavioural trait, struggling behaviour during handling, that indexed boldness, was also assessed. A score of 0 was attributed if the individual did not struggle while a score of 1 was attributed if the individual struggled. This behavioural trait has been shown to be repeatable over time ( $r=0.27$ ) with a tendency to be heritable ( $h=0.15$ ) in

roe deer (Gervais et al., submitted). In addition to the captures occurring at the beginning of the year, post-weaning observations (October-November) of mothers are realised and the number of fawns following them are determined in order to determine recruitment. Thereafter we used this variable as an index of the yearly reproductive success of mothers with two modalities: the mother was not followed by a fawn (0); the mother was followed by at least one fawn (1).

All captures and marking procedures were done in accordance with French and European laws for animal welfare (prefectural order from the Toulouse Administrative Authority to capture and monitor wild roe deer, agreement no. A31113001 delivered by the Departmental Authority of Population Protection, ethical authorisation by French Government).

### **Data collection on offspring**

Initiated in 2004, the capture-mark-recapture program of fawns occurs each year between the end of April and the middle of June. Between 2004 and 2018, newborn fawns (on average 5.8 days old at capture) were caught by hand. The marking procedure was performed once the mothers moved away from the fawns. Fawns were sexed, aged and struggling behaviour during handling was recorded with two modalities (0: did not struggle; 1: struggled). The age of fawns was established in relation to daily monitoring of the mother's appearance (belly size, presence/absence of visible udders) and behaviour (reaction to a whistle mimicking the call of a fawn), and in relation to the fawn's behaviour (Jullien et al., 1992). Birth weight of fawns was determined using the regression slope  $\beta$  of a model fitting an effect of age on fawns' weight such that birth weight = weight - ( $\beta$  \* age). In addition, a salivary sample was taken from each captured fawn, using a cotton swab (SalivaBio Children's Swab; Salimetrics) and conserved at -20°C within 2 hours until further analyses. Samples were collected in order

to further assess baseline cortisol levels. Collection occurred within 10 minutes following the fawn's capture as salivary cortisol is expected to reflect baseline cortisol within a window of 30 minutes following disturbance (Sheriff et al., 2011). Approximately half of the fawns were captured twice, with the recapture occurring on average one week after the first capture, i.e. at 12 days old. We were therefore able to calculate early individual growth rates of each fawn as the weight gain between the two captures, divided by the number of days elapsed.

### **Faecal cortisol metabolites (FCMs) extraction and analyses**

FCMs were extracted following a methanol-based procedure and assayed using a group-specific 11-oxoaetiocholanolone enzyme immunoassay (EIA), as previously described (Möstl and Palme, 2002) and validated for roe deer (Zbyryt et al., 2017). Briefly, each faecal sample was homogenised and  $0.5 \pm 0.005$  g of homogenate was transferred to a glass tube containing 5 ml of a 80% methanol solution. The suspended samples were vortexed at 1500 rpm for 30 min and centrifuged at 2500 g for 15 min (Palme et al., 2013). An aliquot of the supernatant was further diluted (1:10) with assay buffer prior to EIA analysis. Measurements were carried out in duplicate (intra- and inter-assay coefficients of all samples were less than 10% and 15%, respectively) and the results expressed as nanograms per gram of wet faeces (ng/g).

### **Salivary cortisol analyses**

Cotton swabs that were used for the salivary collection during handling of fawns were centrifuged at 1500 g for 15 min and an aliquot of the supernatant was diluted (1:2) with assay buffer prior to EIA analysis with a commercial kit (Item No. 1-3002; Salimetrics). Measurements were carried out in duplicate (intra- and inter-assay coefficients of all samples were less than 10% and 15%, respectively) and results expressed as picograms per millilitre (pg/mL).

## Statistical analyses

We analysed variation in fawn growth rate, birth weight and baseline cortisol using linear models, and we analysed fawn struggling behaviour during handling with logistic generalised linear models. We built five sets of candidate models (Supplementary data 1) testing for a specific hypothesis or possible confounding effects. In each model we included the effects of mother's FCM levels (log transformed), mother's struggling behaviour during handling, mother's weight, quality of the year, and sex of the fawn. In addition to the variables mentioned above, we included the effect of age in models examining variations in fawns' struggling behaviour and baseline cortisol levels.

To select the best models we used a model selection procedure based on the second-order Akaike Information Criterion (AICc, Burnham and Anderson, 2002) and retained the model with the lowest AICc. In addition, we calculated AICc weights (AICcw) to measure the relative likelihood that a given model was the best among the set of fitted models. Given that our study was explorative, following Garamszegi and colleagues (2009), we did not use inferential statistical tests based on P values. Normality of the residuals for the selected models was tested (Shapiro-Wilk test) and visually assessed with histograms. Goodness-of-fit was assessed by calculating  $R^2$  values for linear models, pseudo  $R^2$  ( $1 - [\text{Residual Deviance}/\text{Null Deviance}]$ ) for generalised linear model, and standard residual plot techniques. All analyses were carried out with R version 3.6.0 (R Development Core Team 2016).

## Results

Regarding the potential effects of maternal cortisol levels on the offspring phenotype, none of the traits considered in our analyses were related to maternal FCM levels (Table 1). Instead,

growth rate was higher in offspring from heavier mothers (Fig. 1), and males were heavier at birth compared to females (Table 1). Baseline cortisol levels of fawns were related to the behaviour of their mother (Table 1), such that bolder mothers produced offspring with lower baseline cortisol levels (Fig. 2). However, the model including the effect of the quality of the year was very close ( $\Delta AICc < 0.2$ ) such that baseline cortisol levels of fawns were higher during poor quality years compared to better years ( $-6.26$ ;  $CI = [-12.77, 0.26]$ ). Finally, offspring's behaviour was not linked to mother's phenotype, but rather to quality of the year (Table 1), with bolder offspring being produced during good quality years in term of resources (Fig. 3).

## Discussion

In this study, we aimed to explore the potential consequences of maternal stress on the offspring's phenotype. Overall, our data did not support any negative or positive influence on growth rate, birth weight, boldness and baseline cortisol levels. In line with previous studies, growth rate, a particularly important trait for survival which determines the duration of the period where fawns are vulnerable to external factors (Pontier et al., 1989, Plard et al., 2014) was influenced by mother's weight. In roe deer, maternal morphological traits such as body mass, age, and previous reproductive success are known to generate marked individual differences in female reproductive success (Gaillard et al., 2000). Birth weight, another trait influencing early survival through sensitivity to disease, predation and weather conditions (Singer et al., 1997; Verme 1963), was influenced by the sex of the offspring, males being heavier than females, in line with the sex allocation theory (Trivers and Willard, 1973), even if this results appears quite surprising in a species with low sexual dimorphism. Then we found that in good quality years, bolder offspring were produced. According to the pace of life framework (Réale et al., 2010) bolder individuals are expected to also exhibit higher activity

levels and to be more superficial explorer of their environment than shier individuals. In good quality years, where resources are widespread, bold phenotype must therefore have a higher adaptive value than in bad years where resources are scarce and through exploration with reduced activity would be more adaptive. If fawns are dependent of their mother during the first weeks of their life, they start to feed on their own around five months of age, and their growth rate may therefore be influenced by their ability to exploit resources. Finally, we found that offspring with higher baseline cortisol levels were produced by shier mothers. Within an individual, the coping style framework predicts shier individuals to exhibit higher glucocorticoid levels, and conversely bolder to exhibit lower levels (Koolhaas et al., 1999). In addition, we observed a trend for baseline cortisol levels to depend on the quality of the year, with higher cortisol levels during poor quality years compared to better ones. Combined with our result on the behavioural stress response, this would mean that during years of poor quality (challenging conditions), fawns tended to shy and to exhibit high baseline cortisol levels, which is coherent with the coping-style framework (Koolhaas et al., 1999). In addition, our index of the quality of the year could reflect the state of the mothers during the period of birth, but also of rearing. Indeed, as mentioned in the material and methods section, our index is based on juveniles body mass, which highly depend on the availability of resources during spring (i.e. the period of fawns' birth). Previous studies have shown that quality and availability of resources might influence faecal cortisol levels of adult roe deer (Carbillet et al., in press). Consequently, our results might also reflect a link between cortisol levels of the mother right before and after fawn's birth, and cortisol levels of the fawn, such that mothers with higher FCM levels would produce fawns with higher cortisol levels, which would be in accordance with one of our hypothesis.

However, this last point highlights one limitation of our study. While our study is one of the first to explore the influence of maternal stress on reproduction and offspring phenotype in a wild and large mammal, the timing of our sampling was not ideal. Indeed, mothers' FCM levels were evaluated during winter (January-February), while birth of fawns occurs in spring (May-June). Roe deer exhibit a delayed implantation with a gestation that starts right after the rut (summer), but a placental attachment that occurs only at the beginning of January and is followed by 5 months of normal gestation (Aitken, 1974). This means that our measures of cortisol occurred at the beginning of the possible placental transmission of the hormones to the offspring. While consistent among-individual differences in cortisol levels occurs in roe deer (Carbillet et al. in press), this hormonal trait remains quite labile within a year. A recent comparative analysis across mammals on the effect of maternal stress on offspring growth revealed that the timing of exposure to maternal stress was an important factor (Berghänel et al., 2017) to consider. According to this comparative analysis, most of the studies investigated the effect of maternal stress early during gestation on early growth rate of offspring found no effect. Our results regarding the effects of FCM levels of mother are therefore in accordance with this finding, but also highlight the need to consider different windows of measures to get a better understanding of maternal stress consequences on the offspring phenotype.

Altogether, our results did not find any consequences of maternal stress during early gestation on early phenotypic characteristics of fawns. However, this does not mean that maternal stress has no consequences, but rather that further investigation should be done in order to expand our understanding and to which extent maternal stress may be adaptive or not. Precisely, considering different measures of maternal glucocorticoid levels at different stages of gestation, but also several measures of the offspring phenotype, for instance until dispersal



in the case of roe deer, should help to improve our knowledge on maternal stress consequences in large herbivores.

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| Parameter  | Estimate | Lower CI | Upper CI |
|--|----------|----------|----------|
| <b>Growth rate</b> (n = 28)<br>(R <sup>2</sup> :0.22)              |          |          |          |
| Intercept  | -80.59   | -231.37  | 70.19    |
| Mother's weight (kg)   | 9.67     | 2.84     | 16.49    |
| <b>Birth weight</b> (n = 46)<br>(R <sup>2</sup> :0.11)             |          |          |          |
| Intercept  | 1236.00  | 1094.36  | 1377.64  |
| Sex: males   | 224.81   | 48.37    | 401.242  |
| <b>Baseline cortisol levels</b> (n = 14)<br>(R <sup>2</sup> :0.21) |          |          |          |
| Intercept  | 7.23     | 6.34     | 8.12     |
| Mother's boldness (1)  | -1.33    | -2.69    | 0.03     |
| <b>Boldness</b> (n = 39)<br>(R <sup>2</sup> :0.13)                 |          |          |          |
| Intercept  | -39.09   | -75.45   | -7.28    |
| Year quality (kg)  | 2.14     | 0.35     | 4.19     |

**Table 1** - Characteristics of the selected linear models (growth rate, birth weight, baseline cortisol levels) and generalised linear models (boldness, reproductive success) for explaining variation in reproductive success and the 4 phenotypic traits of fawns considered. Boldness stands for the struggling behaviour during handling (0: no struggle; 1: struggle). R<sup>2</sup> referred to the explained variance of the models. CI stands for 95% Confidence Interval. See text for definition of model sets. CI values that do not overlap 0 are presented in italics.

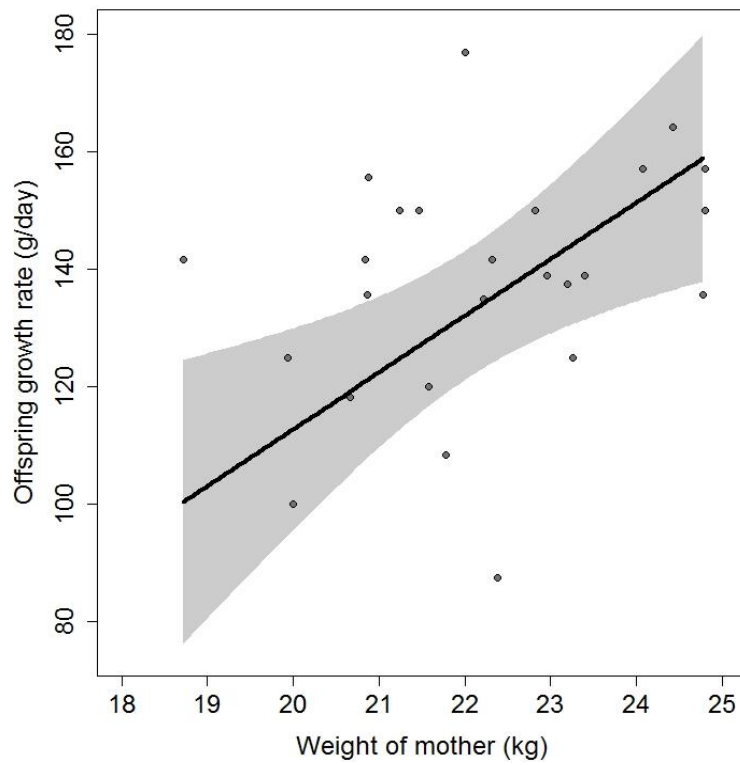


Fig. 1 Relationship between offspring growth rate variation and mother's weight variation. Points represent observed values, lines represent model predictions and shaded area represents the 95% confidence interval.



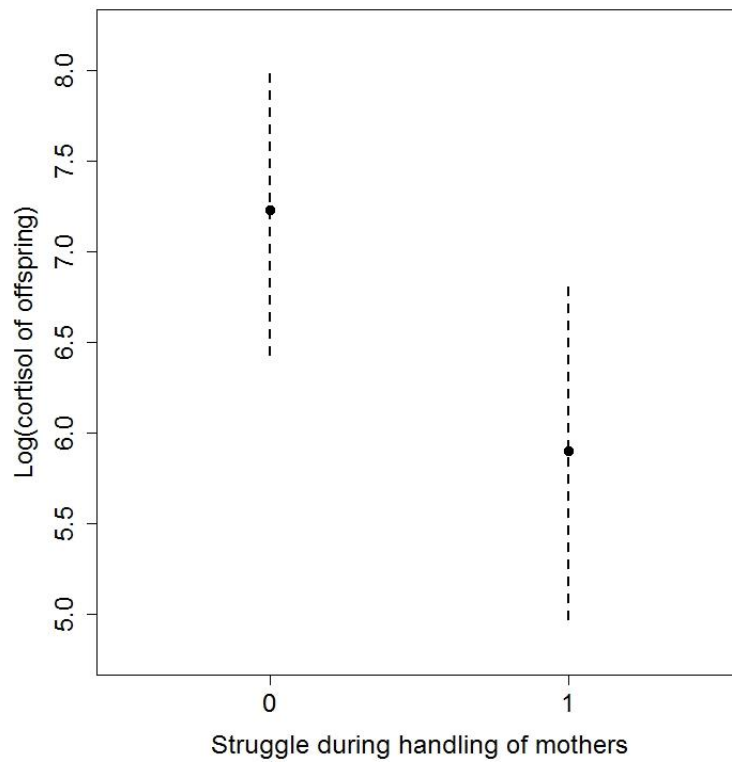


Fig. 2 Relationship between offspring baseline cortisol levels and the struggling behaviour of the mothers during handling. Behaviour during handling was expressed in two classes with a score of 0 (no struggle) and 1 (struggle). Points indicate observed values. Lines represent model predictions and the dashed lines represent the 95% confidence interval.

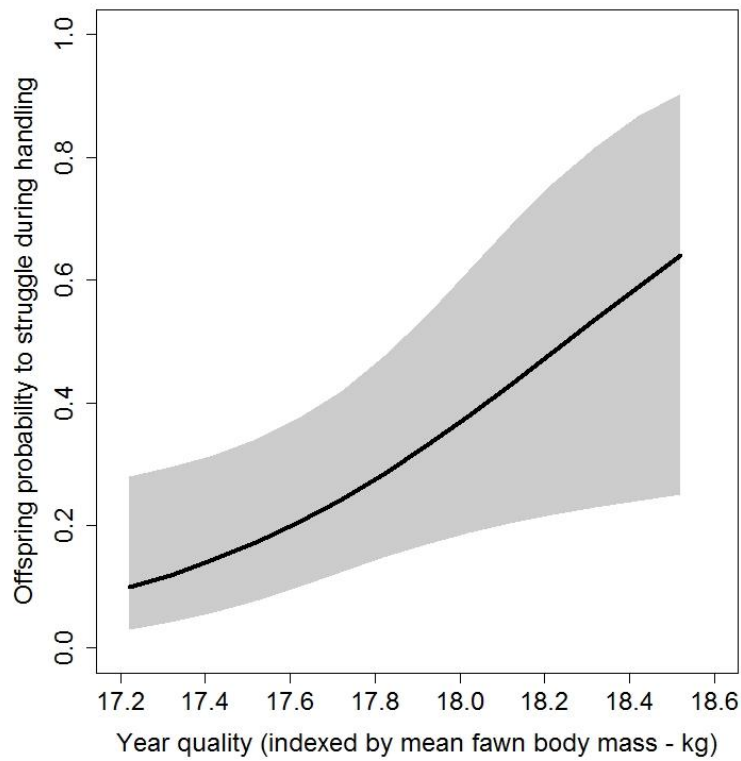


Fig. 3 Probability for an offspring to struggle during handling in relation with the quality of the year. Year quality was indexed using the population average body mass of juveniles (approximately 8 months old) captured during the following winter. Lines represent model predictions and shaded area represents the 95% confidence interval.

**Supplementary data 1:** Performance of the candidate linear models (A, B, C) and generalised linear models (D) fitted to investigate variation in the four phenotypic traits of fawns considered (growth rate, birth weight, baseline cortisol level, struggling behaviour during handling). Candidate models included a single variable or additive effects testing for a specific hypothesis or possible confounding effects. In each model we included the effects of mother's faecal cortisol metabolite (FCM) levels, mother's struggling behaviour during handling (boldness), mother's weight, quality of the year (year quality), and sex of the fawn. In addition to the variables mentioned above, we included the effect of age in models examining variations in fawns' struggling behaviour and baseline cortisol levels. AICc is the value of the corrected Akaike's Information Criterion and K is the number of estimated parameters for each model. The ranking of the models is based on the differences in the values for  $\Delta AICc$  and on the Akaike weights (AICw). Number of individuals available for the analysis are mentioned in brackets.

A) Growth rate variation (n = 28)

| Models            | K | AICc   | $\Delta AICc$ | AICw |
|-------------------|---|--------|---------------|------|
| Mother's weight   | 3 | 274.41 | 0.00          | 0.81 |
| Sex               | 3 | 279.16 | 4.75          | 0.08 |
| Nul               | 2 | 279.80 | 5.38          | 0.05 |
| Year quality      | 3 | 281.08 | 6.67          | 0.03 |
| Mother's FCM      | 3 | 282.09 | 7.68          | 0.02 |
| Mother's boldness | 3 | 282.23 | 7.82          | 0.02 |

B) Birth weight variation (n = 46)

| Models            | K | AICc   | $\Delta AICc$ | AICw |
|-------------------|---|--------|---------------|------|
| Sex               | 3 | 639.67 | 0.00          | 0.74 |
| Null              | 2 | 643.80 | 4.13          | 0.09 |
| Mother's FCM      | 3 | 644.82 | 5.14          | 0.06 |
| Mother's weight   | 3 | 645.09 | 5.42          | 0.05 |
| Mother's boldness | 3 | 646.00 | 6.33          | 0.03 |
| Year quality      | 3 | 646.01 | 6.34          | 0.03 |

C) Baseline cortisol variation (n = 14)

| Models            | K | AICc  | $\Delta AICc$ | AICw |
|-------------------|---|-------|---------------|------|
| Mother's boldness | 3 | 50.04 | 0.00          | 0.29 |
| Year quality      | 3 | 50.19 | 0.15          | 0.27 |
| Null              | 2 | 51.24 | 1.20          | 0.16 |
| Sex               | 3 | 51.99 | 1.94          | 0.11 |
| Age               | 3 | 52.50 | 2.46          | 0.09 |
| Mother's FCM      | 3 | 54.21 | 4.17          | 0.04 |
| Mother's weight   | 3 | 54.33 | 4.29          | 0.03 |

D) Boldness variation (n = 39)

| Models            | K | AICc  | $\Delta AICc$ | AICw |
|-------------------|---|-------|---------------|------|
| Year quality      | 2 | 41.00 | 0.00          | 0.59 |
| Null              | 1 | 44.24 | 3.24          | 0.12 |
| Mother's boldness | 2 | 44.94 | 3.94          | 0.08 |
| Mother's weight   | 2 | 46.16 | 5.15          | 0.04 |
| Sex               | 2 | 46.26 | 5.25          | 0.04 |
| Age               | 2 | 46.26 | 5.25          | 0.04 |
| Weight            | 2 | 46.35 | 5.35          | 0.04 |
| Mother's FCM      | 2 | 46.43 | 5.42          | 0.04 |





## **Chapter 3**

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# **Consequences of baseline stress levels on immunity**





# **Into the wild of the stress-immunity relationship, a case study on roe deer *Capreolus capreolus***

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## Abstract

Due to the dynamic and unpredictable nature of their environment, organisms face many stressful situations in their lifetime. One of the main mechanisms that have evolved to cope with these perturbations is the stress response which triggers the secretion of glucocorticoid hormones. Although short-term elevations of these hormones is often associated with immediate beneficial consequences for individuals, long-term glucocorticoids elevation can compromise survival and reproductive success, and affect key physiological functions such as immunity. While previous works have highlighted the immunosuppressive effect of long-term elevated glucocorticoids, how this relationship may be modulated by individual and environmental characteristics remains largely unknown, especially in wild animals. In this study, we explored the co-variation between baseline cortisol levels (faecal cortisol metabolites, [FCM]) and 12 constitutive indices of innate and adaptive immune functions in wild roe deer living in three contrasting habitats. Using longitudinal data on 564 individuals, we further investigate whether age, behavioural traits and spatio-temporal variations in the quantity and quality of resources may affect the relationship between baseline cortisol levels and immunity. Our data indicates that the immunosuppressive effect of cortisol was evident only in one of the three studied populations, which presented the most unfavourable environment for roe deer. This immunosuppressive effect of cortisol was exacerbated in individuals facing severe environmental constraints, in those with low physical condition, and in older individuals, whereas behavioural traits did not affect the relationship. Our results therefore highlight the importance of considering individual and environmental characteristics in order to better understand the relationship between glucocorticoid hormones and immunity in wild animals. Such an integrative approach may help to explain



the contradictory results found in the literature, but could also increase our understanding of the long-term consequences of elevated glucocorticoid levels on population dynamics.

## Keywords

stress hormones, immune functions, *Capreolus capreolus*, innate immunity, adaptive immunity, faecal glucocorticoid metabolites

## 1. Introduction

One of the main physiological responses organisms have evolved to cope with stressful situations is the stress response (Sapolsky et al., 2000; Wingfield and Romero, 2001). Exposure to stressors stimulates the hypothalamic-pituitary-adrenal (HPA) axis and leads to the production and release of glucocorticoids (i.e. corticosterone and cortisol) into the bloodstream by the adrenal gland cortex (Sapolsky et al., 2000; Sheriff et al., 2011; Hau et al., 2016). This hormonal cascade is then subjected to a negative feedback exerted by glucocorticoids on their own secretion (Romero, 2004; Sheriff et al., 2011), which facilitates a return to a baseline level that ensures the maintenance of daily activities according to the current life-history stage (Möstl and Palme, 2002; Wingfield and Sapolsky, 2003). While short-term elevation of glucocorticoids promotes survival (Sapolsky et al., 2000; Breuner et al., 2008), chronic elevation of glucocorticoids can alter physiological functions and ultimately compromise both survival and reproductive success (Boonstra, 2005). Among these functions, the immunosuppressive effect of glucocorticoids is one of the most described deleterious effects of chronic stress (Martin et al., 2009).

Immunity is a key physiological function in the vertebrates because of its crucial role for survival (Sorci et al., 2008). In vertebrates, the immune system is composed of the innate and adaptive arms (Stanley, 2002), each comprising cellular and humoral effectors (Stanley, 2002). While innate immunity sets up rapidly (within a few hours) and is mostly non-specific, adaptive (memory-based) immune response deals with repeated infections and selectively eliminates pathogens (Lee, 2006). Like any other physiological function, the immune system requires energy (Lee, 2006; Martin, 2009) but costs varies depending on the stage (development, maintenance or activation) and arm of immunity (Lee, 2006). Typically, adaptive immune response is thought to have a relatively lower activation cost than the innate immune response, the inflammatory response being particularly energy demanding (McDade, 2016). Due to its cost, immune function has been hypothesised to trade-off against other other energy demanding functions (Martin, 2009), and long-term elevation of glucocorticoid levels may redirect the energy away from immunity towards functions immediately enhancing survival (Lee, 2006; Martin, 2009).

In accordance with the hypothesis that stress derives resources toward immediately important functions, most empirical studies in this field showed that chronic stress impaired immune functions (Dhabhar and McEwen, 1997; Sapolsky et al., 2000; Dhabhar, 2009), increasing susceptibility to external aggressions. However, most of our knowledge is based on studies conducted on laboratory or domestic animals (Dhabhar et al., 1994; Dhabhar et al., 1995; Wada et al., 2010; Moazzam et al., 2012) while very little is known in wild populations (but see Bourgeon and Raclot, 2006; Brooks and Mateo, 2013). In wild populations, fluctuations of environmental conditions in terms of resources, meteorology, predator risk and disease threats could lead to different outcomes regarding the relationship between stress and immunity. In addition, a large body of literature showed that diverse factors could

influence immune responses (Lee, 2006; Martin, 2009) but also glucocorticoid secretions (Hau et al., 2016). For instance, poor environments (in term of resource quantity or quality) can lead to increased glucocorticoid levels (Fokidis et al., 2012) and decreased concentrations of immune parameters (Dhabhar et al., 1994; Dhabhar et al., 1995). More generally, trade-offs may become apparent in situations of scarce or poor-quality of resources, while they may pass unnoticed when abundant resources provide enough energy to engage in all functions. Behavioural traits may also be related to stress and immune responses such that coping-style (i.e. behavioural and physiological efforts to master stressful situations Koolhass et al., 1999) and immune function could be linked (Lee, 2006). Baseline glucocorticoid levels have also been shown to be age-related (Sapolsky et al., 1983) as well as several immune parameters (Nussey et al., 2012). Therefore, the relationship between immune functions and baseline glucocorticoid concentrations appears complex and may be shaped by several individual and environmental factors whose relative contribution is still poorly understood..

The aim of this study was to analyse variations in baseline glucocorticoid levels and immune functions, and to explore how these relationships were affected by both individual and environmental characteristics in three wild populations of roe deer (*Capreolus capreolus*). Baseline glucocorticoid level was assessed by measuring faecal cortisol metabolites (FCM) which reflects the overall circulating glucocorticoids that an individual has experienced over the preceding days (Palme et al., 2005; Sheriff et al., 2011). Immune function was assessed by measuring twelve immune parameters encompassing both the innate (neutrophils, eosinophils, basophils, monocytes, hemagglutination, hemolysis, alpha-1-globulin, alpha-2-globulin, beta-globulin, haptoglobin) and adaptive (lymphocytes, gamma-globulin) arms of immunity.

Based on our current knowledge, we expected that individuals with higher baseline glucocorticoid levels would exhibit depressed innate and adaptive immunity compared to individuals with lower baseline glucocorticoid levels. More precisely, we expected (i) that high levels of FCMs would be associated with a decrease in leukocyte concentrations (neutrophils, eosinophils, basophils, monocytes), and a decrease of inflammatory components (i.e. alpha-1-globulin, alpha-2-globulin, beta-globulin and haptoglobin) due to their high activation cost (Dhabhar et al., 2012; Brooks and Mateo, 2013; McDade et al., 2016). We also expected a weak effect of FCMs on gamma-globulin and lymphocyte concentrations due to their lower activation cost (Lee, 2006). In addition, we expected that (ii) the effects of baseline glucocorticoid levels on immune parameters would be greater in individuals facing poor environmental conditions such as low availability in food resources, compared to individuals in favourable environments (Hau et al., 2016), (iii) that older individuals would exhibit a more pronounced relationship between FCM levels and their immune parameters than younger individuals (Cheynel et al., 2017), and (iv) that the relationship should be more pronounced for less docile and more exploratory individuals compared to more docile and less exploratory ones (Barber and Dingemanse, 2010).

## **2. Material and methods**

### **2.1 Study sites**

This study was conducted on three wild populations of roe deer living in contrasting habitats.

The Trois-Fontaines population is located in an enclosed forest (1360 hectares) in the north-east of France (48°43' N, 4°55' E). The climate is continental and the soil is particularly rich,

making it a productive forest habitat. This forest offers a homogeneous habitat of high quality resources for roe deer (Pettorelli et al., 2006).

The Chizé population is located in an enclosed forest (2614 hectares) in western France (46°05' N, 0°25' W). The climate is temperate oceanic, with Mediterranean influences. Due to poor soil quality and frequent summer droughts, the forest productivity is low compared to Trois-Fontaines (Pettorelli et al., 2006), making it a relatively poor quality habitat for roe deer in terms of resources (Gaillard et al., 1993). At a finer scale, three areas (sectors) are distinguished according to the quantity and quality of resources (Pettorelli et al., 2001): sector 1 is composed of oaks (*Quercus spp.*) and hornbeam (*Carpinus betulus*) and is considered to be of high quality compared to the other two sectors; sector 2 is composed of oaks and Montpellier maples (*Acer monspessulanum*) and is considered to be of medium quality; and sector 3 is composed of beeches (*Fagus sylvatica*) and is of poor quality. Pettoirelli and colleagues (2003) reported that these differences in habitat resulted in differences in roe deer body mass, with individuals in sector 1 being on average 2 kg heavier than those in sector 3.

Finally, the Aurignac population is located in an agricultural landscape (10 000 hectares), in south-western France (43°13' N, 0°52' E). This site is exposed to an oceanic climate, with summer droughts (Hewison et al., 2007). It provides a highly heterogeneous environment with a fragmented landscape composed of forests, grassland and cultivated fields (see Martin et al., 2018 for details). This study site can also be divided into three sectors according to resource quality and habitat openness (see Morellet et al., 2009 for details). The most open habitats (sector 1) offers more important and high quality food resources for roe deer during most of the year (Abbas et al., 2011), but can also be a source of greater exposure to stressors (Bonnot et al., 2013) compared to the partially wooded area (sector 2) and woodland (sector

3). Hewison and colleagues (2009) have shown a beneficial effect of landscape openness on roe deer reproduction, demographic performance and juveniles body mass, with juveniles in sector 3 weighing on average 2.0 kg less than in sector 2, and 3.1 kg less than in sector 1 (Hewison et al., 2009).

## **2.2 Data collection**

As part of long-term capture-mark-recapture programs initiated in 1975, 1977 and 1996, in Trois-Fontaines, Chizé and Aurignac respectively, 6 to 12 days of drive net captures are organised between December and March each year. At each capture session, between 30 and 100 beaters push the roe deer towards nets deployed over 4 km. Once captured, each animal is marked, weighed (to the nearest 10g), sexed, and age is determined by tooth eruption patterns in Aurignac (Hewison et al., 1999), with 2 age classes: juveniles (< 1 year) and adults (> 1 year). For the Trois-Fontaines and Chizé populations, precise age is known for all individuals studied here, since they were caught for the first time during their first year of life. Blood samples are taken from the jugular vein (up to 1 mL/kg) since 2009 in Aurignac and since 2010 in Trois-Fontaines and Chizé. For each individual, faeces were collected rectally and were stored at -20°C until extraction. In addition, part of the collected blood is transferred to a dry tube, centrifuged and the plasma conserved at -20°C for biochemical analyses. The remaining blood is transferred to a tube containing EDTA for further determination of immune parameters (see below). In addition, the behaviour during handling has been recorded for each individual since 2009 in Aurignac. Behaviour during handling is repeatable over time ( $r=0.27$ ), tends to be heritable ( $h=0.15$ ) in this population, and has previously been used to index docility (Gervais et al., submitted). A score of 0 was attributed to individuals that reacted

in a passive way to handling and did not struggle, while a score of 1 was attributed to individuals that reacted in a non-passive way and struggled.

## **2.3 Measurement of FCMs**

Faecal cortisol metabolite extraction was performed following the protocol developed by Palme and colleagues (2013). For each individual, 5mg of faeces was suspended in 5mL of 80% methanol, vortexed for 30 minutes and centrifuged for 15 min at 2500 g. Supernatant was diluted 1:10 with assay buffer before FCM concentration was determined with a group specific 11-oxoaetiocholanolone enzyme immunoassay as previously described for roe deer (Zbyryt et al., 2017). Measurements were carried out in duplicate (intra- and inter-assay coefficients of all samples were less than 10% and 15%, respectively) and the results expressed as nanograms per gram of wet faeces (ng/g).

## **2.4 Measurement of immune parameters**

Data on immune parameters and FCMs used for Trois-Fontaines were available for the years 2010 to 2017, for Chizé for the years 2013, 2014, 2016 and 2017. For the Aurignac population, for neutrophils, eosinophils, basophils, monocytes and lymphocytes, analyses were performed on data from the years 2012 to 2017. For hemagglutination and hemolysis, analyses were performed on data from years 2013 to 2017, while for gamma-, alpha-1-, alpha-2- and beta-globulin, analyses were performed on data from years 2014 to 2017.

### **2.4.1 Innate cellular immunity**

For this component of immunity, white blood cells (WBC) count was carried out by impedance technology (ABC Vet automaton, Horiba Medical) and the proportion of each WBC type

(neutrophils, monocytes, lymphocytes, eosinophils and basophils) were quantified under microscope (x1000) by counting the first 100 WBC on blood smears, previously stained with a May-Grünwald and Giemsa solution (see Houwen, 2001 for more details). The concentrations of each type of leukocyte were then determined as  $(WBC \times \text{parameter cells count})/100$ . Neutrophil, eosinophil, basophil and monocyte counts were considered to represent mainly innate cellular immunity (Cheynel et al. 2017).

#### **2.4.2 Innate humoral immunity**

Innate humoral immunity was assessed by measuring circulating levels of natural antibodies (NABs) and complement activity of serum. The concentration of NABs was measured by the hemagglutination test (HA) that measures NABs ability to agglutinate exogenous cells (Matson et al., 2005). The complement is a group of proteins that acts in a chain reaction and causes lysis of exogenous cells in presence of an antigen-antibody complex. It is revealed by the ability of proteins to induce hemolysis (HL) (Matson et al., 2005). The HA/HL protocol defined by Matson et al., (2005) in birds has been successfully adapted and previously used in roe deer (Gilot-Fromont et al., 2012). Innate humoral immunity was also evaluated using levels of alpha-1, alpha-2, and beta globulins. The total concentrations of these proteins (in g/L) were quantified by a refractometer followed by an electrophoresis on agarose gel, using an automaton (HYDRASYS). Haptoglobin concentration (in mg/mL), which belongs to the alpha-2-globulin fraction and reflect infection or chronic inflammation, was also measured. Analyses were performed from a dry tube stored at 4°C with a Konelab 30i PLC (Fisher Thermo Scientific) which operates on the principle of spectrophotometry. Unlike other immune parameters, haptoglobin was measured only at Trois- Fontaines and Chizé.



### **2.4.3 Adaptive cellular immunity**

Adaptive cellular immunity was assessed using lymphocyte concentrations, determined by the previously described leukocyte count, which includes both B and T lymphocytes.

### **2.4.4 Adaptive humoral immunity**

The adaptive humoral immunity component was assessed using concentration of gamma globulins, obtained by the protein analysis described above for other globulins. Gamma globulin concentration has been used as an estimator of total antibodies, since they are essentially composed of circulating antibodies (Stockham and Scott, 2008).

## **2.5 Statistical analyses**

For each of the 12 immune parameters, we ran separated analyses for each population, using linear mixed effect models (LMMs). The different immune traits were each treated as a response variable and some of them (counts of eosinophils, basophils, monocytes, lymphocytes, and concentrations of haptoglobin, alpha-1, alpha-2 and beta globulins) were transformed as  $\log(x+1)$  to ensure normality of model residuals. Model selection was carried out by adjusting a reference model that included all biologically relevant variables and interactions to test our hypotheses, and then comparing this model with all its sub-models. The reference model included:

i) Individual variables: baseline stress level (FCM), transformed into logarithms and centred, sex, centred weight, age (considered to have either a linear effect, a quadratic function or a threshold effect for Chizé and Trois-Fontaines). The choice of the function was based on a previous study carried out on the same two populations (Cheynel et al., 2017). In Aurignac,

age was considered with two classes (juveniles and adults). In addition, for Aurignac only, behaviour during handling (struggle) was included in the reference model.

ii) Environmental variables: in Chizé and Aurignac, we considered the sector as a marker of spatial variations in resource quality and quantity. To account for temporal variations of resources, we used a centered year quality index. Quality of a given year was assessed using the average body mass of fawns caught the following winter (Pettorelli et al., 2003).

iii) Methodological variables: the time delay between capture and blood sampling is known to influence the level of some immune parameters such as neutrophil and lymphocyte counts (Carbillet et al., in press), and was thus taken into account (delay, in minutes, centred).

Several plausible interactions based on biological hypotheses were also incorporated in our reference model. First, the interaction between FCMs and sex was included, as the influence of stress should be more pronounced on the immune parameters of females since males increase their fitness by increasing their reproductive success while females increase their fitness through an increase in longevity that necessitates much higher investment in immunity (Bateman's principle; Rolff, 2002). We also included the interactions between FCMs and body weight, and between FCMs and age, because of a possible increase of the effect of FCMs on immune parameters in individuals with the lowest physical condition (Hau et al., 2016) and oldest age (Sapolsky et al., 1983). We also considered interactions between FCMs and sector, and between FCMs and quality of the year, to account for a potential greater effect of baseline stress level on immune parameters under poorer environments or years of poorer quality (Hau et al., 2016). Finally, we included an interaction between FCMs and behaviour during handling to take into account a possible effect of behavioural types on the influence of FCMs, considering that individuals with different behavioural types generally do not invest in the

same arms of immunity (Jacques-Hamilton et al., 2017). An interaction between sex and age was also included in neutrophil models for the Trois-Fontaines and Chizé populations to control for previous results obtained by Cheynel and colleagues (2017). This interaction was also included in the Aurignac population for all immune parameters. Individual's identity and year of capture were included as random effects to avoid pseudo-replication problems (Hurlbert, 1984) and to control for unexplained variance due to among-individual differences and inter-annual variation. In Chizé and Trois-Fontaines populations, the birth cohort was also included as a random effect to account for differences between individuals at the beginning of their lives, because of large variations in environmental conditions from one year to the next, which may have persistent effects (Douhard et al., 2014).

As a result, our reference models were as follow: Immune parameter =  $f(\text{duration} + \text{FCMs} \times \text{sector} + \text{FCMs} \times \text{year quality} + \text{FCMs} \times \text{sex} + \text{FCMs} \times \text{age} + \text{FCMs} \times \text{weight} + \text{FCMs} \times \text{struggle} + 1 | \text{individual} + 1 | \text{year of capture} + 1 | \text{birth cohort})$ .

Overall, we implemented 35 series of reference models, including 12 models for the populations of Trois-Fontaines and Chizé and 11 models for Aurignac (due to the absence of haptoglobin assays). To select the best model describing variation of each immune parameter, we used a model selection method based on Akaike's information criteria (AIC, Burnham and Anderson, 2002). We compared our reference model with all the simpler sub-models for which the AICc difference ( $\Delta\text{AICc}$ ) was less than 2 units (Burnham and Anderson, 2002). For each immune parameter, we selected the simplest model to satisfy parsimony rules (Burnham and Anderson, 2002). For each model selected, the marginal ( $R^2_m$ ) and conditional ( $R^2_c$ ) variance explained by the model were calculated using the `r.squaredGLMM` function of the `MuMIn` package (Barton, 2016). Normality of the residuals of the selected model for each

parameter in each population was tested (Shapiro-Wilk test) and visually assessed. All analyses were performed using R version 3.5.1 (R Development Core Team 2018) and using the lmer function of the lme4 package (Bates et al., 2014) and the dredge function of the MuMIn package (Barton, 2016).

### 3. Results

For the Aurignac population, FCMs concentration was not retained in any model to explain changes in immune parameters. For the Trois-Fontaines population FCMs concentration was retained for a single immune parameter (neutrophil count). For the Chizé population, several immune parameters (neutrophil, basophil counts, concentrations of gamma-, alpha-1 and alpha-2 globulins) were affected by FCM levels (Table 1).

For the Trois-Fontaines population, the neutrophil count was related to the time delay between capture and blood sampling and by FCM level, such that neutrophil concentrations increased with FCM levels (0.614; CI = [0.142; 1.093]) (Table 1; Fig. 1A). For the Chizé population, we found effects of age, quality of the year, sector, FCM level and interaction between FCM level and quality of the year on neutrophil count (Table 1). Unlike Trois-Fontaines, neutrophil concentrations decreased with increasing levels of FCMs, but this negative relationship was modulated by quality of the year. Precisely, as the quality of the year decreased the negative relationship disappeared (-2.496; CI = [-4,866; -0,206]) (Table 1; Fig. 1B). For basophil concentrations, we observed that the relationship with FCM levels was modulated by the quality of the year, such that during good quality years basophil concentrations were positively linked to FCMs, while the the relationship reversed during poor quality years (0.162; CI = [0.055; 0.256]) (Table 1; Fig. 2). Gamma globulin concentrations for

the Chizé population were explained by the age threshold function (set at 4 years according to Cheynel et al., 2017), FCM levels and spatial variation in terms of resources (Table 1). We observed a slight negative relationship between gamma globulin concentrations and FCM levels ( $-0.850$ ; CI =  $[-1.644; -0.020]$ ) (Table 1; Fig. 3A). Similarly, negative effect of FCM levels was also observed on alpha-2-globulin concentrations ( $-0.055$ ; CI =  $[-0.088; -0.024]$ ) (Table 1; Fig. 3B), where the retained model also included weight (Table 1). Finally, variations in alpha-1-globulin concentrations for the Chizé population were best supported by a model that included age with linear and quadratic functions, the time delay between capture and blood sampling, FCM levels, weight, sex, the two-way interaction between age and FCM levels, and the two-way interaction between weight and FCM levels (Table 1). In particular, there was a slight decrease in alpha-1-globulin concentrations as FCM levels increased, and this negative relationship became steeper as individuals were older ( $-0.009$ ; CI =  $[-0.018; -0.001]$ ) (Table 1; Fig. 4A). We also observed that the negative relationship between alpha-1-globulin concentration and FCM levels was attenuated as individuals were heavier ( $3.462e-04$ ; CI =  $[7.778e-05; 6.102e-04]$ ) (Table 1; Fig. 4B).

## 4. Discussion

The first aim of this study was to provide a fine description on how baseline glucocorticoid levels influence immune parameters of both the innate and adaptive arms of immunity, in three free-ranging populations of roe deer living in markedly different ecological conditions. Second, we aimed to identify the extent to which individual and environmental characteristics may affect this relationship. Overall, the relationships between baseline glucocorticoid levels and immune parameters differed among the three studied populations. While FCMs were not associated with any of the immune parameters in Aurignac and only with neutrophils in Trois-

Fontaines, the influence of baseline glucocorticoids was much more pronounced in Chizé. In particular, neutrophil, alpha-1, alpha-2 and gamma-globulin concentrations decreased when FCM levels increased in this population. Given the contrast between environmental conditions in the three populations, we expected such differences in the effect of stress on immunity. In particular, in Chizé, which is the least favourable environment in terms of nutritional resources (Gaillard et al., 1993), we expected any energetic trade-off to be particularly pronounced. Indeed, individuals may not be able to allocate energy simultaneously into immune defences while maintaining other functions (Martin, 2009). In this population, our results highlighted that innate and adaptive component of immunity may be impaired when glucocorticoid levels are high, but also that individual (age and weight) and environmental (temporal variation in resources quantity and quality) characteristics can affect this relationship.

In Trois-Fontaines, we found that neutrophil count increased with the level of stress exhibited by the individuals. This result contradicts our hypothesis of a negative effect of stress on innate immune parameters. However, this result is in accordance with previous works that demonstrated a redistribution of immune cells following an increase in blood cortisol, resulting in a rise in circulating neutrophils (Dhabhar et al., 1994; 2006). In contrast, in Chizé, neutrophil count was negatively related to glucocorticoid levels during years of better quality and quantity of resources, but that this relationship disappeared as the quality of the year decreased. At first glance, this result seems counter-intuitive, but it matches with an alternative theoretical framework proposed by Davis and Maney (2018), who suggested that in poor quality habitat, glucocorticoid levels should be down-regulated to prevent impairment of immune functions. Therefore, during the worst years in Chizé, immunity could be prioritised and independent of glucocorticoid levels that should be minimised with less among-individual variations. Still for the innate arm of immunity, we found that in Chizé, basophil

concentrations were positively related to FCM levels, but only during years when resources were abundant and/or of better quality. When year quality declined, the relationship reversed, such that basophils declined when glucocorticoid levels increased. This positive relationship during favourable years in terms of resources was not expected. However, a possible hypothesis of this effect that remains to be tested could be the presence of ectoparasites, such as ticks, whose abundance in the environment varies according to the environmental conditions and that can be a source of stress (Lightfoot & Norval, 1981). During years of good quality, ticks are expected to be more abundant (Kraemer, 2018), induce higher stress levels, and basophils would be secreted to develop acquired tick resistance (Wada et al., 2010; Karasuyama et al., 2018). On the opposite, during years of low quality, ticks may be less abundant. Moreover, the level of available resources being lower, individuals may not have enough energy to invest in both the immune and stress responses, and a trade-off may be revealed (Lee, 2006; Martin, 2009).

Regarding globulins, we found that alpha-2 and gamma globulins concentrations decreased with increased FCM in the Chizé population. These results are in accordance with our predictions, especially for alpha-2 globulins, which belong to the innate immune system, the most costly arm of immunity (McDade, 2016). Indeed, because of the high activation cost of innate immunity a negative relationship with cortisol levels is expected, and has been highlighted in previous studies on wild species (Matson et al., 2006; Brooks and Mateo, 2013). We obtained a very similar effect for gamma globulins which belongs to the adaptive arm of immunity. Although we expected that the negative relationship with stress levels should be weaker for the adaptive components of the immune system (McDade, 2016), our results are consistent with a previous experimental study on birds (Stier et al., 2009). Knowing that Chizé is a forest with a particularly low productivity, compared to the two other populations

(Pettorelli et al., 2006), limited food availability might explain the negative relationship between an adaptive component of immunity and FCM levels.

Finally, the relationship between FCM levels and alpha-1-globulin concentration depended on body condition and age. Precisely, individuals in lower body condition, and older ones, exhibited a more marked negative relationship. These results are therefore in accordance with our predictions and the hypothesis that individuals in poorer condition might not be able to bear the costs of both stress and immune response (Martin, 2009). Old individuals face the costs of chronic stress and the cost of immune functions, but also a cost associated with cell maintenance (disposable soma theory, Kirkwood, 1977). However, contrary to younger individuals, their metabolism and ingestion capacity are less efficient, leading to a diminution in available energy. A hypothesised by Kirkwood (1977), when resources are limited, natural selection should adjust the distribution of cellular and physiological resources. And this adjustment should occur between the fundamental processes of somatic maintenance and reproduction, in a manner appropriate to the ecological context of the organism. Thus, we can establish that older and highly stressed individuals invest less in immunity than younger individuals, especially in the alpha-1-globulin fraction, which is one of the first effectors of the inflammatory response, but that is highly energy demanding (Klasing and Leshchinsky, 1999; Lee, 2006). In the same way, in accordance with our prediction (iii), roe deer in poor body condition have a more negative relationship between FCM and alpha-1 globulins than animals in good body condition.

Overall, we found that alterations in immune parameters could occur as a result of elevated baseline glucocorticoid levels. However, the direction and magnitude of these effects might depend on the environmental context in terms of resources availability and of the individual



phenotype. Further work is now needed to investigate the fitness consequences of the stress-immunity relationship in relation to individual and environmental phenotypes.

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| Immune trait  | Parameter | Estimate   | Lower CI   | Upper CI   |
|---|-----------|------------|------------|------------|
| Trois-Fontaines   |           |            |            |            |
| Neutrophil count (N = 223)<br>R <sup>2m</sup> : 0,035 ; R <sup>2c</sup> : 0,471 | Intercept | 6,351      | 5,541      | 7,174      |
|   | delay     | 0,003      | 4,412e-4   | 0,005      |
|   | FCMs      | 0,614      | 0,142      | 1,093      |
| Eosinophil count (N = 223)<br>R <sup>2m</sup> : 0,048 ; R <sup>2c</sup> : 0,204 | Intercept | 0,070      | 0,054      | 0,086      |
|   | delay     | -2,183e-04 | -3,389e-04 | -8,659e-05 |
| Basophil count (N = 223)<br>R <sup>2m</sup> : 0,005 ; R <sup>2c</sup> : 0,358   | Intercept | 3,963e-02  | 0,009      | 0,069      |
|   | delay     | -4,944e-05 | -1,342e-04 | 2,313e-05  |
| Monocyte count (N = 223)<br>R <sup>2m</sup> : 0,009 ; R <sup>2c</sup> : 0,537   | Intercept | 0,215      | 0,082      | 0,359      |
|   | delay     | -2,198e-04 | -4,250e-04 | 2,927e-06  |
| Hemagglutination (N = 247)<br>R <sup>2m</sup> : 0,017 ; R <sup>2c</sup> : 0,390 | Intercept | 1,6769     | -0,496     | 3,821      |
|   | ageT      | 0,2857     | 0,049      | 0,517      |
| Hemolysis (N = 244)<br>R <sup>2m</sup> : 0 ; R <sup>2c</sup> : 0,285            | Intercept | 2,333      | 1,764      | 2,799      |
| Gamma-globulin (N = 245)<br>R <sup>2m</sup> : 0,052 ; R <sup>2c</sup> : 0,608   | Intercept | 11,956     | 9,998      | 13,958     |
|   | ageT      | 0,441      | 0,249      | 0,660      |
| Lymphocyte count (N = 223)<br>R <sup>2m</sup> : 0,032 ; R <sup>2c</sup> : 0,519 | Intercept | 1,204      | 1,119      | 1,288      |
|   | weight    | -0,001     | -0,001     | -1,860e-04 |
| Alpha-1-globulin (N = 245)<br>R <sup>2m</sup> : 0,051; R <sup>2c</sup> : 0,688  | Intercept | 1,450      | 1,349      | 1,569      |
|   | weight    | -4,452e-04 | -0,001     | -2,835e-04 |
| Alpha-2-globulin (N = 245)<br>R <sup>2m</sup> : 0,022 ; R <sup>2c</sup> : 0,309 | Intercept | 1,932      | 1,802      | 2,056      |
|   | sex       | -0,086     | -0,147     | -0,024     |
| Beta-globulin (N = 245)<br>R <sup>2m</sup> : 0,133 ; R <sup>2c</sup> : 0,613    | Intercept | 1,803      | 1,678      | 1,929      |
|   | age       | 0,055      | 0,031      | 0,080      |
|   | l(age^2)  | -0,003     | -0,005     | -3,863e-04 |
|   | Intercept | 4,369      | 4,022      | 4,783      |

| Immune trait  | Parameter         | Estimate   | Lower CI   | Upper CI   |
|---|-------------------|------------|------------|------------|
| Haptoglobin (N = 244)<br>R <sup>2m</sup> : 0,109 ; R <sup>2c</sup> : 0,290      | year quality      | -0,525     | -1,106     | 0,037      |
| Chizé   |                   |            |            |            |
| Neutrophil count (N = 270)<br>R <sup>2m</sup> : 0,122 ; R <sup>2c</sup> : 0,466 | Intercept         | 3,687      | 3,022      | 4,320      |
|   | age               | 0,161      | 0,080      | 0,249      |
|   | delay             | 0,003      | 3,638e-04  | 0,005      |
|   | FCMs              | 0,254      | -0,330     | 0,839      |
|   | year quality      | 1,144      | -0,639     | 2,803      |
|   | sector(2)         | 0,524      | 0,003      | 1,021      |
|   | sector(3)         | -0,441     | -1,163     | 0,226      |
|   | FCMs*year quality | -2,496     | -4,866     | -0,206     |
| Éosinophil count (N = 270)<br>R <sup>2m</sup> : 0,092 ; R <sup>2c</sup> : 0,243 | Intercept         | 0,070      | 0,043      | 0,098      |
|   | age               | 0,006      | 0,001      | 0,011      |
|   | delay             | -3,034e-04 | -4,317e-04 | -1,756e-04 |
| Basophil count (N = 270)<br>R <sup>2m</sup> : 0,069 ; R <sup>2c</sup> : 0,320   | Intercept         | 0,032      | -0,032     | 0,103      |
|   | age               | 0,011      | 0,001      | 0,019      |
|   | l(age^2)          | -0,001     | -0,002     | 5,674e-05  |
|   | FCMs              | -0,049     | -0,074     | -0,021     |
|   | year quality      | 0,020      | -0,237     | 0,237      |
|   | FCMs*year quality | 0,162      | 0,055      | 0,256      |
| Monocyte count (N = 270)<br>R <sup>2m</sup> : 0,031 ; R <sup>2c</sup> : 0,511   | Intercept         | 0,423      | 0,242      | 0,588      |
|   | age               | -0,035     | -0,064     | -0,011     |
|   | l(age^2)          | 0,002      | -5,269e-05 | 0,005      |
| Hemagglutination (N = 323)<br>R <sup>2m</sup> : 0,009 ; R <sup>2c</sup> : 0,581 | Intercept         | 5,653      | 3,610      | 7,819      |
|   | ageT              | -0,200     | -0,434     | 0,022      |
|   | Intercept         | 0,174      | -0,535     | 0,968      |



| Immune trait  | Parameter    | Estimate   | Lower CI   | Upper CI   |
|---|--------------|------------|------------|------------|
| Hemolysis (N = 323)<br>R <sup>2m</sup> : 0,480 ; R <sup>2c</sup> : 0,604        | year quality | 7,294      | 4,703      | 9,840      |
| Gamma-globulin (N = 301)<br>R <sup>2m</sup> : 0,092 ; R <sup>2c</sup> : 0,660   | Intercept    | 18,137     | 15,379     | 20,898     |
|   | ageT         | 0,529      | 0,255      | 0,831      |
|   | FCMs         | -0,850     | -1,644     | -0,020     |
|   | sector(2)    | 0,431      | -0,735     | 1,597      |
|   | sector(3)    | -2,739     | -4,332     | -1,223     |
| Lymphocyte count (N = 270)<br>R <sup>2m</sup> : 0,121 ; R <sup>2c</sup> : 0,561 | Intercept    | 1,183      | 1,002      | 1,376      |
|   | age          | -0,081     | -0,119     | -0,040     |
|   | l(age^2)     | 0,005      | 0,001      | 0,009      |
|   | delay        | -0,001     | -0,001     | -1,589e-04 |
|   | sex          | -0,075     | -0,146     | -0,002     |
| Alpha-1-globulin (N = 301)<br>R <sup>2m</sup> : 0,196 ; R <sup>2c</sup> : 0,438 | Intercept    | 1,489      | 1,412      | 1,561      |
|   | age          | -0,027     | -0,049     | -0,004     |
|   | l(age^2)     | 0,002      | 1,913e-04  | 3,752e-03  |
|   | delay        | 2,797e-04  | 1,363e-04  | 4,228e-04  |
|   | FCMs         | 0,009      | -0,027     | 0,050      |
|   | weight       | -2,210e-04 | -4,535e-04 | 4,564e-06  |
|   | sex          | 0,040      | 0,012      | 0,074      |
|   | age*FCMs     | -0,009     | -0,018     | -0,001     |
|   | weight*FCMs  | 3,462e-04  | 7,778e-05  | 6,102e-04  |
| Alpha-2-globulin (N = 301)<br>R <sup>2m</sup> : 0,066 ; R <sup>2c</sup> : 0,111 | Intercept    | 1,942      | 1,900      | 1,977      |
|   | FCMs         | -0,055     | -0,088     | -0,024     |
|   | weight       | -2,950e-04 | -4,931e-04 | -5,594e-05 |
| Beta-globulin (N = 301)<br>R <sup>2m</sup> : 0,211 ; R <sup>2c</sup> : 0,653    | Intercept    | 1,970      | 1,900      | 2,045      |
|   | age          | 0,063      | 0,042      | 0,086      |
|   | l(age^2)     | -0,004     | -0,006     | -0,002     |
|   | sector(2)    | -0,006     | -0,053     | 0,036      |

| Immune trait  | Parameter    | Estimate | Lower CI | Upper CI |
|---|--------------|----------|----------|----------|
|   | sector(3)    | -0,118   | -0,188   | -0,056   |
| Haptoglobin (N = 301)<br>R <sup>2m</sup> : 0,047 ; R <sup>2c</sup> : 0,173      | Intercept    | 4,258    | 3,866    | 4,738    |
|   | sex          | 0,538    | 0,286    | 0,808    |
| Aurignac  |              |          |          |          |
| Neutrophil count (N = 176)<br>R <sup>2m</sup> : 0,045 ; R <sup>2c</sup> : 0,691 | Intercept    | 6,058    | 5,718    | 6,419    |
|   | delay        | 0,006    | 0,001    | 0,011    |
|   | weight       | -0,074   | -0,164   | 0,019    |
| Éosinophil count (N = 175)<br>R <sup>2m</sup> : 0,031 ; R <sup>2c</sup> : 0,200 | Intercept    | 0,065    | 0,047    | 0,084    |
|   | sex          | -0,030   | -0,057   | -0,007   |
| Basophil count (N = 176)<br>R <sup>2m</sup> : 0 ; R <sup>2c</sup> : 0,368       | Intercept    | 0,059    | 0,046    | 0,071    |
| Monocyte count (N = 176)<br>R <sup>2m</sup> : 0 ; R <sup>2c</sup> : 8,206e-07   | Intercept    | 0,114    | 0,100    | 0,131    |
| Hemagglutination (N = 158)<br>R <sup>2m</sup> : 0 ; R <sup>2c</sup> : 0,741     | Intercept    | 3,519    | 2,932    | 4,073    |
| Hemolysis (N = 160)<br>R <sup>2m</sup> : 0 ; R <sup>2c</sup> : 0,560            | Intercept    | 2,650    | 1,655    | 3,641    |
| Gamma-globulin (N = 138)<br>R <sup>2m</sup> : 0,033 ; R <sup>2c</sup> : 0,598   | Intercept    | 12,230   | 11,486   | 13,060   |
|   | age(young)   | -1,157   | -2,257   | -0,116   |
| Lymphocyte count (N = 176)<br>R <sup>2m</sup> : 0,121 ; R <sup>2c</sup> : 0,186 | Intercept    | 1,289    | 1,231    | 1,347    |
|   | weight       | -0,019   | -0,029   | -0,009   |
|   | sex          | -0,081   | -0,149   | -0,016   |
| Alpha-1-globulin (N = 138)<br>R <sup>2m</sup> : 0,307 ; R <sup>2c</sup> : 0,307 | Intercept    | 1,314    | 1,264    | 1,358    |
|   | age(young)   | 0,065    | 0,012    | 0,119    |
|   | sector(1)    | 0,062    | 0,022    | 0,110    |
|   | sector(2)    | 0,006    | -0,043   | 0,062    |
|   | weight       | -0,009   | -0,016   | -0,002   |
|   | year quality | 0,073    | 0,031    | 0,116    |

| Immune trait  | Parameter    | Estimate | Lower CI | Upper CI |
|---|--------------|----------|----------|----------|
|   | sex          | 0,044    | 0,007    | 0,088    |
| Alpha-2-globulin (N = 138)<br>R <sup>2m</sup> : 0,106 ; R <sup>2c</sup> : 0,504 | Intercept    | 1,657    | 1,631    | 1,680    |
|   | struggle     | 0,043    | 0,012    | 0,076    |
|   | year quality | 0,057    | 0,007    | 0,099    |
| Beta-globulin (N = 138)<br>R <sup>2m</sup> : 0,031 ; R <sup>2c</sup> : 0,031    | Intercept    | 1,918    | 1,878    | 1,955    |
|   | age(young)   | -0,059   | -0,111   | -0,001   |

**Table 1** Parameters of linear mixed-effect models selected for each immune parameters of the three roe deer populations studied (Trois-Fontaines, Chizé, Aurignac). The departure model for model selection included the fixed effects of three age functions (linear, quadratic and threshold) for the Trois-Fontaines and Chizé populations or the age class for the Aurignac population (juveniles and adults), faecal cortisol metabolites (FCMs), sex, weight, quality of the year (year quality), time delay between capture and blood sampling (delay), sector for Chizé and Aurignac, behaviour during handling for the Aurignac population (struggle) also with two-way interactions between FCMs and sex, weight, sector, struggling behaviour, and the two-way interaction between sex and age. Individual identity and year of capture were included as random effects as well as the cohort for analyses of the Chizé and Trois-Fontaines populations. R<sup>2m</sup> and R<sup>2c</sup> correspond respectively to the marginal and conditional variance explained by the model, and lower CI and upper CI corresponds to the upper and lower limits of the 95% confidence interval. AICc represents the value of the corrected Akaike information criterion, K is the number of estimated parameters for each model and Weight the weight of Akaike. See text for definition of model sets.

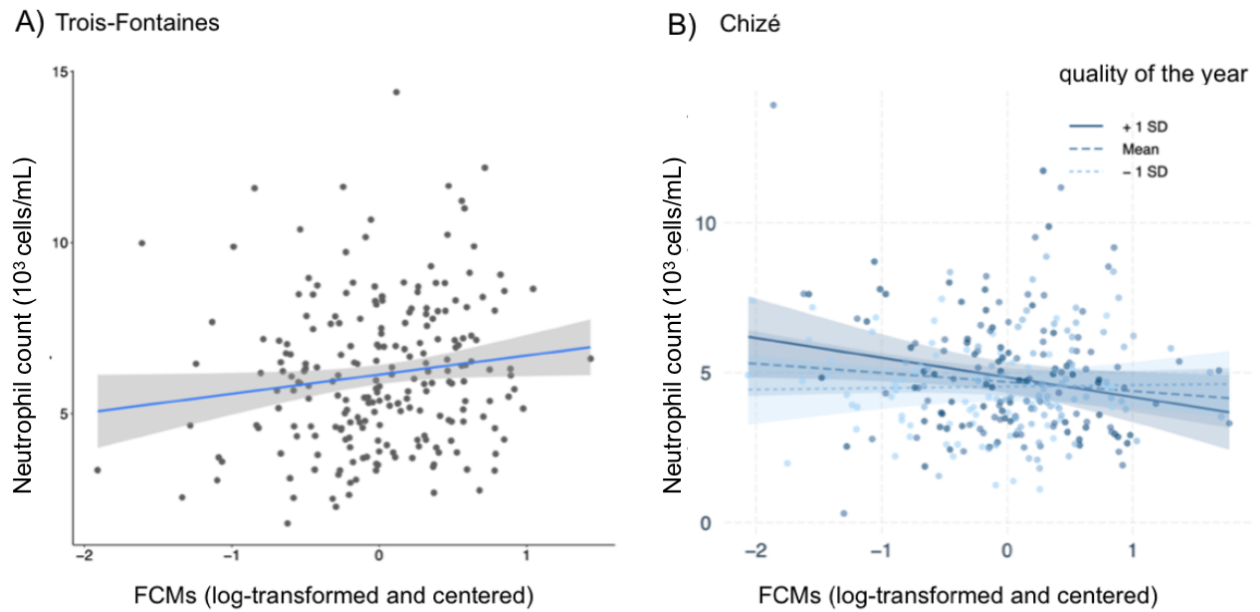


Fig. 1. Relationships between faecal cortisol metabolites (FCMs) and neutrophil count in Trois-Fontaines (A), and neutrophil count according to the quality of the year for the Chizé population (B). Points represent observed values. Lines represent model predictions, "mean" corresponds to the average value of the quality of the year, +1 SD and - 1 SD corresponds to the standard deviation  $\pm 1$  of the mean. Coloured areas represent 95% confidence intervals.

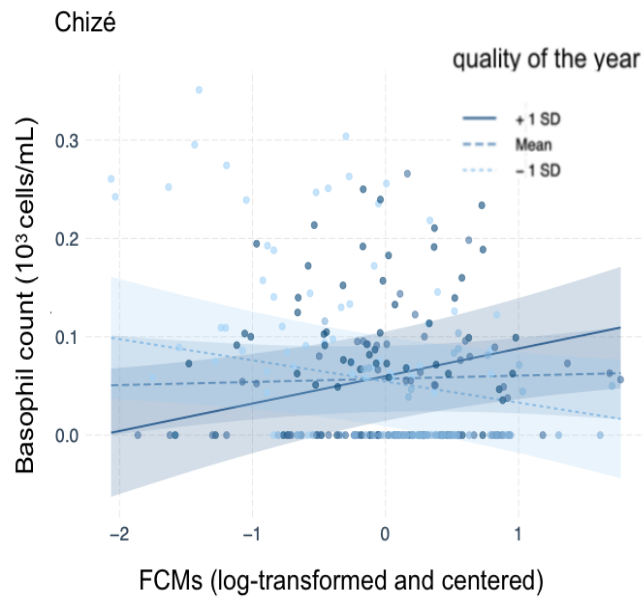


Fig. 2. Relationship between faecal cortisol metabolites (FCMs) and basophil count according to the quality of the year for the Chizé population. Points represent observed values. Lines represent model predictions, "mean" corresponds to the average value of the quality of the year, +1 SD and - 1 SD corresponds to the standard deviation  $\pm 1$  of the mean. Coloured areas represent 95% confidence intervals.

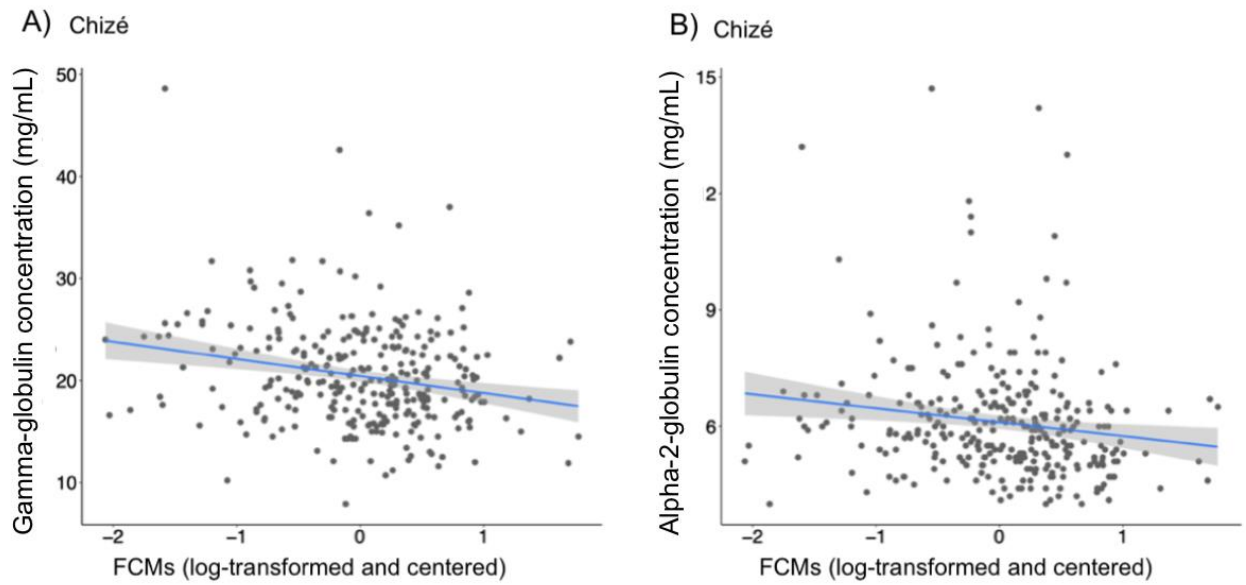


Fig. 3. Relationships between faecal cortisol metabolites (FCMs) and (A) gamma-globulin concentrations and (B) alpha-2-globulin concentrations in Chizé. Points represent observed values. Lines represent model predictions and coloured areas represent 95% confidence intervals.

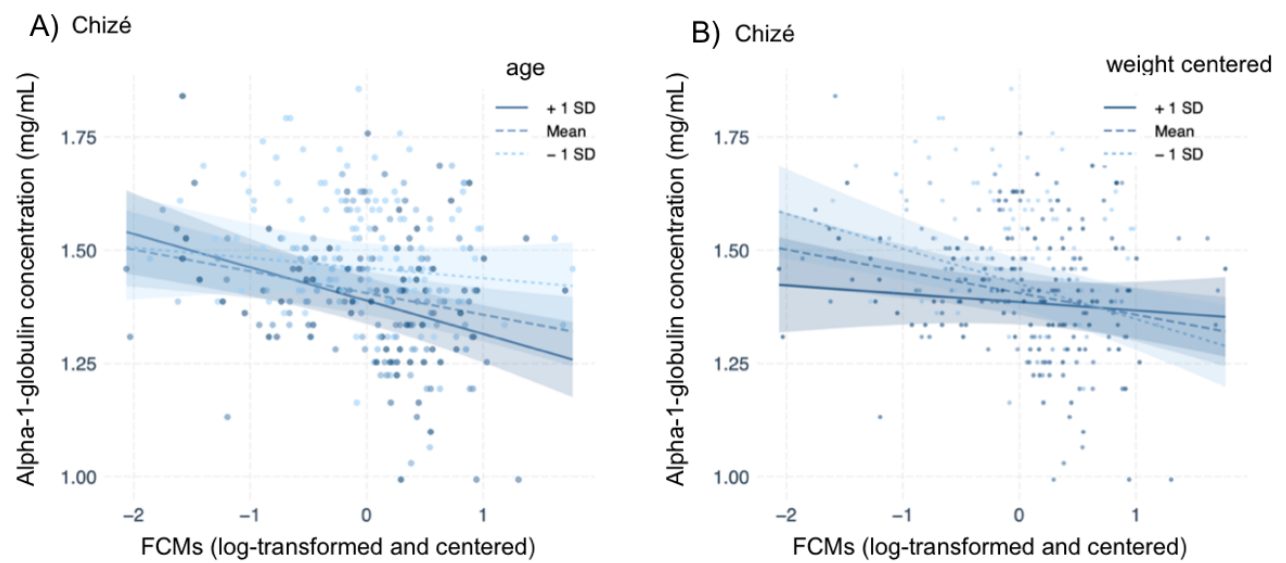


Fig. 4. Relationships between faecal cortisol metabolites (FCMs) and alpha-1-globulin concentrations according to the age (A) and according to the body weight (B) for the Chizé population. Points represent observed values. Lines represent model predictions, "mean" corresponds to the average value of the age (A) or body weight (B), +1 SD and - 1 SD corresponds to the standard deviation  $\pm 1$  of the mean. Colored areas represent the 95% confidence interval.

## **Co-variation between faecal cortisol metabolites, behaviour and immune parameters over time in captive roe deer**

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Key words: stress, glucocorticoids, immunity, behaviour, mammal

## Summary statement

Variations in baseline stress levels of a wild ungulate and a behavioural trait influenced variations of immune parameters of both innate and adaptive arms.

## Abstract

Immunity is a major mechanism allowing organisms to face selective pressures exerted by pathogens. Yet, this mechanism entails energetic and somatic costs and is predicted to trade-off against other physiological functions. For instance, biomedical literature highlighted that long-term elevation of stress hormones such as glucocorticoids might impair immune functions, while short-term elevation might enhance them. Studies on wild animals found contrasting results, and whether the effect of chronically elevated stress hormones on immune parameters is positive, negative, does not exist, and differ between both arms still remains largely unknown. Consequently, we decided to explore the stress immunity relationship on a wide range of immune markers in controlled conditions, taking into account the potential confounding effects of individual characteristics such as behavioural types. By monitoring 14 roe deer *Capreolus capreolus* over an eight-weeks period encompassing two capture events, we assessed how variations over time in faecal cortisol metabolite (FCM)

concentrations following a standardised capture protocol affected immune parameters of both the innate (total white blood cells [WBC], neutrophils, eosinophils, monocytes, basophils, haptoglobin, alpha-1, alpha-2, beta-1 and beta-2 globulins) and adaptive arms (lymphocytes, anti-rabies antibody, gamma-globulins). We also tested whether behavioural traits such as boldness and spontaneous activity were linked to changes in FCM levels and immune parameters, as it would be expected by the pace of life syndrome hypothesis. We found that the nature of the relationship between variations in FCM levels and immune parameters varied according to the arm of immunity, with a negative relationship with some innate parameters (WBC, neutrophils) and a positive relationship with adaptive parameters (lymphocytes, anti-rabies antibodies). In addition, bolder individuals exhibited higher FCM levels and tended to invest less in an immune parameter such as eosinophils. Our results suggest that both innate and adaptive immunity of large mammals may be influenced by their stress level, but also to some extent by their behavioural type. Our analysis highlights the need to consider different immune parameters to understand the complexity of the stress-immunity relationship. We also emphasise the potential of considering among-individual variation in behavioural traits as a modulator of this relationship.

## Introduction

Evolved to cope with pathogens, the immune system is one of the most important mechanisms in vertebrates to improve their survival. This complex system is composed of two complementary arms, the innate immunity (relatively fast and non-specific) and the adaptive immunity (slower at first encounter, but specific), each composed of numerous cells and effectors (Demas, 2004). This system, however, is not cost-free (Lochmiller and Deerenberg, 2000; Demas 2004), and one of the basic assumptions in biology is that there should be trade-

offs between immune defences and other functions that use a common resource and contributes to an individual's fitness (Sheldon and Verhulst, 1996; Rauw, 2012). Glucocorticoid hormones (i.e. cortisol and corticosterone) play a major role in regulating the use of energy (Landys et al., 2006; Busch and Hayward, 2009) and may therefore underlie such trade-offs.

In addition to their role as metabolic hormones, glucocorticoids are also one of the main mediators of the stress response. In response to external or internal stimuli, stress activates behavioural and physiological responses that allow an organism to cope with challenges, and therefore enhance survival (Wingfield, 1998; Romero and Wingfield, 2015). In particular, the activation of the hypothalamic-pituitary-adrenal axis that results in the secretion of glucocorticoid hormones helps organisms to cope with stressful situations by making stored energy available (Sapolsky et al., 2000). However, when glucocorticoid hormones are secreted repeatedly or for long time periods, negative effects on other energetically demanding functions such as reproduction (Boonstra, 2005) and immunity (Lee, 2006) may appear.

Over the past years, several studies investigated the relationship between stress and immunity, mostly in the biomedical domain. Based on these studies it is assumed that short-term elevation of glucocorticoids enhances immune functions (Dhabhar, 2002; Dhabhar, 2014) while chronically elevated glucocorticoid levels are immunosuppressive (Dhabhar and McEwen, 1997; Sapolsky et al., 2000; Dhabhar, 2009). However, studies on wild animals have shown mixed results, suggesting that chronically elevated glucocorticoids levels may be associated with decrease, increase, or no changes in immune functions (Matson et al., 2006; Delehanty and Boonstra, 2009; Merrill et al., 2012; Brooks and Mateo, 2013). Evidence is also accumulating to show that glucocorticoid levels do not affect both arms of the immune system the same manner, such that one arm may be impaired while the other might not be affected

(Bourgeon and Raclot, 2006; Stier et al., 2009). These contrasting results indicate that our understanding of the nature of the stress-immunity relationship in the wild remains limited and fragmentary.

Among the factors likely to modulate this relationship and to help to shed light on the discrepancies, the consideration of behavioural types has been largely neglected. From a theoretical point of view, close links between physiology and behaviour are expected due the underlying energetic basis of both traits (Careau et al., 2008). Accordingly, consistent among-individual differences in behaviour, also called personality (Sih et al., 2004), are related to numerous physiological traits including glucocorticoid secretion and immune function (Koolhaas et al., 1999; Koolhaas et al., 2008; Réale et al., 2010). For instance, a study on wild superb fairy-wrens (*Malurus cyaneus*) showed that individuals showing proactive personality traits (fast exploration of a novel environment) exhibited lower level of natural antibodies (Jacques-Hamilton et al., 2017). Such co-variations between behavioural and physiological traits can be interpreted within the pace of life framework (Réale et al., 2010). Previtali and colleagues (2012) showed that rodent species with slow life histories invested more in adaptive immunity than those with fast life histories that relied more on innate immunity. On the other hand, previous works highlighted that slower or more reactive individuals tend to exhibit higher glucocorticoid levels compared to faster or more proactive ones (Koolhaas et al., 1999; Montiglio et al., 2012; Montiglio et al., 2014). Consequently, it is reasonable to expect that individuals with slower life history strategies have higher glucocorticoid levels and higher investment in adaptive immunity, while those with faster life history strategies have lower glucocorticoid levels and higher investment in innate immunity (Lee, 2006).

In wild-living animals, the levels of glucocorticoid hormones vary over time due to predictable (seasonal) or unpredictable changes in the environment (Wingfield and Romero, 2001). Here, we took advantage of a captive roe deer (*Capreolus capreolus*) population to examine the link between long-term changes in faecal cortisol metabolite (FCM) levels and changes in immune functions before and after a standardised capture stress protocol and how these changes may be related to behavioural profiles. To reach this goal, we measured 12 immune markers to achieve a comprehensive picture of both adaptive and innate immune functions. In particular, in each roe deer, we performed an immune challenge to probe the ability of the adaptive immune system to secrete specific antibodies directed against rabies. For each roe deer, we characterised two behavioural traits indexing boldness and activity levels.

Based on the literature, we expected 1) that changes in FCM levels between the two capture events would co-vary negatively with changes in innate immune parameters and inflammatory markers due to their high activation costs (McDade et al., 2016); 2) that variation in adaptive immune parameters should be less related to variation in FCM levels due to their low costs (McDade et al., 2016). In addition, we expected 3) that FCM levels as well as temporal variations in FCM levels should differ according to individual behavioural traits (Koolhaas et al., 1999; Montiglio et al., 2012), with higher levels and higher variations in shier and less active individuals; and 4) that variation in innate and inflammatory immune parameters would be higher in bolder and more active individuals, while variations in adaptive immune parameters would be higher in shier and less active individuals (Lee, 2006; Réale et al., 2010; Previtali et al., 2012).

## Material and methods

## **Study site**

This study was conducted on a captive population of roe deer living in the Gardouch research station, located in south-west of France, approximately 30 km south-east of Toulouse. The station is located on the slopes of a hill, approximately 230 m above sea level. The climate is oceanic with summer droughts. The station is owned and managed by the French Institute for Agricultural Research (Institut de la Recherche Agronomique - INRA). It consists of 12 enclosures of 0.5 ha, each containing between one to six captive and tame roe deer. Each enclosure is composed of meadow, trees and groves that provide shelter. Each enclosure contains a hut where individuals are supplemented with pellets (600 g per individual per day) and can obtain shelter as well. The current experiment included a total of 16 females, living in their enclosure since several years already, only females, aged from 4 to 13 years old. All roe deer had some degree of habituation to humans but were able to express normal behavioural responses (e.g. vigilance, escape) to stressful situations.

## **Experimental design**

The experimental procedure was carried out between mid-September and mid-November 2019 and is summarised in Fig.1. To assess the baseline stress levels of each individual, we collected faeces every four days for four weeks to further determine faecal cortisol metabolite concentrations (period 1). Faeces were collected immediately after defecation was observed and kept at +4°C for a maximum of 1h before being stored at -20°C until analysis. At the end of the four-weeks period, each roe deer was subjected to a standardised capture stress protocol involving restrained immobilisation. This procedure made it possible to collect faeces from the rectum, to draw blood samples, to inject an anti-rabies vaccine, and to fit collars equipped with accelerometers (see below for details). Collection of faecal sample was

continued every four days for an additional four weeks as describe above (except during the two days following the first capture) to evaluate the effect of capture on the stress patterns for each individual (period 2). At the end of the four-week period, roe deer were recaptured following similar procedure and faeces and blood were collected.

### **Capture protocol and data collection**

Roe deer were directed into their hut (one enclosure at a time) by slowly approaching them. Then each individual was pushed through a trap door into a retention box. Once in the box, animals were lightly tranquilised with an intramuscular injection of acepromazine (Calmivet, Vetoquinol, France; targeted dose of 0.075 mg/kg) administered with a syringe to reduce risk of injury, as recommended by Montané and colleagues (2003). This procedure was successful for all individuals for the first capture, but failed for two individuals that could not be recaptured despite numerous attempts. Our capture stress protocol involved keeping the animal in the box for one hour before initiating physical restraint and sample collection. Individuals were weighed with an electronic balance to the nearest 10 g. Faecal samples were collected rectally and rectal temperature was monitored continuously during handling using an Ecoscan thermometer (YSI 400) and flexible probe (accuracy of 0.2°C). For each individual, we characterised two behavioural traits. First, we recorded whether animals struggled during handling, and this measure was used as an index of boldness (Gervais et al., submitted). A score of 0 was assigned to individuals that did not struggle (shy), while a score of 1 was attributed to those that did struggle (bold) during handling. This behavioural trait during handling has been shown to be repeatable over time ( $r=0.27$ ) with a tendency to be heritable ( $h=0.15$ ) in roe deer (Gervais et al., submitted). The second behavioural trait consisted in determining spontaneous daily activity during four weeks (Réale et al., 2007) with tri-axial

accelerometry technology. To this end, we fitted collars equipped with tri axial accelerometers (Daily Diaries tag) which recorded overall body accelerations at 20 Hz. Activity was assessed by calculating vectors of the dynamic body acceleration (VeDBA) as described in Benoit and colleagues (2019).

Finally, we obtained a functional index of the adaptive immune system activity by injecting a standardized dose of an anti-rabies vaccine (Rabisin<sup>®</sup>, Merial, France, 1ml) administered intramuscularly/subcutaneously during the first capture and by quantifying the circulating specific antibodies after four weeks (see below).

### **Immune parameters measurement and analyses**

Blood samples were taken from each individual from the left jugular vein (up to 20 mL for a 20 kg roe deer) and divided in two. 10ml of blood was collected on EDTA, preserved at 4°C and served to measure total leukocytes count (white blood cell [WBC]. WBC were determined at the Ecole Nationale Vétérinaire de Toulouse (ENVT) with an automat (Sysmex 2000iV, Sysmex). To obtain percentages of each leukocyte type (neutrophil, lymphocyte, basophil, eosinophil and monocyte), a differential cell count was performed on the first 100 WBCs on Wright-Giemsa-stained blood smears by trained technicians (Houwen, 2001). 10ml of blood was collected on a dry tube, centrifuged at 1500 g for 15 min, and the serum was stored at -20°C. Serum was used to quantify circulating alpha-1, alpha-2, beta-1, beta-2, and gamma globulins with a refractometer followed by an electrophoresis on agarose gel. Haptoglobin that belongs to the alpha-2-globulin fraction was also analysed from a dry tube stored at 4°C with an automat (Konelab 30i PLC, Fisher Thermo Scientific). Finally, we quantified the level of anti-rabies antibody following the method described by Cliquet and colleagues (1998) with results expressed as International Units per millilitre (IU/mL). All together, we therefore had



9 markers of innate immunity (total WBC, neutrophils, basophils, monocytes, eosinophils, beta-2 globulins) including 3 inflammatory markers (haptoglobin, alpha-1, alpha-2 and beta-1 globulins), and 3 markers of adaptive immunity (lymphocytes, gamma-globulins and anti-rabies antibody).

### Extraction of FCMs

FCM were extracted following a methanol-based procedure and assayed using a group-specific 11-oxoaetiocholanolone enzyme immunoassay (EIA), as previously described (Möstl et al., 2002) and validated for roe deer (Zbyryt et al., 2017). Briefly, each faecal sample was homogenised and  $0.5 \pm 0.005$  g of homogenate was transferred to a glass tube containing 5 ml of methanol 80%. The suspended samples were vortexed at 1500 rpm for 30 min and centrifuged at 2500 g for 15 min (Palme et al., 2013). An aliquot of the supernatant was further diluted (1:10) with assay buffer prior to EIA analysis. Measurements were carried out in duplicate (intra- and inter-assay coefficients of all samples were less than 10% and 15%, respectively) and the results expressed as nanograms per gram of wet faeces (ng/g).

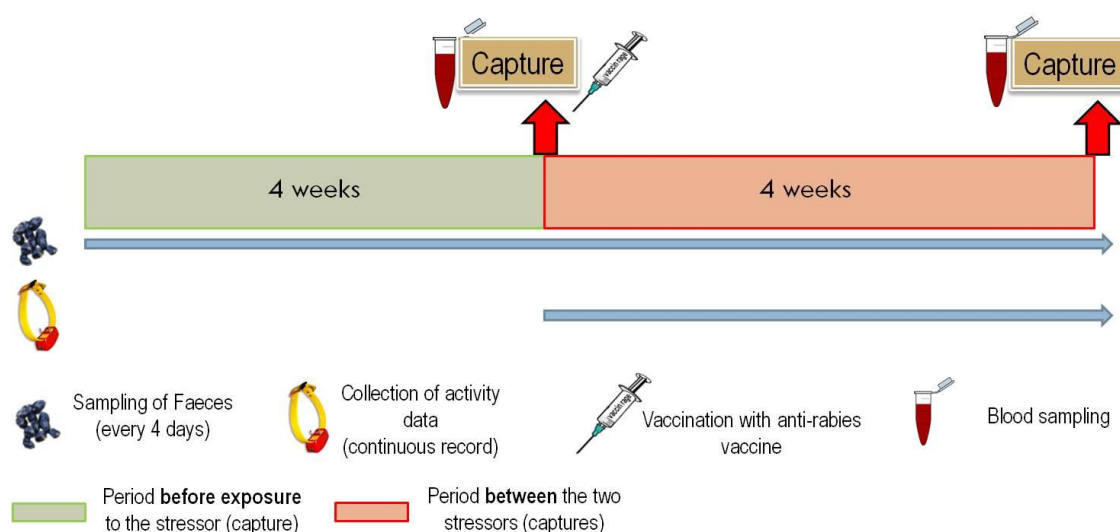


Fig. 1. Summary of the experimental design used in the present study.

## **Statistical analyses**

### **Relationship between FCMs and behavioural traits**

To analyse variation in FCM levels, we performed linear mixed-effects models (LMMs) on 194 observations of 14 individuals. FCM was log transformed to achieve normality of model residuals. We built a set of six candidate models, a null model and five models each including one variable testing for a specific hypothesis or possible confounding effects. Candidate models therefore included, boldness, activity, age, collection number (such that the third faeces collection was numbered three), session (such that session 1 was attributed to samples collected before the first capture event, and session 2 was attributed to samples collected after the first capture). Activity was taken as the mean daily activity through the 4 weeks between the two capture events. In addition, behaviour during handling at the first capture was associated to each samples collected for an individual since values did not differ between the two captures within-individual, except for one individual for which the score passed from 1 to 0. Individual identity and enclosure identity were included as random effects to avoid pseudo-replication issues (Hurlbert, 1984) and to control for unexplained variance due to among-individual differences and among-enclosure variation.

In addition we analysed temporal variations in FCM levels between period 2 and period 1 events using linear models (LMs) on 13 individuals and mean values of FCMs during each period for each individuals. We built a set of six candidate models, a null model and five models each including one variable testing for a specific hypothesis or possible confounding effects. Candidate models therefore included, boldness, activity, age, weight at the second capture as well as difference of weight between the two capture events.

### **Relationship between immune parameter variations, FCM and behavioural traits**

To analyse changes in immune parameters, we performed linear models (LMs) on the differences between immune parameter values at the two captures, for the 13 immune parameters measured. Each of the 13 differences was treated as a response variable and some of them (eosinophils, alpha-1 and alpha-2 globulins, and haptoglobin) were log transformed to achieve normality of model residuals. Our main hypothesis was that changes in immune parameters would follow temporal variations in FCM levels and to behavioural traits. However, other determinants of immune parameters were also included in candidate models. For each response variable, we thus adjusted 12 candidate models, one being the null model and the other 11 models, each including a single effect or two additive effects testing for a specific hypothesis (co-variation with stress: difference of FCM levels between period 2 and period 1; and behaviour : boldness and activity) and possible confounding effects of age, enclosure identity, weight at the second capture as well as difference of weight between the two capture events. Values of FCMs were averaged per individual for each period. We decided to include these two variables in our sets of models because body condition is known to influence immunity, but we had no a priori hypothesis based on the literature on which one of these two variables would be the most relevant to explain immune variations. All candidate models are presented in Table 1.

|         |  |
|---------|--|
| Model 1 | parameter ~ 1                                  |
| Model 2 | parameter ~ age                                |
| Model 3 | parameter ~ enclosure identity                 |
| Model 4 | parameter ~ FCM difference                     |
| Model 5 | parameter ~ weight                             |
| Model 6 | parameter ~ weight + FCM difference            |
| Model 7 | parameter ~ weight difference                  |
| Model 8 | parameter ~ weight difference + FCM difference |
| Model 9 | parameter ~ boldness                           |

|          |                                       |
|----------|---------------------------------------|
| Model 10 | parameter ~ boldness + FCM difference |
| Model 11 | parameter ~ activity                  |
| Model 12 | parameter ~ activity + FCM difference |

**Table 1** - Set of candidate models built for each immune parameter.

To select the best models of variation in FCM levels and temporal variations in FCM levels and immune parameters, we used a model selection procedure based on the second-order Akaike Information Criterion (AICc, Burnham and Anderson, 2002). In addition, we calculated AICc weights (AICcw) to measure the relative likelihood that a given model was the best among the set of fitted models. Given that our study was an exploratory work, following Garamszegi and colleagues (2009), we did not use inferential statistical tests based on P values. Normality of the residuals for the selected models was tested (Shapiro-Wilk test) and visually assessed with histograms. Goodness-of-fit was assessed by calculating  $R^2$  values and standard residual plot techniques. All analyses were carried out with R version 3.6.0 (R Development Core Team 2016), using the lmer function from the lme4 package (Bates et al., 2014).

## Results

### Co-variation between stress levels and behavioural traits

Of the five variables tested, the one that best described changes in FCM levels over the course of the experiment was the effect of boldness (Table 2). On average, individuals that did not struggle during handling (i.e. shy individuals) exhibited 25% higher FCM values than those who struggled (i.e. bold individuals;  $-0.224$ ;  $CI = [-0.401, -0.040]$ ; Fig. 2). Regarding temporal variations in FCMs levels, the same relationship was not observed as our best models was the

null model (Table 3). Age, activity and collection number did not appear to explain the observed variations of FCM levels.

### **Co-variation between immune markers, FCM, and behavioural traits**

The best models explaining changes in immune functions four weeks after capture stress procedure differed among the 13 immune markers (see Supplementary data 1). Changes in immune functions were not related to changes in FCM level, boldness or activity levels for seven of the following immune markers: basophils, alpha-1, alpha-2, beta-1, beta-2, gamma globulins and haptoglobin (Table 4). Two innate markers, WBC (Fig. 3) and neutrophils (Fig. 4) showed clear negative co-variation with FCMs. A positive trend was also observed for a third innate marker of immunity, monocytes (Table 4). Conversely, there was a positive co-variation between FCMs and two adaptive markers: lymphocytes (Fig. 5) and anti-rabies antibodies concentration following an immune challenge (Fig. 6). Variation in eosinophils was related to the degree of boldness, with individuals that struggled during handling (i.e. bold individuals) exhibiting a lower increase in eosinophils between the two capture events compared to those who did not struggle (i.e. shy individuals) (Fig. 7). Variation in haptoglobin was best explained by mean activity levels and shows a weak negative relationship (Table 4; Fig 8). None of the selected models nor of the models within a delta AICc of 1 unit included the additive effect of FCM variation and behaviour (boldness or activity) (supplementary data 1).

## **Discussion**

The aims of this study were twofold. First, we aimed to gain a better understanding on how relatively long-term changes in glucocorticoid hormones affected changes in immune parameters of both innate and the adaptive arms. Second, we tested whether behavioural

traits could underlie the co-variation of immune parameters and glucocorticoid levels. While we found some support for an influence of FCM level fluctuations on the variation of immune parameters, we obtained a mitigated support for the effect of behavioural traits.

Among the innate immune parameters, WBC and neutrophil counts were negatively impacted by a positive change in cortisol levels, while we had a weak positive effect on monocytes. The observed negative relationship is consistent with the previous finding of an immunosuppressive effect of chronic stress on immunity (Dhabhar, 2014), however here this effect appeared only for innate immunity.

With respect to the adaptive arm of the immune system, our result did not supported the finding of the immunosuppressive effect of long-term elevation of glucocorticoids, but instead supported the finding of a positive relationship between a short-term increase in cortisol levels and lymphocytes and antibodies levels. In particular, it has been shown that a short-term elevation in glucocorticoids at the time of vaccination reinforce the efficacy of vaccination (Dhabhar, 2014). A similar scenario may have occurred in our study. The stress of capture, which results in a sudden increase in circulating glucocorticoids, would have strengthened the immune response to anti-rabies vaccine. In addition, considering that the energetic cost of mounting an antibody response is relatively low compared to that of the innate immune response (McDade et al., 2016), such a trade-off between cortisol and antibody levels is not expected. This positive relationship also supports the framework proposed by Lee (2006), who stated that non-specific and inflammatory responses should be down-regulated, while specific defences should be up-regulated in individuals experiencing high energy demands as it is the case with increased cortisol levels. Here, all individuals have been subjected to standardised capture stress, before returning to their usual living condition.

However, this protocol resulted in different outcomes depending on the individuals: some of them had an increased level of FCMs during the second period compared to the first one, which was associated with lower levels of WBC and neutrophil counts, but higher levels of lymphocytes and anti-rabies Abs, thus an immune system directed towards more adaptive immunity. On the contrary, in the individuals showing a lower level of FCMs during the second period, immunity was more oriented toward the innate arm (higher levels of WBC and neutrophil counts), and adaptive immunity may have been down-regulated, as illustrated by lower lymphocyte counts and lesser increase in anti-rabies antibodies. Consequently, it appears that in our study, individuals may have switched their investment from one arm of the immune system to the other according to their fluctuation in stress levels.

We did not find a strong support for the association between behavioural traits and immunity. On the opposite, in accordance with our hypothesis, we observed that bolder individuals exhibited lower cortisol levels compared to shier individuals. This result support previous study on wild (Carbillet et al., under review) and captive (Monestier et al., 2016) roe deer. It is also in accordance with the coping style framework developed by Koolhaas and colleagues (1999) and the pace of life hypothesis (Réale et al., 2010) which states that behavioural and physiological responses to stressful situations are connected. However, we did not find a similar relationship between temporal variations in stress levels and behavioural traits. This lack of such a relationship might be due to our small sample size compared to the one used for the previous analysis on cortisol levels. Regarding co-variations between immunity and behaviour, we found only a weak relationship, with only eosinophil production (innate immunity) being negatively linked to boldness, and a weak trend for a negative relationship between haptoglobin production (inflammatory) and activity. However, this latter relationship appears to be highly dependent on three particular point and its biological

significance remains unsure. We expected bolder individuals to invest more in innate and inflammatory markers compared to shy individuals that were expected to invest more in adaptive immunity (Lee, 2006; Réale et al., 2010). Shy individuals usually have a slower pace of life and favour survival and future reproduction contrary to bolder individuals that favour current reproduction (Réale et al., 2010). Eosinophils are part of the cells that bridge innate and adaptive immune response. In particular, they are involved in Th2 responses, which are part of the adaptive immune system, and are mostly beneficial against repeated infection. They are therefore more likely to be favoured in slower-lived individuals (Lee, 2006).

Overall, our results seems to indicate that temporal variations in cortisol levels might be associated with a re-allocation between innate and adaptive immunity, such that individuals exhibiting increased cortisol concentration might shift from innate to adaptive immunity, while individuals experiencing decreased cortisol concentrations might shift from adaptive to innate immunity. Cortisol is a metabolic hormone that regulates the use of energy. The cost of innate immunity being higher than that of adaptive immunity (McDade et al., 2016), we suggest that the switch observed between arms of immunity may be underlies by energetic trade-offs, which would be congruent with the pace of life hypothesis (Réale et al., 2010).

Finally, still in accordance with the pace of life hypothesis (Réale et al., 2010), we found that behavioural traits might be linked to investment in one arm of the immune system or the other. Considering all of our results that tend to support a co-variation between stress hormones, immunity and to a lesser extent behaviour, it might be interesting that future works aim to investigate how behavioural traits such as boldness, exploration or activity could modulate the relationship between glucocorticoids and immunity. The extents to which co-variations between these traits are influenced by different life history strategies in the wild



also warrant further investigations. These approaches may help to understand the large and unexplained variability in glucocorticoids-immunity relationship observed in wildlife studies.

## **Acknowledgments**

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| Models            | K | AICc  | $\Delta$ AICc | AICw |
|-------------------|---|-------|---------------|------|
|                   |   |       |               |      |
| Boldness          | 5 | 331.7 | 0.00          | 0.47 |
| Session           | 5 | 332.8 | 1.12          | 0.27 |
| Null              | 4 | 334.8 | 3.10          | 0.10 |
| Collection number | 5 | 335.3 | 3.56          | 0.08 |
| Activity          | 5 | 336.3 | 4.58          | 0.05 |
| Age               | 5 | 336.7 | 4.97          | 0.04 |

**Table 2.** Performance of the candidate linear mixed-effect models fitted to investigate variation in faecal glucocorticoid metabolite levels during the eight weeks of the experiment. Candidate models included behaviour during handling (boldness), mean daily vector of the dynamic body acceleration (activity), age, ordered collection number of samples for each individual (collection number), session of sample's collection before or after the first capture (session), and the null model. Individual identity and enclosure identity were included as random effects. AICc is the value of the corrected Akaike's Information Criterion and K is the number of estimated parameters for each model. The ranking of the models is based on the differences in the values for  $\Delta$ AICc and on the Akaike weights (AICw).



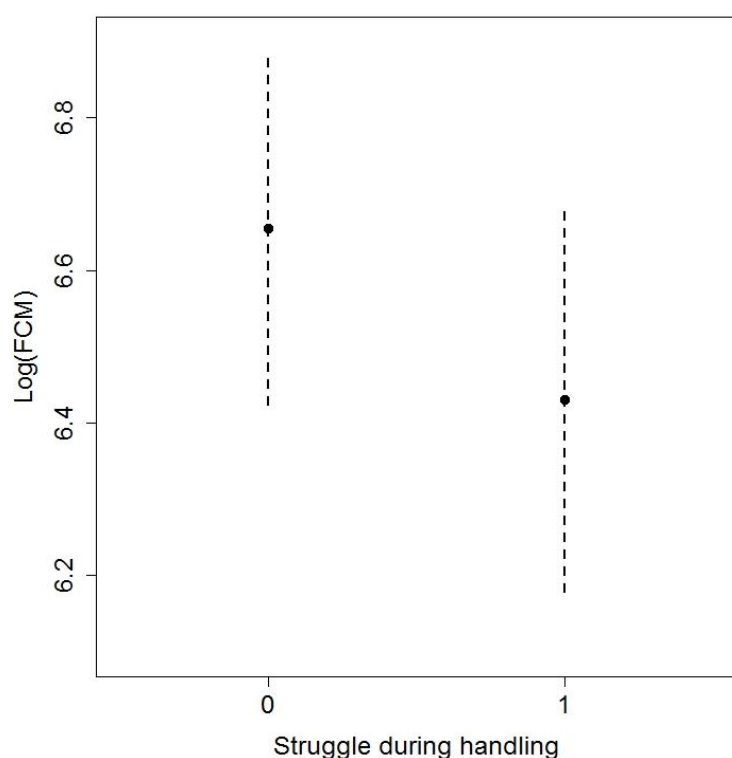
| Models            | K | AICc  | $\Delta$ AICc | AICw |
|-------------------|---|-------|---------------|------|
| Null              | 3 | 195.3 | 0             | 0.59 |
| Weight            | 4 | 199.3 | 3.75          | 0.09 |
| Boldness          | 4 | 199.4 | 3.80          | 0.09 |
| Activity          | 4 | 199.5 | 4.01          | 0.08 |
| Weight difference | 4 | 199.6 | 4.11          | 0.08 |
| Age               | 4 | 199.6 | 4.31          | 0.07 |

**Table 3.** Performance of the candidate linear mixed-effect models fitted to investigate temporal variation in faecal glucocorticoid metabolite levels between the second and first capture events. Candidate models included behaviour during handling (boldness), mean daily vector of the dynamic body acceleration (activity), age, weight at the second capture as well as difference of weight between the two capture events, and the null model. Enclosure identity was included as a random effects. AICc is the value of the corrected Akaike's Information Criterion and K is the number of estimated parameters for each model. The ranking of the models is based on the differences in the values for  $\Delta$ AICc and on the Akaike weights (AICw).

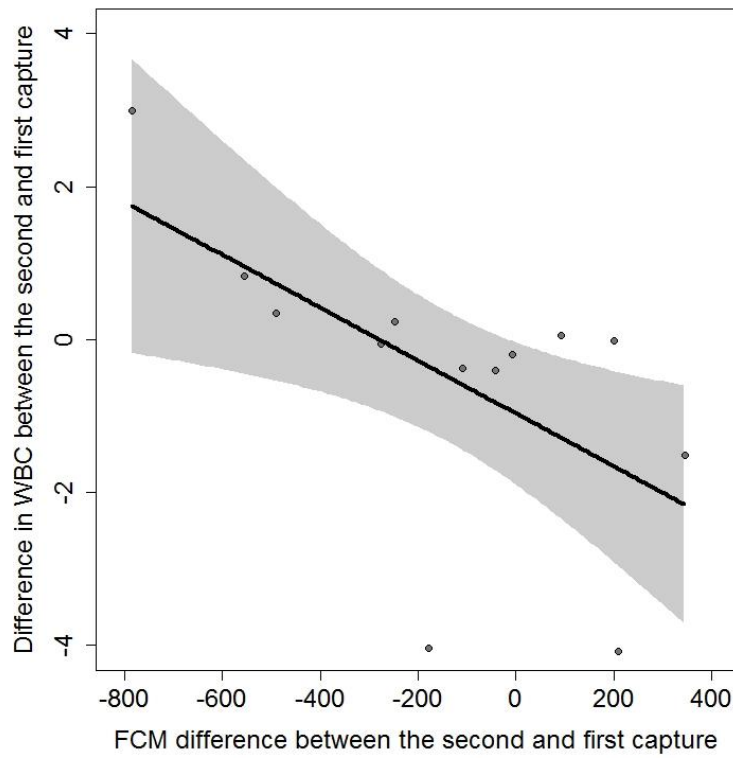
| Parameter   | Estimate | Lower CI | Upper CI |
|---|----------|----------|----------|
| Innate markers of immunity                                  |          |          |          |
| <b>WBC difference</b><br>(R <sup>2</sup> :0.31)             |          |          |          |
| Intercept   | -0.964   | -2.009   | 0.080    |
| FCM difference  | -0.003   | -0.006   | -0.001   |
| <b>Neutrophil difference</b><br>(R <sup>2</sup> :0.32)      |          |          |          |
| Intercept   | -18.826  | -26.764  | -10.888  |
| FCM difference  | -0.027   | -0.050   | -0.004   |
| <b>Monocyte difference</b><br>(R <sup>2</sup> :0.19)        |          |          |          |
| Intercept   | 0.233    | -1.245   | 1.715    |
| FCM difference  | 0.004    | -0.001   | 0.008    |
| <b>Basophil difference</b><br>(R <sup>2</sup> :0.22)        |          |          |          |
| Intercept   | 1.484    | -0.047   | 3.014    |
| Weight  | 2.433    | -0.146   | 5.011    |
| <b>Eosinophil difference</b><br>(R <sup>2</sup> :0.27)      |          |          |          |
| Intercept   | 1.786    | 1.237    | 2.335    |
| Boldness (struggle)   | -0.864   | -1.672   | -0.057   |
| <b>Beta-2 globulin difference</b><br>(R <sup>2</sup> :0.22) |          |          |          |
| Intercept   | 0.127    | -0.039   | 0.293    |
| Weight difference   | -0.264   | -0.543   | 0.014    |
| Inflammatory markers of immunity                            |          |          |          |
| <b>Alpha-1 globulin difference</b>                          |          |          |          |

|  |        |        |        |
|--|--------|--------|--------|
| (R <sup>2</sup> :0.46)                 |        |        |        |
| Intercept                              | -2.950 | -4.955 | -0.944 |
| Weight                                 | 0.116  | 0.040  | 0.193  |
| <b>Alpha-2 globulin difference</b>     |        |        |        |
| Intercept                              | -0.376 | -0.967 | 0.214  |
| <b>Haptoglobin difference</b>          |        |        |        |
| (R <sup>2</sup> :0.32)                 |        |        |        |
| Intercept                              | -2.049 | 0.378  | 3.720  |
| Activity                               | -0.008 | -0.016 | -0.001 |
| <b>Beta-1 globulin difference</b>      |        |        |        |
| (R <sup>2</sup> :0.70)                 |        |        |        |
| Intercept                              | 8.897  | 5.243  | 12.552 |
| Weight                                 | -0.341 | -0.480 | -0.201 |
| Adaptive markers of immunity           |        |        |        |
| <b>Lymphocyte difference</b>           |        |        |        |
| (R <sup>2</sup> :0.35)                 |        |        |        |
| Intercept                              | 16.572 | 11.333 | 21.813 |
| FCM difference                         | 0.019  | 0.004  | 0.034  |
| <b>Anti-rabies antibody difference</b> |        |        |        |
| (R <sup>2</sup> :0.53)                 |        |        |        |
| Intercept                              | 18.568 | 8.169  | 28.967 |
| Weight                                 | -0.624 | -1.020 | -0.228 |
| FCM difference                         | 0.002  | 0.000  | 0.003  |
| <b>Gamma globulin difference</b>       |        |        |        |
| Intercept                              | 1.373  | 1.019  | 1.727  |

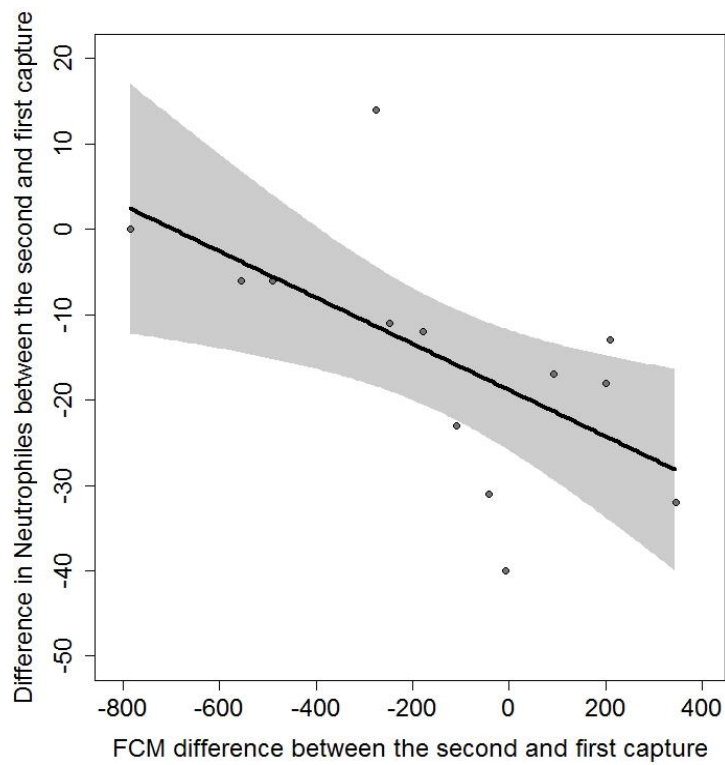
**Table 4.** Characteristics of the selected linear models for explaining variation in the 13 immune parameters considered between the second and first capture events of the experimentation. FCM difference stands for the difference in FCM level values between the second and first capture event; Weight stands for the weight of individuals at the second capture event; Boldness stands for the struggling behaviour during handling (0: no struggle; 1: struggle); Weight difference stands for the difference in weight values between the second and first capture event; Activity stand for the mean daily veDBA values.  $R^2$  referred to the explained variance of the models. CI stands for 95% Confidence Interval. See text for definition of model sets.



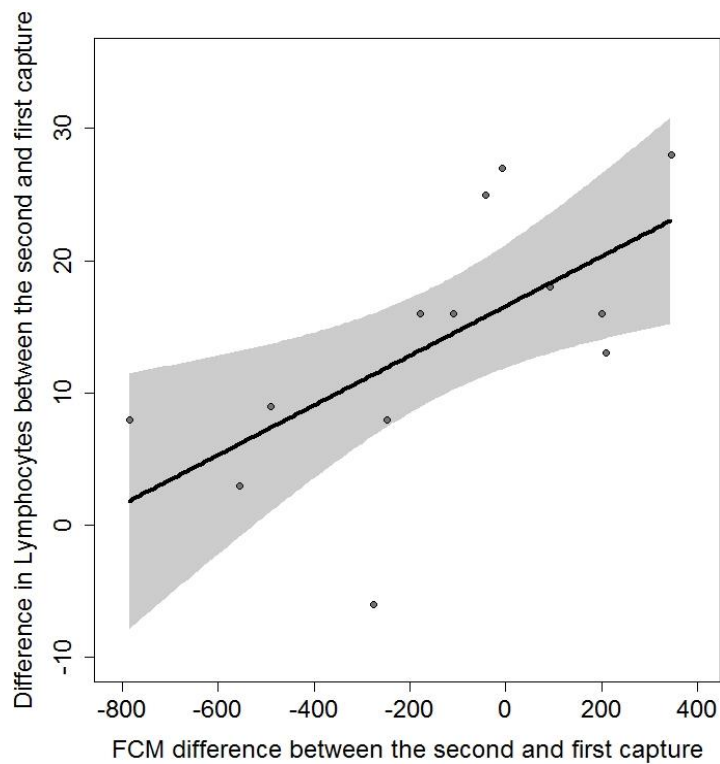
**Fig. 2.** Relationship between faecal cortisol metabolites (FCMs) levels and the behaviour during handling indexing boldness. Behaviour during handling was expressed in two classes with a score of 0 (no struggle) and 1 (struggle). Points indicate observed values. Lines represent model predictions and the dashed lines represent the 95% confidence interval.



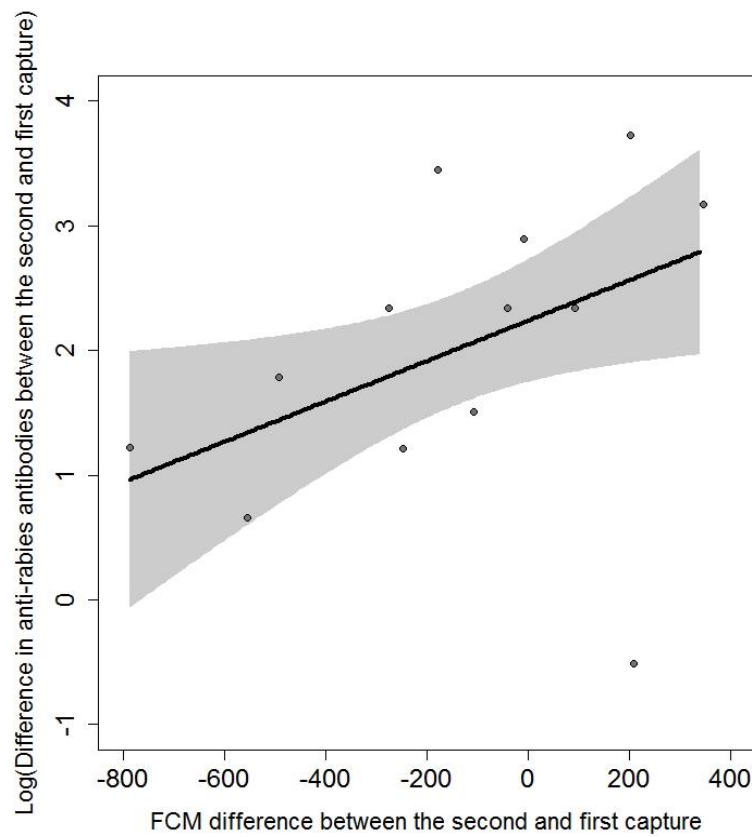
**Fig. 3.** Relationship between white blood cells (WBC) variation between the second and first capture events and faecal cortisol metabolites (FCMs) variation. Points represent observed values, lines represent model predictions and shaded area represents the 95% confidence interval.



**Fig. 4.** Relationship between neutrophils variation between the second and first capture events and faecal cortisol metabolites (FCMs) variation. Points represent observed values, lines represent model predictions and shaded area represents the 95% confidence interval.

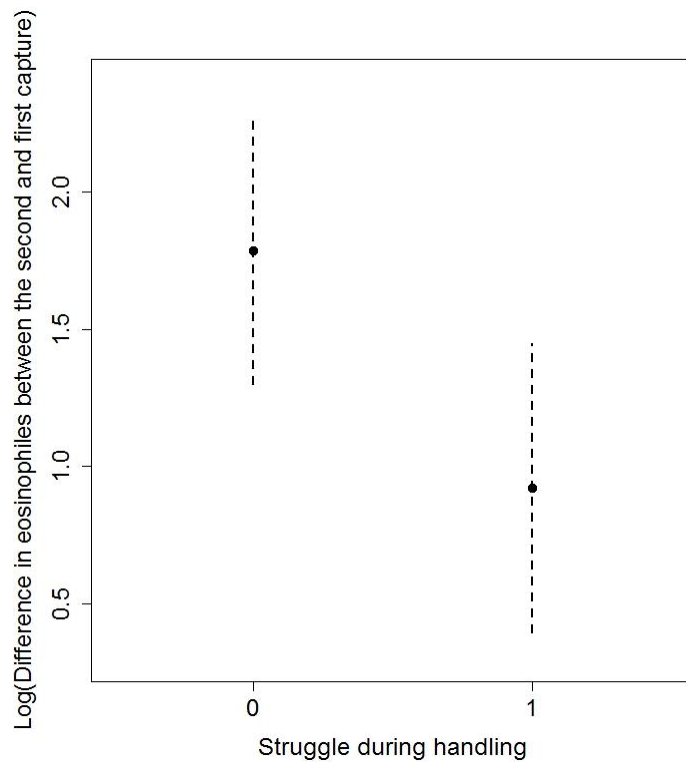


**Fig. 5.** Relationship between lymphocytes variation between the second and first capture events and faecal cortisol metabolites (FCMs) variation. Points represent observed values, lines represent model predictions and shaded area represents the 95% confidence interval.

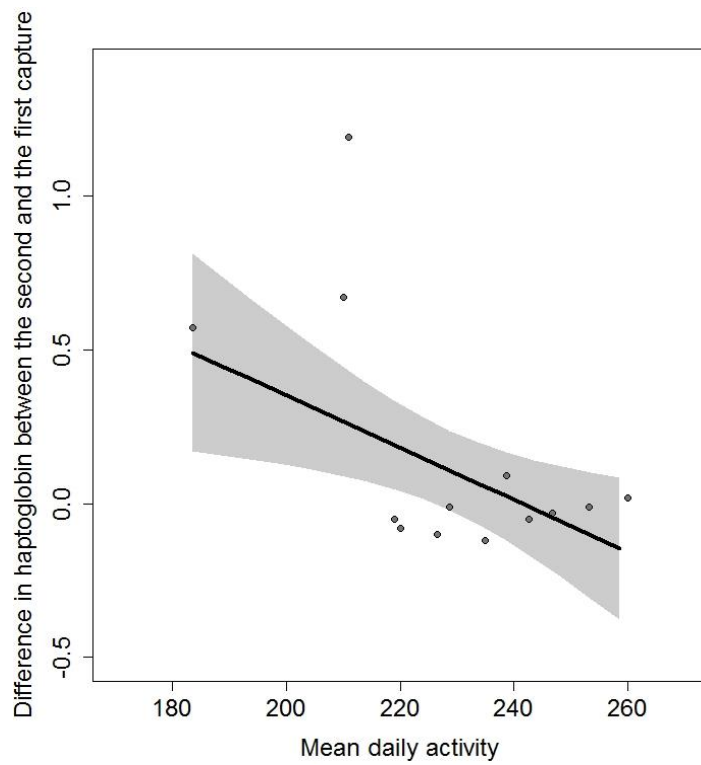


**Fig. 6.** Relationship between anti-rabies antibodies variation between the second and first capture events and faecal cortisol metabolites (FCMs) variation. Points represent observed values, lines represent model predictions and shaded area represents the 95% confidence interval.





**Fig. 7.** Relationship between eosinophils variation between the second and first capture events and the behaviour during handling indexing boldness. Behaviour during handling was expressed in two classes with a score of 0 (no struggle) and 1 (struggle). Points indicate observed values. Lines represent model predictions and the dashed lines represent the 95% confidence interval.



**Fig. 8.** Relationship between haptoglobin variation between the second and first capture events and activity (mean daily veDBA). Points indicate observed values, lines represent model predictions and shaded area represents the 95% confidence interval.

**Supplementary data 1:** Performance of the candidate linear models fitted to investigate variation in the 13 immune parameters between the second and first capture events of the experiment. Candidate models included a single variable or additive effects testing for a specific hypothesis or possible confounding effects. In addition to age, enclosure identity, boldness (struggling behaviour during handling, 0 for no struggle ; 1 for struggle) and activity (mean daily veDBA), difference of FCM levels between the second and first capture events , and weight at the second capture as well as difference of weight between the two capture events. AICc is the value of the corrected Akaike's Information Criterion and K is the number of estimated parameters for each model. The ranking of the models is based on the differences in the values for  $\Delta AICc$  and on the Akaike weights (AICw).

A) WBC variation

| Models                             | K | AICc  | $\Delta AICc$ | AICw |
|------------------------------------|---|-------|---------------|------|
| FCM difference                     | 3 | 54.98 | 0.00          | 0.35 |
| Boldness + FCM difference          | 4 | 56.55 | 1.56          | 0.16 |
| Null                               | 2 | 57.45 | 2.47          | 0.10 |
| Weight + FCM difference            | 4 | 57.56 | 2.58          | 0.10 |
| Boldness                           | 3 | 57.78 | 2.80          | 0.09 |
| Weight difference + FCM difference | 4 | 58.50 | 3.51          | 0.06 |
| Weight                             | 3 | 59.01 | 4.03          | 0.05 |
| Activity + FCM difference          | 4 | 59.28 | 4.30          | 0.04 |
| Weight difference                  | 3 | 59.90 | 4.92          | 0.03 |
| Age                                | 3 | 60.64 | 5.66          | 0.02 |
| Activity                           | 3 | 60.88 | 5.90          | 0.02 |
| Enclosure identity                 | 6 | 77.03 | 22.04         | 0.00 |

## B) Neutrophil variation

| Models                             | K | AICc   | $\Delta AICc$ | AICw |
|------------------------------------|---|--------|---------------|------|
|                                    |   |        |               |      |
| FCM difference                     | 3 | 107.72 | 0.00          | 0.42 |
| Boldness + FCM difference          | 4 | 109.13 | 1.41          | 0.21 |
| Null                               | 2 | 110.48 | 2.76          | 0.11 |
| Weight + FCM difference            | 4 | 111.91 | 4.20          | 0.05 |
| Weight difference + FCM difference | 4 | 111.96 | 4.24          | 0.05 |
| Activity + FCM difference          | 4 | 111.96 | 4.25          | 0.05 |
| Boldness                           | 3 | 113.29 | 5.57          | 0.03 |
| Activity                           | 3 | 113.61 | 5.90          | 0.02 |
| Age                                | 3 | 113.72 | 6.01          | 0.02 |
| Weight difference                  | 3 | 113.94 | 6.23          | 0.02 |
| Weight                             | 3 | 113.95 | 6.23          | 0.02 |
| Enclosure identity                 | 6 | 128.81 | 21.09         | 0.00 |

## C) Lymphocyte variation

| Models                             | K | AICc   | $\Delta AICc$ | AICw |
|------------------------------------|---|--------|---------------|------|
|                                    |   |        |               |      |
| FCM difference                     | 3 | 96.92  | 0.00          | 0.49 |
| Boldness + FCM difference          | 4 | 99.27  | 2.35          | 0.15 |
| Null                               | 2 | 100.17 | 3.25          | 0.10 |
| Weight difference + FCM difference | 4 | 101.04 | 4.12          | 0.06 |
| Activity + FCM difference          | 4 | 101.18 | 4.26          | 0.06 |
| Weight + FCM difference            | 4 | 101.20 | 4.29          | 0.06 |
| Boldness                           | 3 | 103.31 | 6.39          | 0.02 |

|                    |   |        |       |      |
|--------------------|---|--------|-------|------|
| Weight             | 3 | 103.61 | 6.69  | 0.02 |
| Activity           | 3 | 103.62 | 6.70  | 0.02 |
| Age                | 3 | 103.62 | 6.70  | 0.02 |
| Weight difference  | 3 | 103.64 | 6.72  | 0.02 |
| Enclosure identity | 6 | 117.26 | 20.34 | 0.00 |

#### D) Anti-rabies antibody variation

| <b>Models</b>                      | <b>K</b> | <b>AICc</b> | <b><math>\Delta</math>AICc</b> | <b>AICw</b> |
|------------------------------------|----------|-------------|--------------------------------|-------------|
|                                    |          |             |                                |             |
| Weight + FCM difference            | 4        | 41.49       | 0.00                           | 0.50        |
| Weight                             | 3        | 42.34       | 0.86                           | 0.33        |
| Null                               | 2        | 45.75       | 4.27                           | 0.06        |
| Weight difference                  | 3        | 47.47       | 5.99                           | 0.03        |
| FCM difference                     | 3        | 47.61       | 6.13                           | 0.02        |
| Enclosure identity                 | 6        | 49.03       | 7.54                           | 0.01        |
| Boldness                           | 3        | 49.03       | 7.55                           | 0.01        |
| Activity                           | 3        | 49.17       | 7.68                           | 0.01        |
| Age                                | 3        | 49.18       | 7.69                           | 0.01        |
| Weight difference + FCM difference | 4        | 49.31       | 7.83                           | 0.01        |
| Boldness + FCM difference          | 4        | 51.40       | 9.91                           | 0.00        |
| Activity + FCM difference          | 4        | 51.75       | 10.26                          | 0.00        |

## E) Monocyte variation

| Models                             | K | AICc  | $\Delta AICc$ | AICw |
|------------------------------------|---|-------|---------------|------|
|                                    |   |       |               |      |
| FCM difference                     | 3 | 64.01 | 0.00          | 0.32 |
| Null                               | 2 | 64.48 | 0.47          | 0.25 |
| Weight difference                  | 3 | 66.98 | 2.97          | 0.07 |
| Weight difference + FCM difference | 4 | 67.60 | 3.59          | 0.05 |
| Activity                           | 3 | 67.94 | 3.92          | 0.05 |
| Weight                             | 3 | 67.94 | 3.93          | 0.05 |
| Age                                | 3 | 67.94 | 3.93          | 0.05 |
| Boldness                           | 3 | 67.95 | 3.94          | 0.04 |
| Boldness + FCM difference          | 4 | 68.11 | 4.10          | 0.04 |
| Weight + FCM difference            | 4 | 68.30 | 4.29          | 0.04 |
| Activity + FCM difference          | 4 | 68.31 | 4.29          | 0.04 |
| Enclosure identity                 | 6 | 81.86 | 17.85         | 0.00 |

## F) Eosinophil variation

| Models                             | K | AICc  | $\Delta AICc$ | AICw |
|------------------------------------|---|-------|---------------|------|
|                                    |   |       |               |      |
| Boldness                           | 3 | 32.56 | 0.00          | 0.34 |
| Weight difference                  | 3 | 33.08 | 0.52          | 0.26 |
| Null                               | 2 | 34.41 | 1.84          | 0.14 |
| Activity                           | 3 | 35.64 | 3.08          | 0.07 |
| Boldness + FCM difference          | 4 | 36.19 | 3.63          | 0.06 |
| Weight difference + FCM difference | 4 | 37.03 | 4.47          | 0.04 |
| Age                                | 3 | 37.24 | 4.68          | 0.03 |

|                           |   |       |       |      |
|---------------------------|---|-------|-------|------|
| Weight                    | 3 | 37.79 | 5.23  | 0.03 |
| FCM difference            | 3 | 37.81 | 5.25  | 0.02 |
| Activity + FCM difference | 4 | 39.98 | 7.41  | 0.01 |
| Weight + FCM difference   | 4 | 42.08 | 9.52  | 0.00 |
| Enclosure identity        | 6 | 51.55 | 18.98 | 0.00 |

### G) Basophil variation

| <b>Models</b>                      | <b>K</b> | <b>AICc</b> | <b>ΔAICc</b> | <b>AICw</b> |
|------------------------------------|----------|-------------|--------------|-------------|
|                                    |          |             |              |             |
| Weight difference                  | 3        | 67.08       | 0.00         | 0.36        |
| Null                               | 2        | 67.92       | 0.83         | 0.24        |
| FCM difference                     | 3        | 69.85       | 2.77         | 0.09        |
| Weight difference + FCM difference | 4        | 70.07       | 2.98         | 0.08        |
| Age                                | 3        | 70.58       | 3.50         | 0.06        |
| Weight                             | 3        | 71.35       | 4.27         | 0.04        |
| Activity                           | 3        | 71.36       | 4.27         | 0.04        |
| Boldness                           | 3        | 71.38       | 4.30         | 0.04        |
| Weight + FCM difference            | 4        | 74.04       | 6.95         | 0.01        |
| Boldness + FCM difference          | 4        | 74.14       | 7.05         | 0.01        |
| Activity + FCM difference          | 4        | 74.19       | 7.10         | 0.01        |
| Enclosure identity                 | 6        | 83.18       | 16.10        | 0.00        |

#### H) Gamma globulin variation

| Models                             | K | AICc  | $\Delta AICc$ | AICw |
|------------------------------------|---|-------|---------------|------|
| Null                               | 2 | 27.15 | 0.00          | 0.29 |
| Weight difference                  | 3 | 27.95 | 0.80          | 0.19 |
| Activity                           | 3 | 28.74 | 1.59          | 0.13 |
| Boldness                           | 3 | 28.93 | 1.78          | 0.12 |
| Weight                             | 3 | 29.22 | 2.07          | 0.10 |
| FCM difference                     | 3 | 30.50 | 2.35          | 0.05 |
| Age                                | 3 | 30.61 | 3.46          | 0.05 |
| Weight difference + FCM difference | 4 | 31.91 | 4.76          | 0.03 |
| Boldness + FCM difference          | 4 | 32.83 | 5.68          | 0.02 |
| Activity + FCM difference          | 4 | 33.05 | 5.91          | 0.01 |
| Weight + FCM difference            | 4 | 33.52 | 6.37          | 0.01 |
| Enclosure identity                 | 6 | 42.41 | 15.26         | 0.00 |

#### I) Alpha-1 globulin variation

| Models                    | K | AICc  | $\Delta AICc$ | AICw |
|---------------------------|---|-------|---------------|------|
| Weight                    | 3 | -3.70 | 0.00          | 0.68 |
| Activity                  | 3 | -0.72 | 2.99          | 0.15 |
| Weight + FCM difference   | 4 | 0.62  | 4.32          | 0.08 |
| Null                      | 2 | 1.94  | 5.64          | 0.04 |
| Activity + FCM difference | 4 | 3.60  | 7.30          | 0.02 |
| Weight difference         | 3 | 4.72  | 8.43          | 0.01 |
| FCM difference            | 3 | 5.19  | 8.90          | 0.01 |



|                                    |   |      |       |      |
|------------------------------------|---|------|-------|------|
| Age                                | 3 | 5.32 | 9.02  | 0.01 |
| Boldness                           | 3 | 5.35 | 9.05  | 0.01 |
| Weight difference + FCM difference | 4 | 8.71 | 12.41 | 0.00 |
| Enclosure identity                 | 6 | 9.32 | 13.02 | 0.00 |
| Boldness + FCM difference          | 4 | 9.41 | 13.11 | 0.00 |

#### J) Alpha-2 globulin variation

| Models                             | K | AICc  | $\Delta AICc$ | AICw |
|------------------------------------|---|-------|---------------|------|
| Null                               | 2 | 40.44 | 0.00          | 0.41 |
| Activity                           | 3 | 42.64 | 2.20          | 0.14 |
| Weight                             | 3 | 43.46 | 3.02          | 0.09 |
| Boldness                           | 3 | 43.57 | 3.13          | 0.09 |
| Age                                | 3 | 43.78 | 3.34          | 0.08 |
| FCM difference                     | 3 | 43.80 | 3.36          | 0.08 |
| Weight difference                  | 3 | 43.84 | 3.40          | 0.07 |
| Activity + Weight difference       | 4 | 46.69 | 6.25          | 0.02 |
| Weight + FCM difference            | 4 | 47.60 | 7.16          | 0.01 |
| Boldness + FCM difference          | 4 | 47.85 | 7.41          | 0.01 |
| Weight difference + FCM difference | 4 | 48.08 | 7.64          | 0.01 |
| Enclosure identity                 | 6 | 52.64 | 12.20         | 0.00 |

### K) Beta-1 globulin variation

| Models                             | K | AICc  | $\Delta AICc$ | AICw |
|------------------------------------|---|-------|---------------|------|
|                                    |   |       |               |      |
| Weight                             | 3 | 11.90 | 0.00          | 0.87 |
| Weight + FCM difference            | 4 | 15.79 | 3.89          | 0.12 |
| Null                               | 2 | 25.19 | 13.29         | 0.00 |
| Activity                           | 3 | 26.94 | 15.04         | 0.00 |
| Weight difference                  | 3 | 27.70 | 15.80         | 0.00 |
| Boldness                           | 3 | 28.19 | 16.29         | 0.00 |
| FCM difference                     | 3 | 28.65 | 16.75         | 0.00 |
| Age                                | 3 | 28.66 | 16.76         | 0.00 |
| Activity + FCM difference          | 4 | 31.26 | 19.36         | 0.00 |
| Weight difference + FCM difference | 4 | 31.97 | 20.07         | 0.00 |
| Boldness + FCM difference          | 4 | 32.46 | 20.56         | 0.00 |
| Enclosure identity                 | 6 | 35.10 | 23.20         | 0.00 |

### L) Beta-2 globulin variation

| Models                             | K | AICc  | $\Delta AICc$ | AICw |
|------------------------------------|---|-------|---------------|------|
|                                    |   |       |               |      |
| Weight difference                  | 3 | 9.26  | 0.00          | 0.36 |
| Null                               | 2 | 10.13 | 0.87          | 0.23 |
| Weight difference + FCM difference | 4 | 12.06 | 2.80          | 0.09 |
| Weight                             | 3 | 12.38 | 3.11          | 0.08 |
| Activity                           | 3 | 12.41 | 3.14          | 0.07 |
| FCM difference                     | 3 | 13.01 | 3.75          | 0.05 |
| Boldness                           | 3 | 13.32 | 4.05          | 0.05 |

|                           |   |       |       |      |
|---------------------------|---|-------|-------|------|
| Age                       | 3 | 13.52 | 4.25  | 0.04 |
| Weight + FCM difference   | 4 | 16.30 | 7.04  | 0.01 |
| Activity + FCM difference | 4 | 16.34 | 7.08  | 0.01 |
| Boldness + FCM difference | 4 | 16.83 | 7.57  | 0.01 |
| Enclosure identity        | 6 | 25.37 | 16.10 | 0.00 |

#### M) Haptoglobin variation

| Models                             | K | AICc  | $\Delta AICc$ | AICw |
|------------------------------------|---|-------|---------------|------|
|                                    |   |       |               |      |
| Activity                           | 3 | 5.87  | 0.00          | 0.48 |
| Null                               | 2 | 8.51  | 2.64          | 0.13 |
| Activity + FCM difference          | 4 | 9.01  | 3.13          | 0.10 |
| Weight                             | 3 | 9.44  | 3.57          | 0.08 |
| Boldness                           | 3 | 9.64  | 3.77          | 0.07 |
| FCM difference                     | 3 | 10.51 | 4.64          | 0.05 |
| Age                                | 3 | 11.07 | 5.19          | 0.04 |
| Weight difference                  | 3 | 11.70 | 5.83          | 0.03 |
| Weight + FCM difference            | 4 | 12.59 | 6.71          | 0.02 |
| Boldness + FCM difference          | 4 | 12.95 | 7.08          | 0.01 |
| Weight difference + FCM difference | 4 | 14.68 | 8.81          | 0.01 |
| Enclosure identity                 | 6 | 22.02 | 16.15         | 0.00 |



## Discussion and conclusion





Since the middle of the 20<sup>th</sup> century, climate change has caused an increase in extreme climatic events such as periods of severe drought or high precipitations. In addition, changes in landscape, land use, and habitat fragmentation have been observed over the last 30 years due to human activities (Hooke et al., 2012). Consequently, wild animals are increasingly exposed to unpredictable variations in their environment (Romero and Wingfield, 2015).

Since most of these changes are due to human activities (IPCC, 2014), it is likely that wildlife will continue to face such disturbance that can threaten their survival and affect population dynamics (Romero and Wingfield, 2001) during the upcoming years. Therefore, a better understanding (i) of the mechanisms involved in coping with these perturbations (mainly the stress response) and (ii) of their consequences on fitness is a necessary step to evaluate animals' degree of phenotypic plasticity and evolutionary potential in the face of challenging environments. In addition, this knowledge would be of high benefit for the conservation and management of wildlife, as it would allow conservation actions to be taken to avoid the negative outputs of repeated or long-term activation of the stress response.

With this thesis, I focused my research on the stress response, mediated by the HPA axis and which involves glucocorticoid hormones. My general aim was to understand the individual and environmental determinants of among-individual variations in the levels of stress, using an integrative approach. I also aimed to identify some consequences of long-term elevations of glucocorticoids in a long-lived mammal, the roe deer. My results show that the level of baseline stress in roe deer depends on a complex combination of environmental factors and individual (physiological and behavioural) characteristics that occur from the earliest stages of life to adulthood. In addition, I showed that baseline levels of glucocorticoids can influence vital functions such as immunity, but differently according to the availability of resources and individual characteristics such as age, body condition, or behavioural types. The relationship

between glucocorticoids and immunity also depended on the immune arm considered. Altogether, my findings paved the way for future studies to improve our understanding on glucocorticoid hormones, their evolutionary potential, and how they could act as a major trait shaping individual life-history trajectories.

## **1 - Individual and environmental characteristics shape baseline stress levels**

My first goal was to highlight factors that underlie among-individual variations in stress levels. To this purpose, I used a comprehensive approach taking into account both environmental (spatial and temporal heterogeneity in resources quantity and quality) and individual (sex, age, body mass, behavioural response to stressful situations) characteristics in three wild roe deer populations. In addition, I examined whether the effect of anthropogenic activities on glucocorticoid levels could be mitigated by behavioural adjustments, specifically, by increasing the use of refuge habitats. To reach these goals, I carried out two studies in natural conditions (Chapter I).

### **1-1 neutrophils to lymphocytes (N:L) ratio versus faecal cortisol metabolites (FCMs)**

In order to evaluate the influence of environmental and individual characteristics on the stress level of adults, I used two different metrics of stress level, the N:L ratio, and the FCMs. These two measures have different advantages and disadvantages, which I will discuss later in this section. While FCMs are a non-invasive measure of glucocorticoids that reflects the overall concentration of plasma glucocorticoids that an individual has experienced over a short time-window (Palme et al., 2005; Sheriff et al., 2011), N:L ratio is a downstream measure of glucocorticoid secretion. As explained by Andrew Davis and his colleagues (2008), when vertebrates experience a stress response, the rise in circulating glucocorticoid induce an increase in the relative proportion of neutrophils in blood, while the proportion of

lymphocytes decreases. This leads to an increase in the N:L ratio in individuals with high secretion of glucocorticoids, and this measure has been reliably used to index stress levels in a wide range of taxa (Davis and Maney, 2018). Contrary to FCMs that do not necessarily require individuals to be captured and restrained, the measure of N:L ratio requires blood samples, and is therefore a more invasive measure. In addition, while a large number of studies using FMs are available on a wide range of species, there is still a lack of range values for N:L ratio. However a growing database of leucocyte profiles with over 200 species is currently available at the following address: <https://wildlifehematology.uga.edu> (Davis, 2009).

As mentioned previously, while both glucocorticoids and N:L ratio increase when organisms face stressors, they are not always correlated, especially at baseline levels (Davis and Maney, 2018), and hence, are not interchangeable measures. As beautifully showed by Claudia Müller and her colleagues (2011), both glucocorticoids and N:L ratio are not necessarily influenced by the same stressors, and the N:L ratio was sensitive to a wider array of stressors. In addition, my work also showed that N:L ratio (MS1) and FCMs (MS2) are not necessarily influenced by the same factors. While I found a trend for an increase of the N:L ratio with age and higher values in males than females (MS1), it was not the case for FCMs (MS2). As hypothesised in MS1, these effects found with the N:L ratio might not be due to a relationship between stress levels and sex or age in roe deer, but most likely to the immunosenescence patterns of neutrophils and lymphocytes (Cheynel et al. 2017). This highlight the need to take into account different potential confounding factors when using N:L ratio or FCMs to index stress levels.

Another difference that might be important to consider when using the N:L ratio or FCMs to index stress levels is the time-course of both measures. In particular, while the increase in the N:L ratio has been shown to persist over long-periods of time in response to chronic stressors,

the glucocorticoid response has been shown to be less persistent (Gross and Spiegel, 1983; Müller et al., 2011). A recent meta-analysis conducted by Jeffrey Goessling and his colleagues (2015) highlighted two interesting differences between glucocorticoid and N:L ratio responses to long-term stressors. First, they showed that the amplitude of the glucocorticoid response tended to be more elevated than the N:L ratio response. Second, they showed that over 85 days, glucocorticoid response to stressors attenuated, while N:L ratio remained at a high level. To conclude on this methodological aspect, both the N:L ratio and FCMs are valid measures of the stress response. The choice between the two measures will most likely depend on the goal of the study, the possible degree of invasiveness, and the possibility to take into account different confounding factors. Overall, the N:L ratio might be more useful in order to evaluate the effects of long-term stressors, especially long-lasting environmental stressors, but it will probably require to consider a wider range of confounding factors (depending on the species) and will be a more invasive method than measuring FCMs. Conversely, the use of FCMs seems more appropriate to assess the effects of more transient stressors, and is a less invasive measure that does not necessarily require capture and restraint.

### **1-2 The influence of environmental characteristics on stress levels**

First, I found that temporal but not spatial variations in resources quality and quantity influenced stress levels in roe deer (MS1). Precisely, during years of low resource quality, individuals exhibited higher levels of stress. This result was in accordance with previous studies that showed elevated glucocorticoid levels in the face of low food availability (Hellgren et al., 1993), adverse weather conditions (Huber et al., 2003), or poor overall habitat quality (Marra and Holberton, 1998). On the opposite, despite marked local heterogeneity in resources quality and quantity within two study sites (Aurignac: Morellet et al., 2009; Hewison et al., 2009; and Chizé: Pettorelli et al., 2001; 2003), I did not evidence any effect of spatial



variation in resources on stress levels. As discussed in the first manuscript (MS1), it is possible that roe deer get adapted more easily to their predictable local environment (spatial variation in resources) than to global year to year unpredictable variations of resources (temporal variation in resources). If the ability to respond to such unpredictable changes appears positive, the expected rise in extreme climatic events in the upcoming years may represent as a serious threat for wild animals. Indeed, these unpredictable events such as severe drought, storms, flood etc... are likely to affect the availability of food resources and generate large temporal variations which might increase the level of stress of individuals for long-period of times.

Second, I highlighted that human activities, which are often considered as a form of predation (Frid and Dill, 2002), were not necessarily always associated with elevated concentrations of glucocorticoid hormones in wild animals. While a large body of literature found a negative association (see Dantzer et al., 2014 for a review), here, I showed that landscape structure and habitat use modulate the relationship between glucocorticoids and human disturbance. Precisely, the amount of refuge habitats, such as woods or hedgerows, and the selective use of these refuges by roe deer, allowed them to mitigate the physiological effects of human activities.

Similarly, night seemed to act as a refuge, as proximity with human sources of stress during the night was not associated with higher baseline glucocorticoid levels. While previous works clearly established that wild animals modified their spatial (Coulon et al., 2008; Martin et al, 2018) and temporal (Bonnot et al., 2013; Gaynor et al., 2018) behaviour in the face of human activities, I provided evidence that these behavioural adjustments may help wild animals to mitigate the costs of living in close proximity to humans. In a context of increased habitat fragmentation (IPCC, 2014) and intensification of agricultural land transformation (Hooke et

al., 2012), this results suggest that a cohabitation that does not generate stress remains possible between human activities and wild animals, as far as consideration is given to the preservation of key landscape features such as refuge habitats, that allow behavioural adjustments of wild animals.

### **1-3 The influence of individual characteristics on stress levels**

In addition to environmental characteristics, the work conducted within the first chapter of this thesis highlighted that individual's phenotype contributes to shape stress levels. Precisely, I found that baseline stress levels increase with age. While this result has been only observed for the N:L ratio, it remains unsure to which extent it revealed changes in patterns of stress levels or if it reflects age related remodelling of immune functions. However, I briefly discuss the implication of this possible increase of the stress level with age, as it could have important consequences. An increase of glucocorticoids has previously been observed in humans (Bauer, 2005) and rats (Sapolsky et al. 1983). A possible explanation to this phenomenon could be that a repeated stimulation of the HPA axis all along life would reduce the efficacy of the negative feedback. Consequently, ageing individuals should be less able to rapidly restore the initial baseline glucocorticoid levels and their baseline glucocorticoid levels should progressively increase (Romero, 2004). Such an increase in baseline glucocorticoid levels may have harmful consequences for individuals as it has been hypothesised to lead to a reduced capacity to answer to acute stressors (Romero et al., 2009), but may also be linked to reduction in telomere length (see Angelier et al., 2017 for a review; Lemaître et al., submitted), a phenomenon associated with health and mortality in humans (Blackburn et al., 2015) and non-model species (Wilbourn et al., 2018).

I found that among-individual differences in behaviour during a stressful situation (a capture event) were another individual characteristic that was related to stress levels. This result was

consistently observed in three out of the six manuscripts presented within this thesis (MS1, MS3 and MS6), and in three out of the four populations I studied. Precisely, in both wild (MS1) and captive (MS6) adult roe deer, I found that individuals exhibiting bold or proactive behaviours had lower stress levels than those exhibiting shy or reactive behaviours. Interestingly I found the opposite relationship in fawns (MS3). As presented in the introduction section, a framework developed by Jaap Koohlaas and his collaborators (1999) proposed that the physiological and behavioural stress responses may be tightly linked, such that individuals exhibiting active behaviour during stressful situations would exhibit low glucocorticoid levels, while individuals exhibiting a passive behaviour are expected to have high glucocorticoid levels. If my results on adults are coherent with this framework, it is not the case for fawns. Does this mean that the relationship between glucocorticoids and behaviour changes over the life of an individual? To date, very few studies have examined the ontogenetic processes of consistent among-individual differences in behavioural traits (i.e. personality) in vertebrates (but see Stamps and Groothuis, 2010; Günther et al., 2014). The same applies in a lesser extent to the ontogeny of the stress axis (but see Wada, 2008 for a review), but none have investigated the evolution of the glucocorticoids-behaviour relationship from early-life to adulthood. However, environmental factors acting upon individuals during early ontogeny might influence the development of both personality traits and stress physiology by adaptively shaping the individual's phenotype. But it may be possible that the degree of development of both the HPA axis and behaviour differ over time, which might lead to a differential co-variation between these two traits depending on the life-history stage (newborns versus adults). It was not possible to investigate the ontogeny of co-variation between glucocorticoids and behaviour during my PhD because the sample size was too small. My results warrant further investigations, first to confirm my results, with additional/other

behavioural traits because the relationship between glucocorticoids and behaviour may depend on the behavioural trait considered (Clary et al, 2014). It would also be interesting to evaluate the ontogeny of both stress physiology and personality and to investigate how and when a change in the direction of the relationship between both occurs. With our long-term capture-mark-recapture program, it will be possible to have data on cortisol concentrations and behavioural response to capture on the same individual at 10 days, 1 year, 2 years and even at older ages. Ideally, we would need to collect data at a finer time scale between 10 days and 1 year of age, for instance right after weaning (at 5-6 months) when the fawn becomes more independent from its mother in order to better understand when and why a potential change in the relationship occurs.

Another question that has not been fully addressed in the literature is: is there a cause-and-effect relationship between glucocorticoids and behaviour? Some studies on laboratory and domestic species have showed that the suppression of adrenocortical activity led to a reduction of freezing behaviour or fear (Rooszendaal et al., 1996). On the opposite, it has been shown that repeated application of corticosterone resulted in prolonged tonic immobility (Jones et al., 1988). To date, data on wild animals remain scarce, but also suggest that changes in glucocorticoid hormones may well drive, at least to some extent, the plasticity of behavioural traits (Dosmann et al., 2015; Baugh et al., 2017a). Indeed, glucocorticoid levels are by nature a labile trait that fluctuates in response to environmental cues. This led Conor Taff and Maren Vitousek (2016) to develop the framework of endocrine flexibility (i.e. the phenotypic plasticity in endocrine traits within an individual that occur in response to unpredictable changes) and to highlight the need for further research investigating the causes and consequences of variations in glucocorticoid hormones. Indeed, if glucocorticoid hormones have the potential to influence the expression of behavioural traits, the endocrine

flexibility could therefore be a mechanisms driving behavioural plasticity, an important component of the response of wild animals to unpredictable changes. Overall, results from the studies mentioned above suggest that a causal relationship between glucocorticoids and behavioural traits might exist, although it is possible that the co-variation between both may also, in part, be due to common causes. Consequently, how can we explain that we do not always find such a relationship, as it was the case in the Trois-Fontaines population (in this thesis), or in several other studies on other wild animals (Qu et al., 2018; Westrick et al., 2018)? It is this lack of connection between glucocorticoid levels and behavioural traits that brought Jaap Koolhaas and his colleagues (2010) to propose a second framework where these two traits would be independent from each other (the two-tier model). Specifically, the two-tier model proposes that coping-styles can be based on two independent axis linking behaviour and physiology. The first axis represents qualitative response to stressful situations and can be composed of behavioural traits such as activity, exploration or boldness. This first axis would be related to the sympathetic autonomic nervous system (adrenaline). The second axis represents quantitative response to stressful situations and is composed of other behavioural traits thought to be related to emotionality (e.g. docility or panic). These behavioural traits are supposed to be related to the HPA axis and the secretion of glucocorticoids.

Considering the existence of studies with mixed outcomes, we may ask to which extent the glucocorticoids-behaviour relationship might actually depend on the environmental context individuals are facing. Indeed, while most studies held on laboratory animals support the first view of coping-styles, the majority of studies conducted on wild animals found evidences supporting the second view (Ferrari et al., 2013; Grace and Anderson, 2014; Baugh et al., 2017b; Qu et al., 2018; Westrick et al., 2019). One major difference between these two

conditions is that laboratory studies used selection lines, while in the wild, there are usually more variability in both physiological and behavioural traits. In addition, as explained by Shaun Killen and his colleagues (2013), if individuals differ in their reaction norms to an environmental stressor, but that the reaction norm differs between behavioural and physiological traits, then the correlation between both traits will depend on the environment in which the measure is made. Taking this into consideration might help to understand the discrepancy found in the literature, as in the results presented here on adults, or between adults and fawns. In heterogeneous landscapes roe deer are not randomly distributed in their home range, but habitat use depends on their behavioural type (Bonnot et al., 2014). Precisely, bold individuals are expected to be frequent in open and risky habitats while shy individuals occur preferentially in refuge habitats such as woods. An experimental approach allowing to manipulate the repartition of different behavioural types in the landscape could help to elucidate to which extent the environmental context may play a role in the observed relationship between glucocorticoids and behavioural traits. Alternatively, using a within-individual reaction-norm approach in which the strength of the link between glucocorticoids and behavioural traits would be measured across an environmental gradient could also be an option in order to understand this complex relationship. A better understanding of the potential connection between these two traits still remains to be achieved, especially considering the fact that both glucocorticoids (Breuner et al., 2008; Bonier et al., 2009) and behavioural traits (Dingemanse and de Goede, 2004; Groothuis et al., 2008) have been linked to reproductive success and survival.

Overall, glucocorticoid hormones appear to be influenced by environmental factors such as availability of resources, proximity with human activities or amount of refuge habitats. But these hormones also appeared to be related to individual characteristics, and particularly

behavioural traits. Considering the current and expected increase in unpredictable environmental changes, it may be important to further investigate the nature of the relationship between glucocorticoid hormones and behavioural traits, especially to understand the potential for wild populations to adapt to a changing world. If the hormonal pleiotropy hypothesis that underlies the coping-style framework proposed by Jaap Koolhaas and his collaborators in 1999 is verified, then it could facilitate adaptive phenotypic integration by enabling the expression of an adaptive suite of traits (Ketterson et al. 2009; Dantzer and Swanson 2017). In this case, an early-life shaping of the HPA axis could be adaptive as it would allow an individual to exhibit a suite of behaviours that will enhance its performance early in life. Alternatively, if glucocorticoids enhance the expression of two traits, but selection acts in opposite directions on the two traits, hormonal pleiotropy could constrain the independent evolution of two traits and lead to mal-adaptive phenotypes. On the opposite, if the two-tier model of coping-style is supported, it would imply that there are two separate axes (i.e. one with a correlation between behaviour and glucocorticoids; and a second one without correlation), and this would suggest the potential for some behavioural traits to be an independent target of selection, potentially facilitating rapid adaptation to new environmental challenges (Ketterson et al., 2009).

## **2 - Early-life glucocorticoids and maternal effects**

The second goal of this thesis was tightly related to the points discussed above, but with the specific aim to gain a better understanding of the early-life sources of glucocorticoid level variations, including of maternal origin. I also aimed to evaluate the potential consequences of elevated maternal glucocorticoids on the early-life characteristics of the fawns, including its glucocorticoid levels. While some studies investigated the link between maternal and

offspring glucocorticoid levels (Hayward and Wingfield, 2004; Seckl and Meaney, 2004), I am not aware of a study that addressed this question considering environmental and individual factors that may underlie among-individual variations of offspring glucocorticoid levels in the wild. Do levels vary with early-age? Is there already a link between glucocorticoid levels and behavioural response to a stressor? Do human disturbance in the first weeks of life affect baseline glucocorticoid levels? To date, these questions remain poorly or not documented, while an understanding of these early-life variations could be valuable to better understand the trajectories of individuals early in life, those that can have long-term consequences.

To investigate these questions, first, I used individual (age, sex, body mass, behavioural response to a stressor), but also environmental (proximity with human activities and presence of refuge habitat within the home range) characteristics of fawns in order to link them to baseline glucocorticoid levels. Secondly, I investigated the consequences of elevated maternal glucocorticoids on the offspring's phenotype, including birth weight, growth rate, behavioural response to a stressor, and baseline glucocorticoid levels.

### **2-1 Measurement of salivary cortisol in newborns**

Although it is a common technique for assessing circulating glucocorticoids in humans, the use of saliva remains relatively scarce in wild animals. This paucity probably belongs to the difficulty to obtain salivary samples from un-trained and/or non-captive animals. Indeed, the few studies that measured salivary glucocorticoids on wild species were studies conducted on captive and trained animals (Millspaugh et al., 2002; Cross and Rogers, 2004; Pearson et al., 2008). However, the potential to use this technique on wild animals appear attractive, as it may be less invasive than blood sampling. Furthermore, glucocorticoid levels in saliva are not expected to increase until 30 minutes after the onset of a stressor (Kirschbaum and Hellhammer, 1989). The baseline stress level can therefore be measured in saliva if that is



taken less than 30 minutes after capture. In addition, unlike in faeces that contains metabolites, saliva contains the native steroid and reflects plasma concentrations of free glucocorticoids (Sheriff et al., 2011), which represents the biologically active fraction of glucocorticoids (Rosner, 1990). To my knowledge, only one study managed to measure salivary glucocorticoids on free-ranging animals (Higham et al., 2010 on monkeys). Being able to collect salivary samples on fawns appeared as the best option, yet challenging. Nonetheless, thanks to the effort from the field team, we managed to collect salivary samples with enough amount of saliva, and then we were able to measure native cortisol. The use of such a technique in free-ranging animals may not be easy on adults without captures (although it worked with restrained adult roe deer), but it may open new avenue for future researches, particularly on newborns.

## **2-2 Early-life glucocorticoid variations**

I found that the environment exerted a strong influence on the baseline cortisol concentrations of very young fawns (less than 16 days old, MS3). Precisely, my results highlighted that baseline salivary cortisol concentrations increased as fawns were located closer to anthropogenic structures. This effect of human activities on early-life cortisol concentrations may have long-term consequences on adult life and ultimately on population dynamics. Indeed, as it has been documented in the literature, the inhibition of growth and immune function may occur in response to elevated cortisol concentrations during development (Wada and Breuner, 2007; Stier et al., 2009), which can influence survival, but also reproductive success. However, it is important to keep in mind that my results are still preliminary, and that they need to be confirmed with few more years of data. In particular, this could allow one to evaluate, not only the effect of human activities, but also the quality of the year in terms of resources, which could indirectly influence baseline stress levels of the

fawns. Indeed, our index of the quality of the year (the juveniles body mass) is highly correlated to the availability of resources during spring (Pettorelli et al., 2003), thus reflecting the state of the mothers during the period of birth, but also during rearing. Consequently, low values of our index indicate low resources availability, especially during spring. As shown in the MS1 and MS2, low values of this index were associated with high FCMs and NL ratio in adults. Therefore, an association between our index of the quality of the year and cortisol in fawns could possibly reflect an association between cortisol concentration of the fawn and the one of its mother before and after birth of the fawns. These phases are particularly important to consider regarding the potential effects of maternal glucocorticoids on the fawn's phenotype, including development of its HPA axis (Seckl and Meaney, 2004; Berghänel et al., 2017). Indeed, in their recent comparative analysis, Andreas Berghänel and his collaborators showed that maternal glucocorticoids affected growth rate of the offspring only when considering samples collected during late, but not early gestation. In addition, it would be interesting to use more data regarding the location of the fawns in order to have a finer evaluation of its spatial use of its habitat, especially regarding the use of refuge habitats, as I was able to do it on adults. If refuge habitats are able to provide the same mitigation of the effect of human activities on the stress level of fawns, the ability and propensity to use these refuges might heavily shape the life-history trajectory of an individual. Finally, considering a longer time-scale than just the first 15 days of life would be useful in order to increase our understanding of the early-life development and shaping of the stress axis. In the upcoming years, it should be possible to have an interesting dataset including data from juveniles (captured at approximately 8 months) that were also captured when they were few weeks fawns. As discussed in the previous section, study on the ontogeny of the stress axis in wild free-ranging mammals remains relatively scarce to date.

In addition to human activities, I also found that individual characteristics such as behavioural traits were linked to cortisol concentrations early in life. I found a positive association between calling behaviour at capture and salivary cortisol concentrations in fawns, while the effect was in the opposite direction to that found in most of the literature (Koolhaas et al., 1999) including in adult roe deer (MS1 and MS6). This result needs further investigation, confirmation, and the relevance of the behavioural trait considered could be discussed. If I considered the calling behaviour as an active (called) or passive (did not call) behavioural response to a stressful event (the capture), the use of another behavioural trait (struggling behaviour) have been tested, but was not related to cortisol. Here, it is possible also possible that calling behaviour may reflect more emotionality than proactivity (sensu Koolhaas et al., 2010). In this case, calling behaviour would be expected to be positively correlated to glucocorticoid levels, but not struggling (that would reflect proactivity). This highlight an aspect mentioned earlier, the fact that the relationship between glucocorticoids and behaviour may be different depending on the behavioural trait considered (Clary et al, 2014). Once again, further investigation is required with more behavioural traits. However, if an association between cortisol and behavioural traits exist early in the life of an individual, this could suggest that a part of the future pace-of-life of an individual could already be shaped during early-life. As exposed by Denis Réale and his collaborators (2010), the pace of life of an individual results in the co-evolution of a series of connected traits (both physiological and behavioural) with a particular life-history strategy (slow versus fast). The existence of an opposite relationship between fawns and adults regarding glucocorticoids and behaviour may suggest a certain degree of plasticity in these traits, and may depend of the early ecological conditions (Dammhahn et al., 2018) and perception of these conditions. But this may also suggest that the pace-of-life of an individual may not be fully determined at birth (i.e. not fully

genetic), but may instead be shaped during the early-life of an individual. If such an early-programming of the future pace-of-life of an individual would occur, a first step would be to add other measures of other traits to evaluate more co-variations. For instance, it would be possible to measure immunity in fawns at the two capture events (spaced by approximately 7 days), and study to which extent the early-life immune parameters co-vary with glucocorticoids and behavioural traits. And then, evaluate how the co-variation between these traits evolves between the early and juvenile stages. In addition, data from the Aurignac population may allow one to evaluate to which extent these early-life co-variations may shape other important life-history trait in terms of population dynamics, such as dispersal, which occurs (or not) during the juvenile stage in roe deer (Debeffe et al., 2012). Indeed, studies in other taxa, such as reptiles, demonstrated that glucocorticoid levels may influence the propensity to disperse at a postnatal (increasing dispersal) or at a prenatal (decreasing dispersal) stage (de Fraipont et al., 2000; Meylan et al., 2004; Silverin, 1997). In addition, in roe deer, it has been shown that dispersal may also be related to other behavioural traits such as activity (Debeffe et al., 2014), and a recent study highlighted that dispersal was not necessarily dichotomous, but that discrete alternative behavioural tactics may occur during this period (Ducros et al., 2019). If glucocorticoid hormones have the potential to influence behavioural traits, including dispersal decision, it may be possible that the different ontogenetic patterns of the stress axis-behaviour relationship may underlie the observed discrete pattern of dispersal.

### **2-3 Effects of maternal glucocorticoids**

Regarding the possible consequences of elevated maternal glucocorticoids on the fawns' phenotype, I found no conclusive results (MS4). As it is always the case with non-significant results, it is hard to tease apart if there is really no effect of maternal stress in this species, or

if the study lacked statistical power. The latter is an evident option to consider regarding this part of my work, as sample sizes were relatively small ( $n = 30$  on average). Another possible limitation of my work to investigate the link between maternal glucocorticoids and phenotype of the offspring was the timing of sampling. As explained in the corresponding manuscript (MS4), mother's samples were collected during winter (January-February), while fawns come to life between late April and until June. While mother were captured at the beginning of gestation, a recent study showed that glucocorticoid concentrations of mothers were more likely to influence the offspring's phenotype at the end of gestations in mammals (Berghänel et al., 2017). In order to improve the design of this study and obtain complementary results, it would be useful to collect mother's samples closer to the birth of fawns. Ideally, regular sampling of faeces from mothers in the month prior to fawns' birth should be conducted, as well as regular sampling until fawns are weaned. In addition, it would be useful to capture the fawns at weaning in order to complement the two other captures made during the first 15 days of life and that made at the juvenile stage, and therefore have a broader view of their early-life trajectories. Such a design would allow investigating the pre- (through placenta) and post-natal (through milk or modified maternal care) effects of maternal glucocorticoids on the offspring, and this, not only during early-life, but also post-weaning, consequently taking into accounting potential compensatory mechanisms (Strange et al., 2016). As presented in the introduction of this thesis, a growing body of literature supports the maternal programming of the offspring in terms of glucocorticoids, and its adaptive value (Sheriff and Love, 2013; Sheriff et al. 2017b; Sheriff et al. 2018). While some empirical studies showed positive consequences of maternal stress on the phenotype of the offspring, especially on growth rate (Dantzer et al., 2013), others pointed out that the ecological context (Sheriff et al., 2017a) and timing of measurement (Strange et al., 2016) may play an important role in the appreciation

of the adaptive value of the maternal programming (Sheriff et al. 2018). Consequently, the possibility to observe an adaptive or mal-adaptive effect of maternal glucocorticoids might depend on the study design, and particularly on the timing of sampling of both the mother and the offspring.

That being said, not only the early environment or the maternal effect can influence early-life glucocorticoid levels. While challenging to study in wild populations, the genetic basis of glucocorticoids might also play a non-negligible role. This is one of the next important steps of my work, especially since heritable variations in traits are a prerequisite for evolution. Within this thesis I showed that baseline glucocorticoid levels were moderately repeatable ( $R \sim 0.30$  in MS1 and MS2) in roe deer. Repeatability is often used as an approximation of the upper limit of heritability (Lessells and Boag, 1987; but see Dohm, 2002), and my results could therefore suggest that glucocorticoid secretion patterns have a potential for evolutionary change in response to selection. This result would be in line with recent studies on birds showing an heritability comprised between 0.13 and 0.25 (Jenkins et al, 2014; Stedman et al., 2017; Bezier et al, 2019). In a context of a changing world (IPPC, 2014), while phenotypic plasticity in glucocorticoid levels may generate short-term responses to environmental change, the genetic basis of these hormone levels are the ones that will generate rapid evolutionary adaptations. Considering that glucocorticoids influence the expression of around 10% of the genome (Le et al., 2005), evaluation of their evolutionary potential appears as a key component in order to understand if and how wild populations may adapt to changes in their environment. Further work is therefore necessary to evaluate the heritability of glucocorticoids in the roe deer populations and more largely in wild animals. This could be achieved thanks to new statistical and methodological approaches. Indeed, variance partitioning may allow to disentangle the genetic (additive genetic variance) and non-genetic

(residual variance) components of the total phenotypic variance, but also other components such as the variance due the environment or maternal effects. In addition, pedigree-free approach does not require large numbers of genotyped individuals across several generations, but instead relies on genotyping thousands of molecular markers to generate estimates of among individuals relatedness. Overall, mixed-effect models and variance partitioning can help to understand the levels at which a trait (e.g. glucocorticoid levels) varies (Dingemanse and Dochtermann, 2013), while pedigree-free approaches are now available in wild animals, and in particular in the Aurignac population of roe deer (Gervais et al., 2019), allowing one to estimate more easily the heritability of a trait such as glucocorticoid level.

### **3 - Consequences of glucocorticoids on immunity**

In the final part of this work, I investigated the potential consequences of glucocorticoid levels on immunity, a vital function for vertebrates (Sorci et al., 2008; Demas et al., 2011). First, I investigated the relationship between baseline glucocorticoid levels and the expression of eleven immune parameters reflective of both the innate and adaptive arms of immunity in three populations of wild roe deer, and how environmental and individual characteristics could modulate this relationship. Second, I aimed to understand the effect of glucocorticoid hormones changes on immune functions, but also, if behavioural traits could underlie the co-variation of immune parameters and glucocorticoid levels.

#### **3-1 Glucocorticoids and immunity in contrasted environments**

First, I found that the relationship between glucocorticoid levels and immunity was highly dependent on the ecological conditions: a clear relationship was evidenced only in one of the three populations (MS5). Specifically, I found that in Chizé, the population exhibiting the poorest resources (Gaillard et al., 1993), levels of immune parameters were negatively

correlated with baseline glucocorticoid levels. In a situation where low resources are available, trade-offs between two traits sharing a common and limiting resource can be more easily revealed than in richer environments (Sheldon and Verhulst, 1996). This could explain why such trade-offs did not appear in the two other populations which present high levels of resources for roe deer (Gaillard et al, 1993; Abbas et al., 2011). Again, this result highlights the need to consider the ecological context when assessing the potential effects of stress hormones on other functions. For instance, if this study had only been conducted in Aurignac, we would have concluded that glucocorticoids were not related to immune levels, whereas we would have concluded that glucocorticoids have a negative effect on several immune parameters if the study was conducted only in Chizé. A recent publication highlighted the role of context-dependency on the intensity and direction of the relationship between glucocorticoids and fitness (Schoenele et al. 2018). It seems likely that the same scenario could happen for other traits such as immunity. Indeed, depending on the intensity of pathogens pressure, the different amount of energy is mobilised for the immune system. Consequently, depending on the amount of food resources available in the environment and the pathogen pressure, negative, positive, or no relationship between glucocorticoids and immunity can be expected. In addition, this work revealed that the relationship between glucocorticoids and immunity not only depends on ecological conditions, but also on individual characteristics, such as age and body condition. Specifically, the slope of the relationship was much more negative as individual were older or lighter. All individuals must face costs associated with cell maintenance (disposable soma theory; Kirkwood, 1977). However, contrary to younger individuals, older ones have a lower metabolism and their capacity to acquire nutrients is less efficient, leading to a diminution in available energy. Consequently, when those individuals are facing stressors, a negative relationship between glucocorticoid levels and immunity might



be more easily revealed as energy must be allocated to support processes crucial to immediate survival, at the cost of immunity. Nevertheless, it is obvious that the environmental conditions encountered by roe deer in the three populations do not encompass all the possible environmental conditions, and that they are therefore not sufficient in order to depict a general pattern (Pigeon et al., 2013). In addition, the environmental and individual effects we have detected on physiology may vary depending on species, highlighting the need of further investigations. More studies conducted on diverse taxa and in diverse ecological contexts may help increasing our understanding of the effects of elevated glucocorticoid hormones on immunity. A better understanding of such a relationship appears important in understanding the extent to which individuals and populations with elevated glucocorticoid levels may be more susceptible to pathogens because of weaker immune functions (Noble 1962; Eberhardt et al., 2013), which could subsequently affect population dynamics.

### **3-2 Glucocorticoids, immunity and behaviour**

Finally, my work has shown, in a large mammal, that innate and adaptive immunity may be affected by glucocorticoid levels, but also, to some extent, by behavioural types (MS6). However, it should be noted that not all of the immune parameters I tested were influenced by glucocorticoids. This first observation highlights the need to consider various immune parameters to understand the complexity of the stress-immunity relationship. Second, I found that temporal variations in cortisol levels might be associated with a switch in investment between innate and adaptive immunity. Indeed, I observed that individuals for whom FCMs increased over time also showed an increase in adaptive immune parameters (lymphocytes, anti-rabies antibody). Conversely, individuals that exhibited a decrease in FCMs showed an increase in innate immune parameters (neutrophils and total white blood cells). Altogether, these results suggest that individuals exhibiting increased cortisol concentration might switch

from innate to adaptive immunity, while individual experiencing decreased cortisol concentrations might switch from adaptive to innate immunity. The switch of immune arm observed here may be due to energetic trade-offs between both arms of immunity. Such switch is congruent with the pace-of-life hypothesis (Réale et al., 2010). However, while the pace-of-life hypothesis states that some individuals invest in immunity whereas others do not (Réale et al., 2010), a more recent framework have expended this view. Kelly Lee (2006) proposed that individuals invest in both arms of immunity, but that the investment should be partitioned according to the pace-of-life of the individual, with slow ones investing more in adaptive immunity while fast individuals should invest more in innate immunity. Considering that slow individuals usually exhibit high reactivity of the HPA axis (Koolhaas et al., 1999; Réale et al., 2010), while fast individuals exhibit low reactivity, my results appear to partially support this view proposed by Kelly Lee, with slow individuals exhibiting high secretion of cortisol and a more pronounced investment in adaptive immunity, while fast individuals with a low secretion of cortisol exhibit a more pronounced investment in innate immunity.

While the experimental approach allowed me to go beyond time-point estimations of stress and immune levels to understand the relationship between these two, the small sample size did not really allowed to test for an interaction between stress, immunity and behavioural types. Indeed, most work in wild animals usually looks at time-point data and/or comparison of groups (stressed versus non-stressed) to investigate whether or not glucocorticoids are negatively related to immunity (Matson et al., 2006; Delehanty and Boonstra, 2009; Merrill et al., 2012; Brooks and Mateo, 2013). However, the fact of not taking into account the behavioural type of an individual might lead to erroneous results depending on the immune parameter studied. Why should we expect a trade-off between antibodies and other functions that promote survival (leading to a negative relationship between glucocorticoids and

antibody) in fast individuals that do not heavily invest in this arm of immunity? The same question applies to slow individuals with an innate immune parameter.

Going further, these results might also suggest that the development of the HPA axis and shaping of early-life glucocorticoid levels may influence the development of early-life immunity, especially in terms of higher investment in one arm or the other. Indeed, I showed previously that among-individual differences in glucocorticoid levels might exist in early-life in roe deer. These early differences could very likely result from among-individual differences with respect to the ability to secrete glucocorticoids, which could influence the development of the immune system. This influence could also be indirect considering that glucocorticoids can also influence behavioural traits. Indeed, the development of the immune system also depends on the antigenic stimulations that individuals face early in life, which can be due to its spatial behaviour. This path remains to be investigated, and could increase our understanding of the effects of glucocorticoids, but more generally, on the ontogeny of the pace-of-life of an individual.

This part of my thesis on the relationship between glucocorticoids and immunity allowed me to conduct both observational and experimental approaches. While the former allowed me to benefit from three long-term monitoring of wild populations with a large amount of data, investigating specific questions that required several captures of roe deer was not possible. In this sense, I would say that it was a real opportunity to be able to use an experimental facility. This approach allowed me to develop and set a protocol to answer a particular question that required several clustered captures and sampling, but also an intensive monitoring of individuals with collection of faeces every four days. This would have never been possible in any of three natural populations. However, experimental approach also have limitations, and I could not use a large number of individuals ( $n = 13$ ). In addition, experimental studies on roe

deer are nothing comparable to studies on laboratory rodents, and one cannot manipulate them or the environment the same manner. This obviously implies some limitations on the possibilities for experimental settings. However, I would consider that in my case, both approaches have been complementary and allowed me to investigate different questions.

### **3-3 Other possible health consequences of glucocorticoid levels**

Within my thesis, I focused on immune functions in order to evaluate the consequences of glucocorticoid levels. However, stress may have consequences on other traits such as reproduction and survival. Previous studies on the relationship between glucocorticoid hormones and fitness led to mixed results, and studies on mammals are still under-represented in the wild (Bonier et al., 2009). Evaluating such consequences may require long-term monitoring of populations, and our study system may therefore be suitable to investigate such questions in a close future. In addition, a comprehensive approach taking into account environmental and individual characteristics when investigating such a relationship may be an important, and could help to understand discrepancies between positive, negative, or no relationship, that can be found in the literature (Schoenle et al., 2018).

Focusing on other proximate consequences of glucocorticoid hormones on health-related traits, investigating the relationship between glucocorticoids and the microbiome appears as a promising path. The microbiome refers to the resident microbial communities of an individual. Recent evidence suggests that bacterial diversity may be positively related to the health of the host (Lozupone et al., 2012; Cénit et al., 2014), while a low diversity seems to be linked to poor health (Avila et al., 2009). Recently, the HPA axis has been hypothesised to be one of the mediators of the link between the host and its bacterial microbiome (Cryan and O'Mahony, 2011). Few studies have investigated the link or the effect of glucocorticoid hormones on the bacterial microbiome in the wild, but results seem to indicate that higher

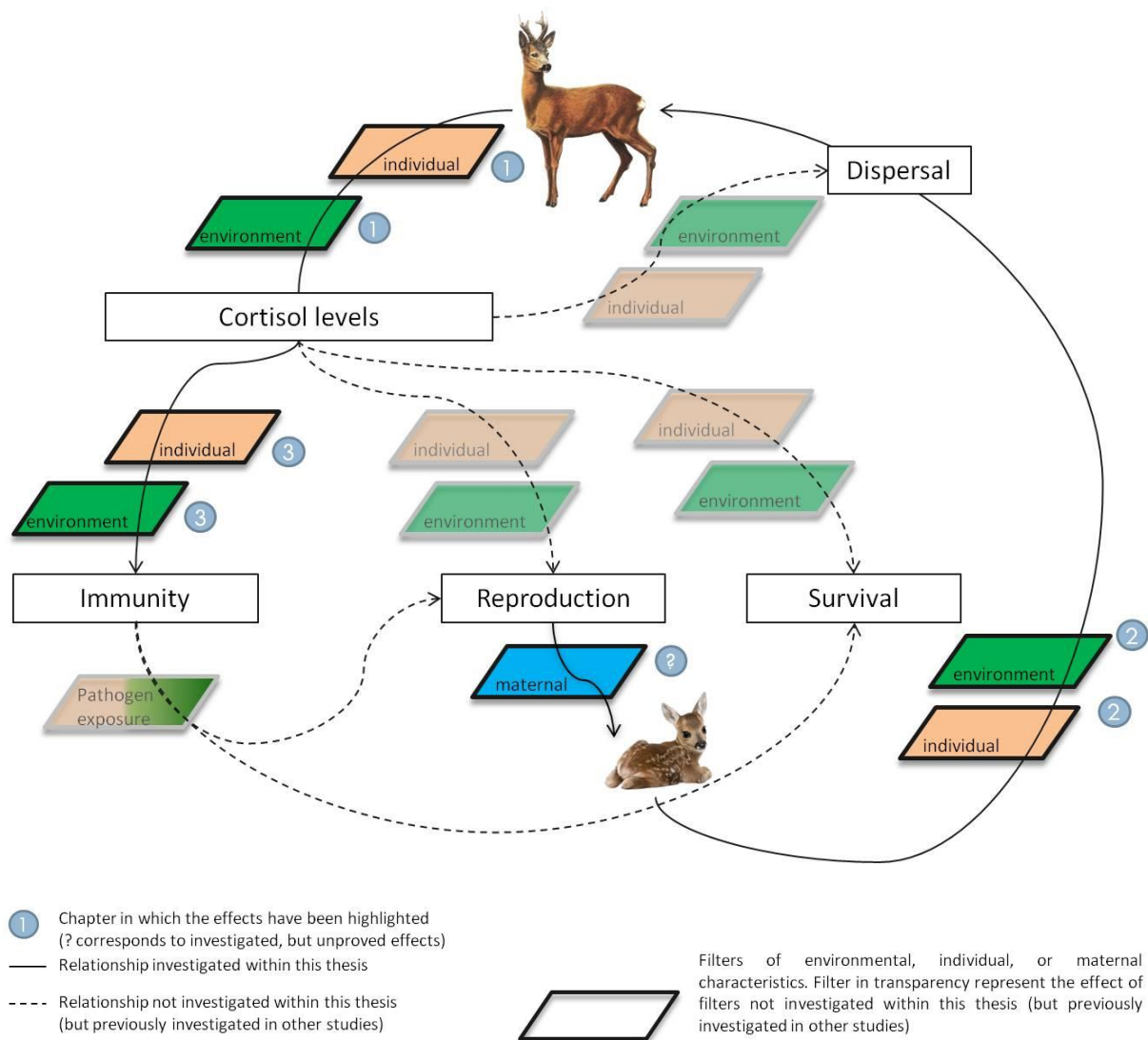
glucocorticoid levels are correlated with lower bacterial diversity (Stothart et al., 2016; Noguera et al., 2018). Glucocorticoid hormones can therefore potentially affect an individual's health by other means than the immune system. Further investigations are needed to explore this path.

#### **4 – Conclusion**

The general aim of this thesis was “to use a comprehensive approach to identify the main determinants of glucocorticoid levels and to investigate their consequences in a long-lived mammal, the roe deer”. Indeed, in a context of increasing unpredictable changes in the environment (human activities, extreme climatic events), it appears important to evaluate the degree of phenotypic plasticity and evolutionary potential of wild animals. My results show that the stress level, and in particular glucocorticoid hormones, exhibited by wild animals is a trait with a certain degree of plasticity. A synthetic representation of my results is presented in the Figure 13. Levels of glucocorticoid hormones appeared to be shaped by the environment, particularly human activities and climatic yearly fluctuations, but were also related to individual characteristics such as behavioural traits. Interestingly, these results were observed both in adults, and in the first weeks of life. In addition, I showed that immunity may be shaped by glucocorticoid concentrations, and that glucocorticoids could influence the arm of immunity in which individuals may invest to. With the expected increase in environmental perturbations in the upcoming years, it is likely that the fluctuation in baseline glucocorticoid levels increases as well. If baseline levels of glucocorticoids adaptively shape the phenotype of the individual, these hormones may therefore appear as an important mechanism allowing individuals to face a challenging environment. Subsequent works are needed to support these findings, and the investigation of the heritability of glucocorticoids appears as an important

step in understanding the potential for wild animals to evolve and adapt to changes in their environment, particularly those imposed by humans. In addition, while the work presented here focused on baseline glucocorticoid levels, a similar approach may be needed on response levels. As presented in the introduction, this measure is complementary, has other functions than baseline levels, and may have different consequences.

As my work on this thesis is coming to an end, a particular question seems to me very exciting and promising: Do glucocorticoid hormones have the potential to shape the pace-of-life of individuals? As mentioned all along this discussion, several trails remain to be explored regarding the effects of maternal glucocorticoids, the early-life effects of glucocorticoids, and their co-variation with other traits. However, my work suggested evidence for a relationship between glucocorticoids and several traits that are included within the pace-of-life hypothesis (Réale et al., 2010). Traits that may themselves influence other phenotypic traits. For instance, I showed that glucocorticoids were related to some behavioural traits and may modulate the investment between both arms of immunity, and it is likely that it influences life-history traits such as dispersal as well. The relationship between glucocorticoids on the age at first reproduction or longevity should also be investigated for a more comprehensive understanding, but glucocorticoids clearly appear as a potential mechanism of phenotypic integration and prime candidate to be a driver of the pace-of-life of individuals.



**Figure 13** – Summary of the results brought by this thesis. Arrows correspond to the effect of a trait on the others (except for the transition between fawn and juvenile/adult stage). Numbers inside circles corresponds to the chapter that investigated and highlighted the depicted results and effects; the “?” corresponds to a question that I investigated, but that remains to be confirmed and deeply explored. Parallelograms correspond to filters that may influence the outcome of a trait on the others. “Environment” corresponds to different variables, such that in chapter 1 it refers to the proximity with human activities, and to the temporal variations of resources from one year to the one; in chapter 2 it refers to the proximity with human activities; and in chapter 3 it refers to the temporal variations of

resources from one year to the one. “Individual” correspond to different variables, such that in chapter 1 it refers to age and behavioural response to the capture event; in chapter 2 it refers to the behavioural response to the capture event; and in chapter 3 it refers to age and body condition. “Maternal” correspond to maternal variables such that body mass, glucocorticoid level and behavioural response to the capture event. Parallelograms without numbers and in transparency correspond to effects that have not been investigated within this thesis and that still remain to be fully explored in wild animals.

To be continued...







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Ma deuxième belle rencontre, Delphine. Enfin retrouvaille pour être exact, puisque aussi incroyable que ça puisse paraître, nous avons déjà partagé des travaux en master. J'avoue que le jour où j'ai appris qu'on allait être dans le même labo j'ai bien rigolé tellement cela était improbable. Deux étudiants de Paris qui attendent un an avant de démarrer une thèse et qui atterrissent dans le même labo à Toulouse, et même dans le même bureau, ce n'est pas courant je pense :) En tout cas je ne regrette pas du tout que les choses se soient passées comme ça car j'ai pu apprendre à te connaître et découvrir une belle personne pleine de qualités. Comme Laura tu es attentionnée et à l'écoute, et je te remercie aussi pour ton soutien durant l'épreuve difficile que j'ai dû traverser et durant l'ensemble de ma thèse. C'est assez évident que l'on a plein de points communs et ça nous auras sans doute bien aidé à tenir sans trop de vagues malgré la proximité dans notre petit bureau durant ces trois ans. Entre moments difficiles et moments plus joyeux, on aura partagé beaucoup de choses qui auront forgées une belle amitié qui là aussi durera longtemps je l'espère. De toute façon, tant que l'on n'a pas vu tous les Harry Potter et les Miyazaki ensemble on est obligé de rester en

contact ;) Quel dommage ! ;) Là aussi les soirées, les randos, les journées au Muséum ou à la cité des sciences resteront de très bons souvenirs ! Tout pareil pour nos nombreuses discussions sur des sujets très divers et variés :) Bon là aussi il va falloir que je fasse attention à ne pas trop m'étaler. Comme pour Laura, accroche-toi pour les semaines à venir ! Ca peut paraître compliqué, mais tu as toi aussi tout ce qu'il faut pour réussir, donc ça va bien se passer. Conseil d'expert, ne stresse pas plus que nécessaire (ça reste adaptatif ;p).

Si je parle de Delphine je parle forcément de Bastien aussi :) Merci à toi pour tous les bons moments que l'on a pu partager, particulièrement durant la dernière année de thèse. Notre petit weekend rando où nous aurons eu la visite d'un petit chevreuil durant le dîner et la rencontre furtive d'un cerf juste avant restera un super moment ! Et maintenant que je vais avoir du temps on va pouvoir les multiplier !

On se rapproche de la fin, et là je voudrais dire un immense merci à ma boule de poils !! Atlas, tu ne liras jamais ces lignes (quoi que), mais sache que si je n'aurais jamais pensé avoir un petit chat durant ma thèse, je ne regrette absolument pas d'avoir craqué (merci Laura ! Et merci Thomas pour cette journée où j'ai fait la connaissance du petit tigre). Ton amour débordant, ton énergie assez exceptionnelle, ta curiosité et tout simplement ta présence auront changé ma dernière année de thèse pour le meilleur.

Papy, mamie, vous m'avez tellement transmis durant toutes ces années. Vous avez toujours été là pour moi, et vous avez été une véritable force. Papy, je sais que tu ne seras probablement pas là le jour de ma soutenance, mais merci pour ton soutien durant cette dernière année en particulier. La communication n'est pas toujours évidente entre nous, mais ta présence (même depuis Paris) m'auras été d'une grande aide morale. J'espère que ce travail t'aura rendu fier. Mamie, je te dédie forcément ce travail. Tu étais tellement heureuse que je puisse décrocher cette thèse. J'espère que de là où tu es tu seras fière de moi et du travail

que j'ai pu accomplir. J'aurais tellement aimé que tu puisses assister à cela. Sache que pas un jour ne s'est écoulé sans que je pense à toi depuis que tu nous as quitté. Malgré la douleur permanente depuis ce jour, tu seras resté une vraie force pour moi. Merci beaucoup pour tout !!

Enfin, merci aux chevreuils pour leur participation (contrainte j'en suis bien conscient) à ces travaux et à l'avancé de la science. Merci également à tous ceux que j'ai oublié (car il y en a toujours) mais qui auront aussi contribué à faire de cette thèse et de ces trois années ce qu'elles ont été. Parmi les oubliés je pense entre autres à Thierry Boulinier avec qui j'ai eu la chance d'échanger lors de deux réunions du REID, et surtout qui m'aura hébergé chez lui pendant cet évènement.

Encore une fois, un très grand merci à tous pour l'ensemble des rencontres que j'ai pu faire et des moments partagés durant ces trois années !!



# Appendix 1

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**How and where to measure glucocorticoids?**





Measurement of glucocorticoid levels can be achieved by using several biological samples. Often the use of one matrix over an other one will depend on the species and what we want to measure, response level, baseline level, cumulative level. Here I only give a very brief overview of the different methods with their advantages and disadvantages, but a comprehensive review has been published on this topic by Michael Sheriff and his colleagues (2011).

|  | <b>Blood</b>  | <b>Saliva</b>   | <b>Faeces/Urine</b>  | <b>Hair/Feather</b>  |
|--|---|---|--|--|
| <b>Time frame</b><br><br>(is the measure reflecting stress-induced or baseline levels) | Immediate snapshot<br><br>Baseline level and possibly response level  | Immediate snapshot<br><br>Baseline level and possibly response level  | Integrative measure over a species-specific amount of time<br><br>Baseline level and possibly response level | Integrative measure over a long period of time (thought to be around weeks)<br><br>Baseline level  |
| <b>Conservation</b><br><br>(delay and conservation methods)                            | Up to 72h at ambient T°<br><br>Plasma and serum for years at minus 20°C   | Up to 15 days at ambient T°<br><br>Up to several month at 5°C<br><br>For year at minus 20°C                                   | Dried or lyophilised<br><br>Minus 20°C as fast as possible   | For years at ambient T°  |
| <b>Pros</b>  | Direct measure of glucocorticoids<br><br>Allow to measure several other physiological components such as immune parameters                        | Levels increase 20 to 30 minutes following onset of the stressor, making baseline levels measurement easier than in the blood | Non invasive method<br><br>Integrative measure<br><br>Also allow detection of parasites                      | Non invasive method<br><br>Integrative measure that reflect the cumulative exposure of an individual to glucocorticoids                          |
| <b>Cons</b>  | To assess baseline levels, measures need to be realised within three minutes following onset of the stressor<br><br>Sampling is a stressor itself | Concentrations may be low<br><br>Difficult to use with wild animals because it requires them to chew a cotton swab            | Does not allow to access many other physiological parameters such as with blood<br><br>Fresh faeces needed   | New method that still need validation in several species<br><br>Possible absorption of glucocorticoids not coming from the blood into hair shaft |



## **Appendix 2**

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**Other manuscripts I was involved in**





# Does body growth impair immune function in a large herbivore?

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**Author Contributions:** JFL, JMG, EGF conceived the study and designed methodology; all authors collected the data; LC, EGF, BR, HV performed the immunological measures; LC and FD analysed the data; LC wrote the first draft of the paper and all authors contributed critically to the drafts and gave approval for the final version.

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## Abstract

According to the principle of allocation, trade-offs are inevitable when resources allocated to one biological function are no longer available for other functions. Growth, and to a lesser extent, immunity are energetically costly functions that may compete with allocation to reproductive success and survival. However, whether high allocation to growth impairs immune system development during the growing period or immune system performance during adulthood is currently unknown in wild mammals. Using three roe deer (*Capreolus*

*capreolus*) populations experiencing contrasting environmental conditions, we tested for potential costs of growth on immune phenotype over both the short-term (during growth), and the long-term (during adulthood) over the course of individuals' life. We investigated potential costs on a set of twelve immune traits that reflect both innate and adaptive responses, and compared them between sexes and populations. Although fast growth tended to be associated with low levels of some humoral traits (globulins) during growth and some cellular immune traits (i.e. eosinophil and neutrophil counts) during adulthood, evidence for a trade-off between growth and other immune components was limited. Unexpectedly, no detectable growth costs on immunity were found in females from the population experiencing the least favorable environment. We discuss our findings in the light of the complex interplay between resource allocation strategies among reproduction, maintenance and immunity, in relation to local environmental conditions experienced by roe deer.

**Keywords:** Body mass, Eco-immunology, Life history, Roe deer, Trade-off

## Introduction

Growth is a key life history trait that shapes reproductive success and both short-term and long-term survival in most organisms. Developing a large body rapidly can buffer the effects of fluctuating environmental conditions (Dmitriew 2011) and allows juveniles to spend less time at the critical early-life stage, when they are particularly vulnerable to predators (Metcalf and Monaghan 2003, Ronget et al. 2018). Large mature males are generally more successful in intra- and inter-sexual competition, and have thereby higher reproductive success than small males (Andersson 1994), while large females often exhibit higher fecundity than small ones (Roff 1992). A large body size can be attained either by an extended developmental period, an accelerated growth rate, or both (Metcalf and Monaghan 2003). However, growth entails substantial energy and nutrient requirements (Wieser 1994), which are expected to have deleterious consequences on individual physiological performance and survival during prime-age adulthood and old age (Arendt and Wilson 1997, Blanckenhorn 2000, Dmitriew 2011).

Physiological and survival costs associated with rapid growth have been documented for several decades, notably in some of the first contributions to the evolutionary biology of aging (Williams 1957). Such relationships are embedded in the principle of allocation (Cody 1966), which states that individuals must allocate the limited amount of energy they acquire from their environment among the competing functions of growth, reproduction and survival. Most studies on vertebrate populations performed in the wild to date have focused on the energy allocation trade-off between current reproduction and subsequent survival or reproduction (Stearns 1992, Lemaître et al. 2015, Gélín et al. 2015). So far, the long-term effect of fast growth has been investigated much less. Yet, high energy allocation to body growth could be

at the expense of energy allocated to the maintenance of cellular and physiological functions (McDade 2005). In the wild, restriction of food resources likely exacerbates energy allocation trade-offs. Harsh environmental conditions and low levels of food resources slow down growth (Arendt and Wilson 1997) and lead to delayed sexual maturity or reduced adult size, or both (Bonenfant et al. 2009). However, compensatory growth (*sensu* Hector and Nakagawa 2012) can occur, enabling juveniles to recover partially or fully from food limitation during early life. This compensatory growth is also associated with various and pronounced physiological costs, both over the short- and long-term throughout the course of an individual's life (Metcalf and Monaghan 2001). Thus, although it is possible to recover a normal growth trajectory at some life stage, slow growth during early life can have profound, pervasive and long-standing effects on individual adult performance (Metcalf and Monaghan 2001). The costs of compensating for a bad start may include a faster rate of telomere attrition (Geiger et al. 2012), decreased physiological condition during adulthood (e.g. low tolerance to food restriction, Dupont-Prinet et al. 2010), an increased rate of senescence (e.g. more rapid loss of body mass in late adulthood, Douhard et al. 2017) and even a shortening of lifespan (Lee et al. 2013).

Immunity is a key physiological trait that potentially mediates life history trade-offs between growth and reproduction or between growth and survival (Sheldon and Verhulst 1996, Lochmiller and Deerenberg 2000, Zuk and Stoer 2002, Lee 2006). Immune function determines an individual's sensitivity to infections and diseases, and thus affects both reproductive success and survival in the wild (Sheldon and Verhulst 1996, Schmid-Hempel 2003). The immune system is also a major physiological mechanism involved in cellular renewal and repair, and as such, is an essential component of somatic maintenance (McDade 2005). So far, the majority of studies that have focused on the trade-off between growth and

immunity have been performed in birds (e.g. Soler et al. 2003, Brommer 2004, Mauck et al. 2005). Of these, experimental work based on food supplementation has shown that the immune response is resource dependent (Soler et al. 2003, Brommer 2004), a prerequisite for an energy allocation trade-off to occur. However, experiments based on parasite challenge or immune stimulation in young birds revealed that immune challenge affects the immune profile and body condition, but does not limit growth (Hörak et al. 2000), and may even result in accelerated wing development (Saino et al. 1998, Szàp and Møller 1999). These previous studies generally based on the manipulation of immunity in growing individuals found limited impact on their growth, possibly because the amount of energy required for the immune response is much lower than that required for tissue growth (Klasing 1998). On the contrary, it is not known whether high allocation to growth could be costly over the short-term (i.e. for immune development) or over the long-term (i.e. for immune performance during adulthood). Specifically, rapid growth is more likely to compete with the most energy demanding traits such as cellular immunity than with less costly ones such as humoral functions (Klasing 2004). In addition, rapid growth may also carry a higher cost in terms of timing of immune development because individuals with low immune function in early life may accelerate their immune development later in life (Mauck et al. 2005, Rossi et al. 2013).

Here we tested for potential costs of *early* growth on immunity in young and adult roe deer, and of *late* growth on adult immunity. We collected data in both sexes on 12 immune traits encompassing both the innate and adaptive components of the immune response for 300 roe deer sampled during their growing period, and for 196 roe deer sampled during adulthood. These animals originated from three populations which have been intensively monitored using Capture-Mark-Recapture for over 20-30 years (Gaillard et al. 2013, Hewison et al. 2009). The populations are subjected to very different environmental conditions, which

leads to marked differences in life history (notably growth rate, see Gaillard et al. 1997) and immunological (Cheynel et al. 2017) traits. Based on the principle of allocation and knowledge gained from empirical studies, we predicted that (i) a negative association should occur between *early* growth and immune components over the short-term (i.e. during the growing period) in young roe deer, in particular for adaptive immunity that develops more progressively compared to innate immunity; (ii) a negative association should occur between *early* and/or *late* growth and adult immune traits over the long-term (i.e. during adulthood); (iii) immune costs of *late* growth should be higher than those of *early* growth; (iv) the trade-off between growth and long-term immunity should be stronger for the most costly immune functions (in particular, cellular innate immunity, e.g. neutrophils, monocytes) than for less costly ones (typically, antibody response); (v) the trade-off should be strongest in the population experiencing the poorest environmental conditions. Finally, we also controlled for sex-specific effects because studies performed in laboratory and natural systems have reported that levels of immune performance generally differ between sexes, being higher in females than in males (Zuk and McKean, 1996). However, we expected the association between either *early* or *late* growth with immunity to be similar in males and females because roe deer only display a low intensity of sexual selection (Andersen et al. 1998), and because no clear sex differences were reported in physiological traits and intensity of parasitism in the studied populations (Cheynel et al. 2017).

## Material and methods

### Study populations and data collection

We used data from three populations of roe deer: two are located in enclosed forests in north-eastern France (48°43'N, 4°55'E, 1,360 ha) at Trois-Fontaines, and in western France (46°50'N,



0°25'W, 2,614 ha) at Chizé; and the third population is located at Aurignac, in a rural site in the southwest of France (43°17'N, 0°53'E, 7,500 ha).

The Trois-Fontaines population is exposed to a continental climate characterized by cold winters and warm rainy summers. This site has rich soils and provides high habitat quality for roe deer. In contrast, the Chizé population has a temperate oceanic climate with Mediterranean influences, and frequent summer droughts combined with poor soils, resulting in limited forest productivity (Pettorelli et al. 2006). Hence, fawn survival, female fertility, adult body mass (Gaillard et al. 2013), age-specific telomere length (Wilbourn et al. 2017), and many markers of immune performance (Cheynel et al. 2017) are consistently lower at Chizé than at Trois-Fontaines. Aurignac population has a oceanic climate with summer droughts and is a mixed landscape of woodland patches, meadows and crops offering high quality resources for roe deer (Abbas et al. 2011).

As part of the long-term Capture-Mark-Recapture program initiated in the late 70's for the two forest populations and in 2001 for the Aurignac population, 6-12 days of capture occur between December and March each year (see Gaillard et al. 1993 and Morellet et al. 2009 for details about the capture sessions). In May-June newborn fawns are also captured annually. Roe deer caught within their first year of birth (i.e. either as newborn or as 8 months-old individuals during winter captures) are identified using tooth eruption patterns (Hewison et al. 1999). At capture, we recorded body mass (to the nearest 50g) and performed a basic clinical examination. We collected blood samples from the jugular vein (up to 20 mL for a 20 kg roe deer). We performed cell counts within 52 hours of sampling on whole blood preserved on EDTA at 4°C. We also extracted and conserved serum at -20°C for measures of functional activities (see below).

The two forest populations of roe deer (Trois-Fontaines and Chizé) were used for the analyses of both short-term and long-term costs of rapid growth on immune performance. The population of Aurignac was included for the analysis investigating the short-term consequences of rapid growth on immune performance only. Indeed, at Aurignac, the monitoring program differs from the two others since the exact age of most adults is unknown (Hewison et al. 2009).

### **Dataset**

We used data from known-aged individuals that were blood-sampled at least once during their lifetime between 2010 and 2016. Roe deer usually gain most of their adult body mass (more than 90%) within the first two years of life but reach their full adult mass only at about 4 years of age (Hewison et al. 2011). When not faced with strong food limitation, females start to give birth at 2 years of age (Gaillard et al. 1992) while most males father their first offspring during their fourth year of life (Vanpé et al. 2009). Adult immune performance was measured as the median of each immune trait during adulthood (i.e. between 4 and 9 years of age). Based on these age-classes, data on immunity were collected for a total of 300 growing animals (i.e. sampled at 8 months of age) in the three populations (see details in Table 1). At Aurignac, we only used data for growing roe deer (i.e. at 8 months of age,  $n = 41$ ) since the sample size for known-aged adults was too small to perform a detailed analysis ( $n = 9$ ). Data on immunity were also collected for a total of 196 prime-aged adults (i.e. sampled between 4 and 9 years of age) at Chizé and Trois-Fontaines. Data were approximately equally distributed between sexes (Table 1). These immune measures on roe deer at Trois-Fontaines and Chizé have already been the subject of a previous analysis on immunosenescence patterns (see Cheynel et al. 2017).

Owing to the relatively recent start of immunity assessments in the monitoring programs (2010) and to the imperfect detection rate during the capture-recapture season (i.e. recapture rate of about 0.5; Gaillard et al. 1993), only 32 out of the 196 adults with immunity measures also had immunity measures during their growing period (Table 1). Growth measures (i.e. *early* and *late* growth, see below for a definition of these metrics) were based on records of both body mass at first capture (at about 8 months of age) and on adult body mass (between 4 and 6 years of age, before body mass senescence starts; Douhard et al. 2017).

### ***Immunity measures***

We measured a set of 12 immune traits to depict both the innate and adaptive responses, represented by both humoral and cell-mediated components (Roitt et al. 1998). We assessed the cellular part of immunity by measuring the total white blood cell (WBC) count and assessing the composition of the white blood cell population (five different cell types, measured in  $10^3$  cells/mL), based on the identification of the first hundred WBC in Wright-Giemsa-stained blood smears (Houwen 2001, Gilot-Fromont et al. 2012). Neutrophils and monocytes are phagocytic cells of the innate response. Basophils and eosinophils participate in the inflammatory response and play a role in the innate response against parasites, but eosinophils are also involved in the adaptive response. Finally, lymphocytes include T and B cells, B cells being specifically involved in the production of antibodies. Innate humoral immunity was assessed by measuring both the circulating levels of natural antibodies (NABs) and the complement-mediated cell lysis activity. We used the hemagglutination-hemolysis (HAHL) assay (Matson et al 2005, see also Gilot-Fromont et al. 2012 for a previous study on roe deer). In this assay, the HA score (measured in titre) measures the ability of NABs to agglutinate exogenous cells and provides a proxy of the NABs concentration, and HL score

measures the ability of these NABs to cause hemolysis. Innate humoral immunity also includes numerous proteins involved in acute and chronic inflammatory processes. We thus measured the levels of alpha1-globulin, alpha2-globulin and beta-globulin, which include several acute phase proteins of the inflammatory response. Total protein content (in g/L) was first assessed by refractometry, followed by automatic agarose gel electrophoresis (HYDRASYS, Sebia, Evry, France) that separates albumin and the 4 fractions of globulins (alpha1, alpha2, beta, and gamma). We also measured the specific level of haptoglobin (HAP in mg/mL), a protein that belongs to the alpha2-globulin fraction which is synthesized in cases of chronic infection or inflammation. Haptoglobin analyses were performed on a Konelab 30i automaton (Fisher Thermo Scientific, Cergy-Pontoise, France) using phase Haptoglobin assay (Tridelta Development LTD, County Kildare, Ireland) chromogenic kit. Contrary to the other immune parameters, haptoglobin was only measured at Chizé and Trois-Fontaines. The humoral component of the adaptive immunity response was assessed by measuring the concentration of gamma-globulins (see above for details about the electrophoresis protocol), i.e., immunoglobulins, which represent the majority of circulating antibodies. Detailed protocols for measuring each of the immune parameter are provided in Online Resource 1. For adult animals with repeated measures of immune traits between 4 and 9 years of age (82 individuals sampled twice or more), we estimated within-individual repeatability of our immune measures and their 95%-confidence interval (CI) using the R package rptR (Nagakawa and Schielzeth 2010). We found that the majority of adult immune traits were consistent over time (between 0.22 and 0.41 for the various forms of white blood cells, between 0.03 and 0.46 for various forms of globulins and between 0.07 and 0.42 for HAP, results provided in Online Resource 1).

### ***Growth measures***

*Early* growth (i.e. adjusted body mass at 8 months of age, which was labelled as "early mass" in Douhard et al. 2017) and *late* growth (i.e. a compound of *early* growth and growth from 8 months of age to adulthood) were defined and calculated as in Douhard et al. (2017). Most births take place around mid-May (80% within less than 25 days, Gaillard et al. 1993), while annual winter captures occur from mid-December to early March. We could not measure postnatal growth rate *per se* (i.e. growth between birth and 8 months) because the exact birth date was unknown for those fawns that were first marked during the winter captures. We indexed *early* growth as fawn mass at winter capture (i.e. about 8 months of age) adjusted to the median date of capture (i.e. January 27th). This adjustment is required because fawns in the studied populations continue to grow during their first winter (Hewison et al. 2002), although their mass at capture is mostly accumulated product of growth from birth to early October when weaning takes place (Andersen et al. 1998). From the body mass data, the average daily mass gain throughout the winter capture period was  $12 \pm 0.005$  (SE) g.day<sup>-1</sup> at Chizé,  $24 \pm 0.008$  g.day<sup>-1</sup> at Trois-Fontaines (Douhard et al. 2017), and  $30 \pm 0.005$  g.day<sup>-1</sup> at Aurignac (linear regression with date of capture as the sole covariate; no sex-differences was detected). As individual variation in *early* growth was partly due to variation in environmental conditions during the year of birth (Gaillard et al. 1996), we further adjusted *early* growth relative to the average fawn mass of each cohort in each population.

*Late* growth corresponded to the post-weaning growth from about 8 months to 4 years of age. When calculating *late* growth we accounted for the variation in mass at 8 months of age because fawns that grow slowly early in life subsequently tend to grow faster than fawns that grow fast early in life. This *late* growth was thus measured within each sex and each population as the residuals of the linear regression between early growth and subsequent mass gain, which corresponds to the difference between adult body mass (i.e. the median

body mass between 4 and 6 years of age) and early growth. As such, *early* growth and *late* growth were statistically independent.

### **Assessing the covariation among immunity traits**

We performed a principal component analysis (PCA) on the 12 immune traits to identify the main axes of variation that determined their correlation structure. We thus examined whether immune traits could be clustered into a few biologically meaningful and independent components (corresponding to PCs). We examined covariation among the 12 immune traits in both growing and adult animals to assess the consistency of the immune phenotype over the lifetime at Chizé and Trois-Fontaines, and to identify potential differences in relation to sex and population (see Fig. 1 and Table 2). We did not include Aurignac data in the PCA analysis because haptoglobin concentration was not quantified in this population. In the analyses, we removed individuals that contributed abnormally to the PCs, possibly because of a severe inflammatory state (i.e. 2 and 1 individuals that contributed to 28.7% and 17% of the first axis among growing and adult animals, respectively).

Finally, to assess whether ‘slow’ and ‘fast’ growing roe deer displayed an overall difference in immune traits, we calculated the average PC coordinates of the fastest growers (top 25%,  $n = 66$  for growing and  $n = 37$  for adult animals) and of the slowest growers (bottom 25%,  $n = 66$  for growing and  $n = 37$  for adult animals) after separating them within sex and population (see Fig. 1). To test whether slowest growers differed in overall immune function from fastest growers, we used a non-parametric PerMANOVA test (‘adonis2’ function in the R package *vegan*; Oksanen et al. 2013).

The PCA was performed using the R package ‘ade4’ (Dray and Dufour 2007). Differences in average trait measures between sexes and populations were assessed using a

two-way ANOVA followed by Tukey's post hoc test using the multcomp R-package (Hothorn et al. 2008) (Table 2).

### **Assessing and testing for short-term and long-term growth costs on immunity**

We investigated, in both growing and adult animals, the effects of growth on the two first axes of covariation in immunity traits (i.e. first and second PCs, which roughly correspond to continuums of the level of globulins (PC1) and of the cellular part of immunity (PC2); see Fig. 1). Growth effects corresponded to *early* growth in growing animals, and to both *early* growth and *late* growth in adults. Firstly, we assessed the magnitude and the precision of these effects by using 'effect size' and their CI (Nakagawa and Cuthill, 2007). Secondly, we performed a model selection procedure for retaining or not these effects using an information-theoretic approach (Burnham & Anderson, 2002).

Within each sex and population, we calculated 'effect size' as partial correlation coefficients which measure the standardized effect of one growth measure on an immune trait while controlling for the potential effects of other traits. To obtain the different effect sizes, for each immune trait we first fitted the mixed-effect model including *early* growth, *late* growth (only in adults), and average cohort fawn mass as fixed factors and cohort as a random effect on the intercept (i.e. additive model). We then used equation (24) in Nakagawa and Cuthill (2007 p. 82) for mixed-effect models to calculate effect size. We calculated CI of effect sizes following Nakagawa and Cuthill (2007)'s recommendations (i.e. using the 'es calculator', see <http://cebcp.org/practical-meta-analysis-effect-size> for further information). We also calculated the effect size of growth on each of the 12 immune traits separately (see Online Resource 2 for growing and adult roe deer). As data for growing roe deer at Aurignac could not be included in the PCA analysis (see above), we assessed effect size of *early* growth only

on individual immune traits separately in this population (results presented in Online Resource 2).

Using the information-theoretic approach, we fitted, and selected among, several candidate linear mixed-effect models of immunity traits that included or not *early* and *late* growth as covariates (supplementary methods in Online Resource 3, and best models selected in Online Resource 4 for growing animals and adults). We investigated a possible population-, or sex-specific effect of growth on the immune phenotype by fitting models including either a three-way interaction (i.e. growth (*early* or *late*)  $\times$  population  $\times$  sex) or a two-way interactions involving growth (see Online Resource 3 for the full list of models). Models were ranked based on the Akaike Information Criterion corrected for small sample size (AICc, Burnham & Anderson, 2002) in the R package MuMIn (Bartoń, 2016). We selected the model with the lowest AICc. When the difference in AIC (denoted  $\Delta\text{AICc}$ ) of two competing models was less than 2, we retained the model with the lowest number of parameters by following accordance with parsimony rules. In addition, we calculated AIC weights ( $w_i$ ) to measure the relative likelihood that a given model was the best among the set of fitted models. All linear mixed-effect models were fitted using restricted maximum likelihood ('lmer' function in the R package lme4; Bates et al., 2015) to estimate model parameters. As data for growing roe deer at Aurignac could not be included in the PCA analysis (see above), we selected separately models explaining each immune trait in this population (Online Resource 4). All analyses were carried out in R version 3.4.1 (R Core Team, 2017).

## Results

### Covariation among immune traits during growth and adulthood



The main axis of covariation among immune traits (PC1) accounted for 24.3% and 23.5% of the total inertia for growing and adult roe deer, respectively (Fig. 1). In both cases, PC1 was mostly influenced by the five globulin concentrations: gamma- ( $r = 0.81$  and  $0.75$  in growing and adult roe deer, respectively), beta- ( $r = 0.90$  and  $0.90$ ), alpha1- ( $r = 0.85$  and  $0.80$ ), alpha2-globulin ( $r = 0.71$  and  $0.55$ ), and haptoglobin ( $r = 0.38$  and  $0.57$ ). PC1 could thus be directly interpreted as a continuum of the humoral part of innate immunity. In the following analyses, we used PC1 as a continuum for globulin levels.

PC2 captured around 13.2% and 13.3% of the total inertia in growing and adult roe deer, respectively (Fig. 1). It mostly reflected variation in cellular immunity. In both growing and adult roe deer, PC2 was positively correlated with markers of natural antibodies (HL:  $r = 0.72$  and  $0.75$ ; HA:  $r = 0.69$  and  $0.46$ ), neutrophil count ( $r = 0.38$  and  $0.29$ ) and lymphocyte count ( $r = 0.14$  and  $r = 0.45$ ). PC2 was negatively correlated with monocyte ( $r = -0.34$  and  $-0.55$ ), eosinophil ( $r = -0.29$  and  $r = -0.18$ ) and basophil ( $r = -0.12$  and  $-0.35$ ) counts. Our analyses reveal clear consistency between the immune phenotype of growing and adult individuals.

The similarity in the structure of correlations between immune traits (Fig. 1) most likely resulted from high and consistent between-population differences in the level of immune traits at both life stages (Table 2). As expected, both growing animals and adults had higher globulin levels at Chizé than at Trois-Fontaines. In contrast, both growing animals and adults had higher neutrophil and lymphocyte counts at Trois-Fontaines than at Chizé (Table 2). In growing animals, no sex-difference was detected for most immune traits except for lymphocytes, which were higher in females than in males. In adult animals, we detect sex-differences for eosinophils, which were higher in females than in males in adults of both populations; and for haptoglobin, which were higher in males than in females (Table 2). Finally, the growth type (*i.e.* slow or fast) did not influence the immune profile of both young

and adult roe deer (Fig. 1). Thus, *early* growth in growing ( $F=1.37$ ,  $df=12$ ,  $132$ ,  $p=0.21$ ) and adult ( $F=0.80$ ,  $df=12$ ,  $74$ ,  $p=0.56$ ) roe deer and *late* growth in adult roe deer ( $F=0.40$ ,  $df=12$ ,  $132$ ,  $p=0.79$ ) did not display any detectable difference between slowest (i.e. bottom 25%) and fastest (i.e. top 25%) growing roe deer.

### **Short-term effects of *early* growth on immunity traits in growing roe deer**

#### **Effect size**

In growing roe deer with known measures of *early* growth ( $n = 300$ , see Table 1), effect sizes of *early* growth on immunity components were mostly negligible (Fig. 2). At Trois-Fontaines and Chizé, no relationship was found between *early* growth and globulins level (PC1, Fig. 2), cellular immunity (PC2, Fig. 2) or immune traits separately (Online Resource 2). At Aurignac, effect sizes were only calculated on the immune traits separately (as explained in the Methods section) and we found a negative effect of *early* growth on neutrophil count in males (effect size:  $-0.48$  [ $-0.76$ ;  $-0.06$ ]). The magnitude of this effect was, however, not consistent with that observed at Chizé or Trois-Fontaines.

#### **Selected models**

Based on this approach, we only found support for an overall negative relationship between *early* growth and PC1 across both sexes and populations (model selection in Online Resource 3 and selected models detailed in Online Resource 4). More specifically, the selected model for the two main immune traits contributing to PC1 included a negative effect of *early* growth, that is for alpha1-globulins at Chizé (Fig. 3(a), slope of  $-0.11 \pm 0.04$ ,  $P < 0.01$ ) and gamma-globulins in all three populations (Fig. 3(b), slope of  $-0.36 \pm 0.09$ ,  $P < 0.001$ ).

### **Long-term effects of *early* and *late* growth on immunity traits in adult roe deer**

#### **Effect size**

Based on effect size of *early* and *late* growth in adult roe deer from both Trois-Fontaines and Chizé (124 out of 196 roe deer available to characterize immunity during adulthood, Table 1), evidence for substantial long-term growth costs on immunity was overall weak and heterogeneous (Fig. 4 and Online Resource 2). In both Trois-Fontaines and Chizé, although several immune traits studied separately were negatively associated to early or late growth (Online Resource 2), no significant association was observed between *early* or *late* growth and either the globulins level (PC1) or cellular immunity (PC2).

### **Selected Models**

The model selection procedure did not reveal any effect of our growth metrics on the different immune traits except for two immune traits that strongly contributed to PC2 (model selection in Online Resource 3 and selected models detailed in Online Resource 4): the eosinophil count in males (model selected: [*early* growth x sex], Online Resource 4) and the neutrophil count (model selected: [*early* growth x pop + *late* growth], Online Resource 4). More precisely, the eosinophil count was negatively affected by *early* growth in males in the two populations (Fig. 5(a), slope of  $-0.03 \pm 0.01$ ,  $P < 0.01$ ). The neutrophil count was negatively affected by *early* growth at Trois-Fontaines only (Fig. 5(b), slope of  $-0.24 \pm 0.11$ ,  $P < 0.05$ ) and by *late* growth in both populations (Fig. 5(c), slope of  $-0.22 \pm 0.09$ ,  $P < 0.05$ ).

## **Discussion**

We tested for potential short-term and long-term costs of growth on immunity in a large herbivore in three free-ranging populations facing markedly different ecological conditions. Overall, we found only limited evidence for the expected costs of growth on roe deer

immunity, both over the short-term during immune development (at 8 months), and over the longer-term during adulthood (between 4 and 9 years of age). In growing animals, we found a negative effect of *early* growth on the levels of some globulins (gamma-globulins in all populations and alpha1-globulins at Chizé). Similarly, we also found a slightly negative effect of *early* and *late* growth on some immune traits in adult animals. In adult males of two populations, neutrophil and eosinophil counts were lower in individuals which had grown fast. In females, adults of both populations showed a negative relationship between late growth and neutrophil count, while only females from Trois-Fontaines appeared to pay a cost of high *early* growth (on neutrophil count). Long-term growth costs thus generally tended to affect the cellular part of the immune response, mostly the number of neutrophils and eosinophils. The fact that the various components of the immune system were differentially affected by growth is consistent with our hypothesis that immune functions are not equally costly to produce and maintain. As reported by previous studies (Klasing 2004), our results support that innate and cellular components of immunity are more costly to produce over the long-term than adaptive and humoral immune components. Consequently, although some cellular immune traits might be impaired by fast growth, the evidence for growth costs on other immune components is far from evident in roe deer. Our results also highlight that, contrary to our expectations, high *late* growth that potentially includes compensatory growth does not lead to higher costs for immunity than *early* growth. Finally, although Trois-Fontaines forest is clearly a more suitable habitat for roe deer than the Chizé forest (based on higher wood production, plant productivity and homogeneity of the forest, see details in Pettorelli et al. 2006), roe deer at Chizé did not seem to pay higher growth costs on immunity than those at Trois-Fontaines.

Obviously, our study had some limitations that might partly account for the overall findings of an absence of a trade-off between growth and immunity in roe deer. The two main axes of our PCA only accounted for 30% of the total variation in the immune measures, indicating that a substantial proportion of variation remains. Low resolution in our immune measures might account for this low explanatory power of the PCA because measuring immunity in wild species is challenging. Indeed, access to wild animals is difficult, and standardised measurement techniques have still to be developed for non-model species such as roe deer. The immune measures in our study provide a general pattern of energy allocation to the two components of the immune response, which is consistent over the long-term, as indicated by the substantial within-individual repeatability we found in adults. Although our measures were taken without considering the previous history of pathogen exposure, these baseline levels have been shown to be related to the response to previous challenges (Schneeberger et al. 2014), and to be strongly associated with survival in some mammals in the wild (Rossi et al. 2013 on wild boar *Sus scrofa*, Garnier et al. 2017 on Soay sheep *Ovis aries*). However, short-term variation in some immune parameters in response to specific immune challenges may obscure the observed pattern, in particular, for parameters displaying short-term responses such as haptoglobin or alpha-globulins. Some of these measures could also be improved to assess more precisely immune allocation, for example, by discriminating different populations of lymphocytes, and/or by conducting functional immunological tests. Lastly, our assessment of growth could potentially be improved and a more detailed assessment of the growth curve might have enabled a better detection of trade-offs. Since we did not have repeated measures of body mass in our populations, we used mass gain between 8 months of age and adulthood as a proxy of growth. Although this prevented us from deriving a very detailed growth trajectory, it still provided relevant information on individual allocation

to growth for our model species. Indeed, roe deer display a monomolecular pattern of growth, involving a peak growth rate at birth consistently decreasing with increasing age (Portier et al. 2000).

Besides the methodological limitations reviewed above, a possible explanation for the absence of a clear trade-off between growth and immunity in the present study might be due to the lack of information on reproductive effort. While the patterns of covariation between growth and immune performance that have been reported so far are highly variable (see e.g. Saino et al. 1998, Hõrak et al. 2000, Mauck et al. 2005), most studies (including ours) ignored the fact that available resources have to be partitioned among growth, reproduction, and a broad range of physiological functions related to survival (Stearns 1992, Kirkwood and Rose 1991). High allocation to reproduction during early life is generally associated with long-term fitness (Lemaître et al. 2015, but see Panagakis et al. 2017) and physiological costs (e.g. Bauch et al. 2013, Hanssen et al. 2005) in wild vertebrates. Although most studies have been performed in females, recent evidence suggests that the same might be true in males (Lemaître et al. 2014, Beirne et al. 2015). Therefore, a decline in immune performance might, in some species, be only apparent in individuals that allocate substantially to both growth and reproduction (e.g. gestation and lactation in females or energy allocation to traits associated with sexual competition in males). Studies on laboratory or farm animals that have been intensely selected for high productivity (growth or reproduction) have shown that, despite *ad libitum* access to nutrient-dense food, a down-regulation of immune function can occur when immunity is not purposely selected for (Van der Most et al. 2011). The trade-offs involving reproduction might be particularly pronounced in roe deer females that rely almost exclusively on available food resources during breeding and do not store body reserves to meet the energetic requirements of reproduction (i.e. income breeder reproductive tactic,

Andersen et al. 2000). Cumulative costs of growth and reproduction might even be more pronounced in harsh environments such as Chizé, where roe deer females show higher between-year variation in reproductive success, than in Trois-Fontaines or Aurignac (Gaillard et al. 2013). Females of long-lived iteroparous species faced with harsh conditions are selected to favour their own survival over their offspring survival (Gaillard and Yoccoz 2003), which could explain the absence of costs paid on immunity by females at Chizé and their lower allocation to reproduction when faced with food shortage (Gaillard et al. 1992). The trade-off between growth and immune development may also be stronger in degraded habitats because habitat quality and biodiversity both contribute to shape pathogen burden and parasite selective pressure (Civitello et al. 2015, Young et al. 2013).

Optimal strategies relative to the maintenance or the regulation of the immune system also depend a lot on the pathogen pressure of the environment, and the optimal allocation of resources among different components of immunity is context-specific. Parasites and pathogens generate a threat to survival and infections are one of the causes potentially jeopardizing juvenile survival (Sams et al. 1996, Lynsdale et al. 2017). The 'antigen-exposure' hypothesis points to ecological variation in pathogen pressure as being a key determinant of observed variation in immunity (McDade 2016). This hypothesis predicts that, in environments with high pathogenic pressure, overall allocation to immune function will be greater, and/or that adaptive immunity will be prioritized over innate immunity if the costs of increasing allocation to all components of the immune function are exceedingly large. At Chizé, young roe deer faced high parasite pressure, for instance of *Trichuris sp.* (Cheynel et al. 2017). It is therefore crucial for them to allocate energy to immunity during body development. This strong allocation in immunity from the early stages until adulthood could explain the overall absence of a trade-off between growth and immunity, especially at Chizé.

At Trois-Fontaines, roe deer are less exposed to parasites and have more resources available to mount efficient immune response. Additionally, the high parasite pressure on young roe deer at Chizé could result in the selective disappearance of lower quality individuals and especially young roe deer unable to allocate to both immunity and growth, a selection expected to be weaker at Trois-Fontaines. Finally, if nutritional resources are limited, as is expected to be the case at Chizé, immune components that require fewer resources both in the short-term but also in the long-term should be favoured. Even if allocating preferentially to adaptive immunity requires the payment of upfront costs, especially when food is limited, adaptive immunity should be favoured in the long run in anticipation of deferred future benefits and given the lower maintenance and per-response activation costs, compared to the innate responses (McDade 2016). In line with previous studies (Gilot-Fromont et al. 2012, Cheynel et al. 2017) these findings support the assertion that roe deer allocate more to adaptive immunity from the youngest age at Chizé than at Trois-Fontaines.

In this study, we showed that young roe deer pay only negligible costs of growing rapidly in terms of immune performance. However, it is likely that high allocation to growth, especially late in the growing period (after weaning), can affect physiological functions other than immunity, which might have deleterious consequences on fitness components later in life. For instance, roe deer growing fast late in the growing period suffered from a steeper rate of mass senescence than slower growers (Douhard et al. 2017). These findings illustrate the complexity of detecting physiological costs and trade-offs in the wild, especially when immune responses are involved (Sandland and Minchella 2003).

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## **Compliance with ethical standards**

### **Conflict of interest**

The authors declare that they have no conflict of interest.

### **Ethical approval**

All applicable institutional and/or national guidelines for the care and use of animals were followed. For Trois-Fontaines and Chizé populations, the protocol of capture and blood sampling of roe deer under the authority of the Office National de la Chasse et de la Faune Sauvage (ONCFS) was approved by the Director of Food, Agriculture and Forest (Prefectoral order 2009-14 from Paris). All procedures were approved by the Ethical Committee of Lyon 1 University (project DR2014-09, June 5, 2014). For the Aurignac population, the study was permitted by the land manager of both sites, the Office National des Forêts (ONF) and

prefecture of Haute Garonne (Partnership Convention ONCFS-ONF dated 2005-12-23). All procedures were approved by the Ethical Committee 115 of Toulouse.

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**Table 1:** Sex- and population-specific sample sizes.

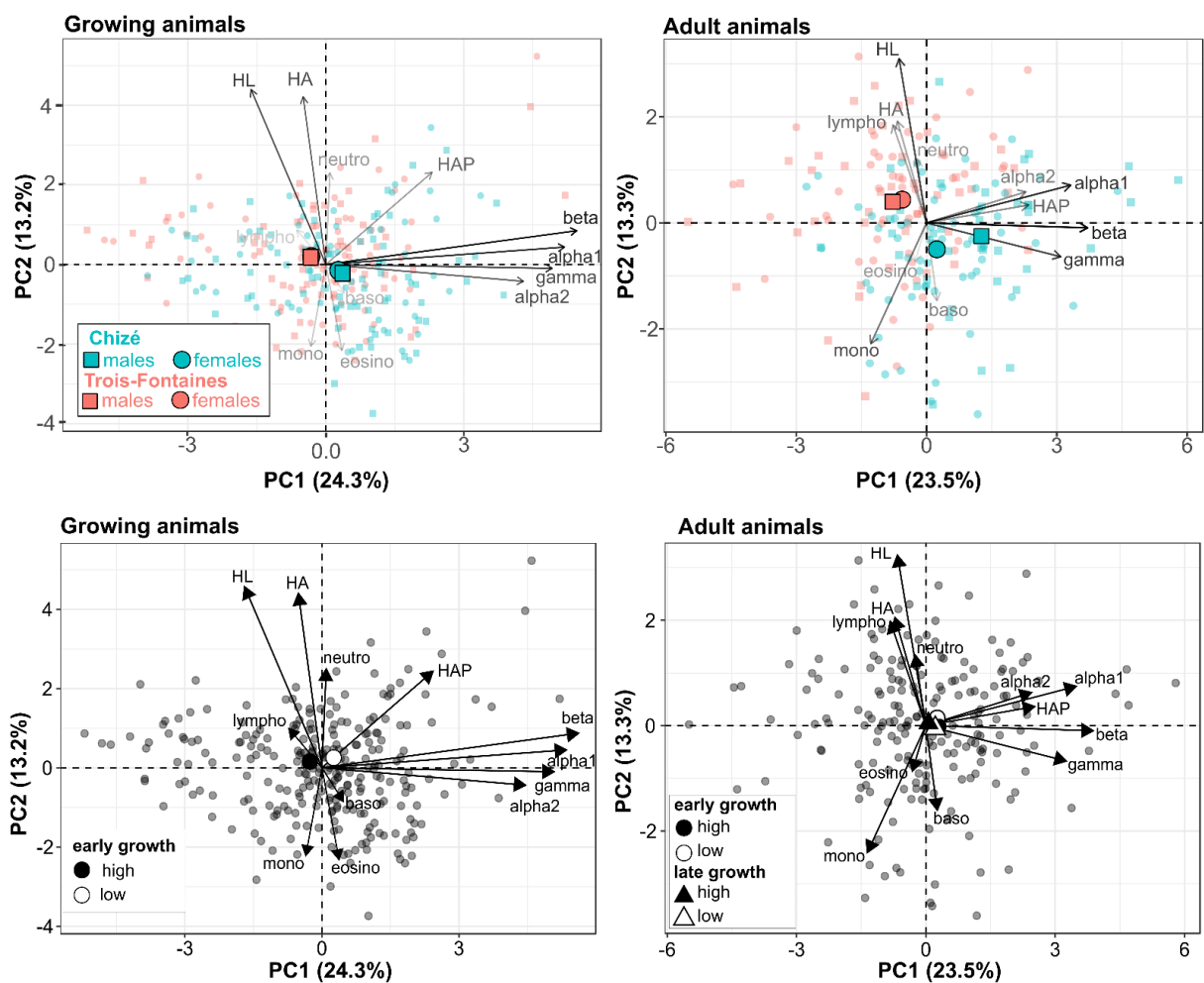
| Subset   | Chizé      |            | Trois-Fontaines |            | Aurignac   |            | Total        |
|--|------------|------------|-----------------|------------|------------|------------|--------------|
|  | Females    | Males      | Females         | Males      | Females    | Males      |              |
| Immunity measures at 8 months of age<br>(individuals with measure of early growth)                           | 59<br>(59) | 71<br>(71) | 68<br>(68)      | 61<br>(61) | 20<br>(20) | 21<br>(21) | 300<br>(300) |
| Immunity measures during adulthood [4 – 9 years of age]<br>(individuals with early and late growth measures) | 61<br>(43) | 39<br>(28) | 51<br>(33)      | 45<br>(20) | -          | -          | 196<br>(124) |
| Immunity measures during growth and adulthood<br>(individuals with growth measures)                          | 6<br>(3)   | 10<br>(2)  | 11<br>(11)      | 5<br>(5)   | -          | -          | 32<br>(21)   |

**Table 2:** Average sex-specific immune measures observed in the three roe deer populations, at Aurignac, Chizé and Trois-Fontaines.

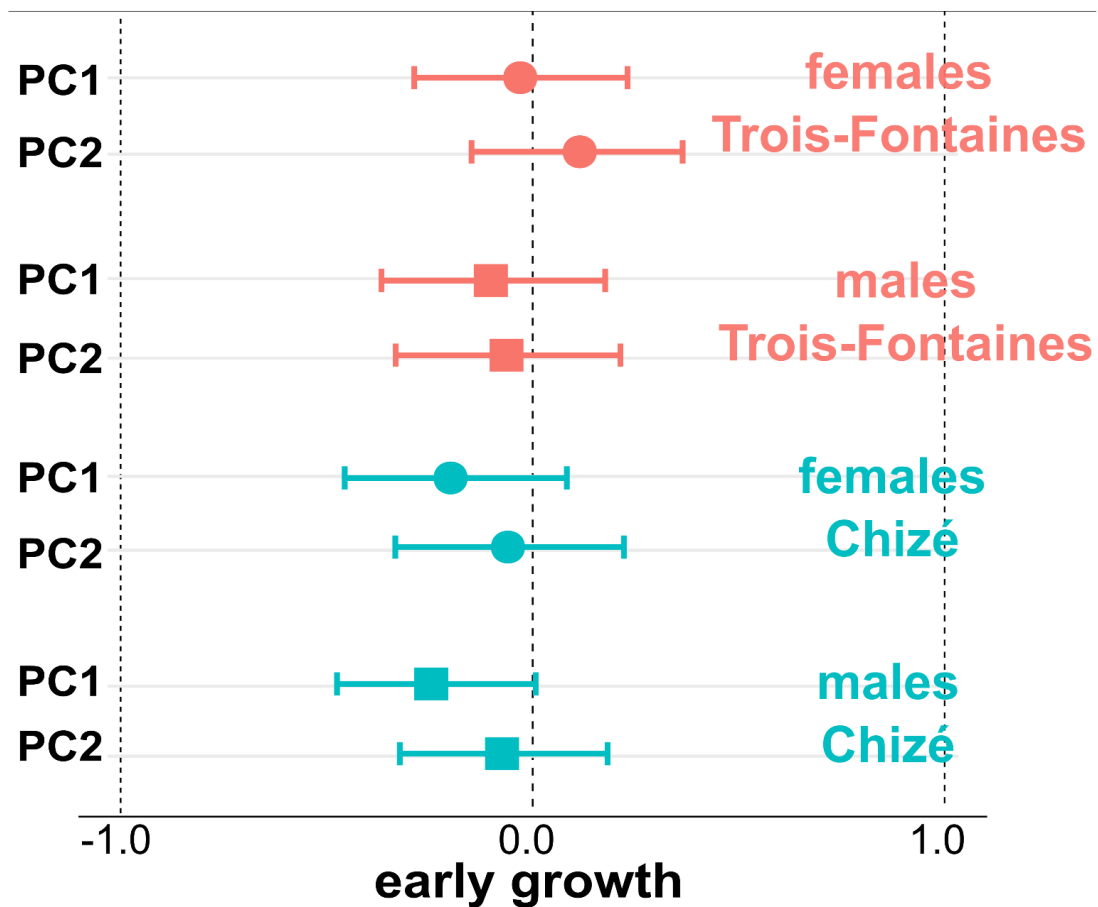
| Immune trait            | Chizé               |                    | Trois-Fontaines     |                     | Aurignac            |                     | Effects    |     |             |
|-------------------------|---------------------|--------------------|---------------------|---------------------|---------------------|---------------------|------------|-----|-------------|
|                         | Females             | Males              | Females             | Males               | Females             | Males               | Population | Sex | Interaction |
| <b>Growing animals</b>  |                     |                    |                     |                     |                     |                     |            |     |             |
| Globulins levels (PC1)  | 0.27 <sup>a</sup>   | 0.36 <sup>a</sup>  | -0.32 <sup>a</sup>  | -0.33 <sup>a</sup>  | -                   | -                   | **         | -   | -           |
| Cellular immunity (PC2) | -0.15 <sup>a</sup>  | -0.22 <sup>a</sup> | 0.20 <sup>a</sup>   | 0.18 <sup>a</sup>   | -                   | -                   | *          | -   | -           |
| Alpha1-globulin         | 3.59 <sup>a</sup>   | 3.60 <sup>a</sup>  | 3.57 <sup>a</sup>   | 3.58 <sup>a</sup>   | 3.48 <sup>a</sup>   | 3.37 <sup>a</sup>   | -          | -   | -           |
| Alpha2-globulin         | 5.55 <sup>ab</sup>  | 6.09 <sup>ac</sup> | 5.91 <sup>a</sup>   | 5.56 <sup>ab</sup>  | 4.47 <sup>bc</sup>  | 4.30 <sup>b</sup>   | ***        | -   | -           |
| Haptoglobin             | 0.13 <sup>a</sup>   | 0.14 <sup>a</sup>  | 0.15 <sup>a</sup>   | 0.16 <sup>a</sup>   | -                   | -                   | -          | -   | -           |
| Beta-globulin           | 5.99 <sup>a</sup>   | 5.89 <sup>a</sup>  | 5.59 <sup>a</sup>   | 5.52 <sup>a</sup>   | 5.14 <sup>a</sup>   | 5.30 <sup>a</sup>   | ***        | -   | -           |
| Gamma-globulin          | 18.24 <sup>a</sup>  | 17.88 <sup>a</sup> | 13.31 <sup>b</sup>  | 14.11 <sup>b</sup>  | 10.08 <sup>c</sup>  | 9.69 <sup>c</sup>   | ***        | -   | -           |
| Neutrophil count        | 4.34 <sup>ac</sup>  | 4.06 <sup>a</sup>  | 5.80 <sup>d</sup>   | 5.67 <sup>d</sup>   | 5.53 <sup>bcd</sup> | 5.89 <sup>bd</sup>  | ***        | -   | -           |
| Monocyte count          | 0.42 <sup>ade</sup> | 0.45 <sup>ad</sup> | 0.33 <sup>cd</sup>  | 0.26 <sup>ce</sup>  | 0.08 <sup>bc</sup>  | 0.17 <sup>bce</sup> | ***        | -   | -           |
| Basophil count          | 0.06 <sup>a</sup>   | 0.09 <sup>a</sup>  | 0.05 <sup>a</sup>   | 0.05 <sup>a</sup>   | 0.06 <sup>a</sup>   | 0.07 <sup>a</sup>   | -          | -   | -           |
| Lymphocyte count        | 2.38 <sup>abd</sup> | 2.03 <sup>a</sup>  | 2.69 <sup>bef</sup> | 2.37 <sup>abd</sup> | 3.00 <sup>cdf</sup> | 2.72 <sup>ace</sup> | ***        | *   | -           |
| Eosinophil count        | 0.06 <sup>a</sup>   | 0.07 <sup>a</sup>  | 0.05 <sup>a</sup>   | 0.07 <sup>a</sup>   | 0.05 <sup>a</sup>   | 0.04 <sup>a</sup>   | -          | -   | -           |
| HA                      | 4.59 <sup>a</sup>   | 4.59 <sup>a</sup>  | 4.10 <sup>a</sup>   | 4.08 <sup>a</sup>   | 3.70 <sup>a</sup>   | 3.83 <sup>a</sup>   | ***        | -   | -           |
| HL                      | 1.78 <sup>a</sup>   | 1.98 <sup>a</sup>  | 2.02 <sup>a</sup>   | 2.30 <sup>a</sup>   | 3.05 <sup>b</sup>   | 3.33 <sup>b</sup>   | ***        | -   | -           |
| <b>Adult animals</b>    |                     |                    |                     |                     |                     |                     |            |     |             |
| Globulins (PC1)         | 0.237 <sup>b</sup>  | 1.263 <sup>a</sup> | -0.56 <sup>c</sup>  | -0.78 <sup>c</sup>  | -                   | -                   | ***        | -   | **          |
| Cellular immunity (PC2) | 0.50 <sup>a</sup>   | 0.25 <sup>a</sup>  | -0.43 <sup>b</sup>  | -0.40 <sup>b</sup>  | -                   | -                   | ***        | -   | -           |
| Alpha1-globulin         | 3.03 <sup>a</sup>   | 3.35 <sup>b</sup>  | 3.16 <sup>ab</sup>  | 3.12 <sup>ab</sup>  | -                   | -                   | -          | -   | *           |
| Alpha2-globulin         | 5.83 <sup>a</sup>   | 5.76 <sup>a</sup>  | 5.70 <sup>a</sup>   | 5.35 <sup>a</sup>   | -                   | -                   | -          | -   | -           |
| Haptoglobin             | 0.21 <sup>ab</sup>  | 0.78 <sup>a</sup>  | 0.14 <sup>b</sup>   | 0.17 <sup>c</sup>   | -                   | -                   | **         | *** | **          |
| Beta-globulin           | 7.19 <sup>ab</sup>  | 8.36 <sup>a</sup>  | 6.61 <sup>b</sup>   | 6.44 <sup>c</sup>   | -                   | -                   | ***        | *   | **          |
| Gamma-globulin          | 20.28 <sup>a</sup>  | 20.89 <sup>a</sup> | 15.20 <sup>b</sup>  | 14.53 <sup>b</sup>  | -                   | -                   | ***        | -   | -           |
| Neutrophils             | 5.18 <sup>b</sup>   | 5.05 <sup>b</sup>  | 6.42 <sup>a</sup>   | 6.01 <sup>ab</sup>  | -                   | -                   | ***        | -   | -           |
| Monocytes               | 0.31 <sup>a</sup>   | 0.31 <sup>a</sup>  | 0.32 <sup>a</sup>   | 0.35 <sup>a</sup>   | -                   | -                   | -          | -   | -           |
| Basophils               | 0.08 <sup>a</sup>   | 0.08 <sup>a</sup>  | 0.05 <sup>a</sup>   | 0.07 <sup>a</sup>   | -                   | -                   | -          | -   | -           |
| Lymphocyte count        | 1.62 <sup>b</sup>   | 1.62 <sup>b</sup>  | 2.33 <sup>a</sup>   | 2.12 <sup>a</sup>   | -                   | -                   | ***        | -   | -           |
| Eosinophil count        | 0.11 <sup>a</sup>   | 0.09 <sup>a</sup>  | 0.11 <sup>a</sup>   | 0.06 <sup>a</sup>   | -                   | -                   | -          | *   | -           |
| HA                      | 3.96 <sup>a</sup>   | 3.90 <sup>a</sup>  | 4.04 <sup>a</sup>   | 4.20 <sup>a</sup>   | -                   | -                   | -          | -   | -           |
| HL                      | 1.84 <sup>b</sup>   | 2.07 <sup>ab</sup> | 2.25 <sup>ab</sup>  | 2.47 <sup>a</sup>   | -                   | -                   | *          | -   | -           |

Level of statistical significance denoted by asterisk: \* $P < 0.05$ , \*\*  $< 0.01$ , \*\*\*  $< 0.001$ . Means within rows sharing a common character in their superscript are not statistically different ( $P > 0.05$ ) based on pairwise comparisons (Tukey's test).

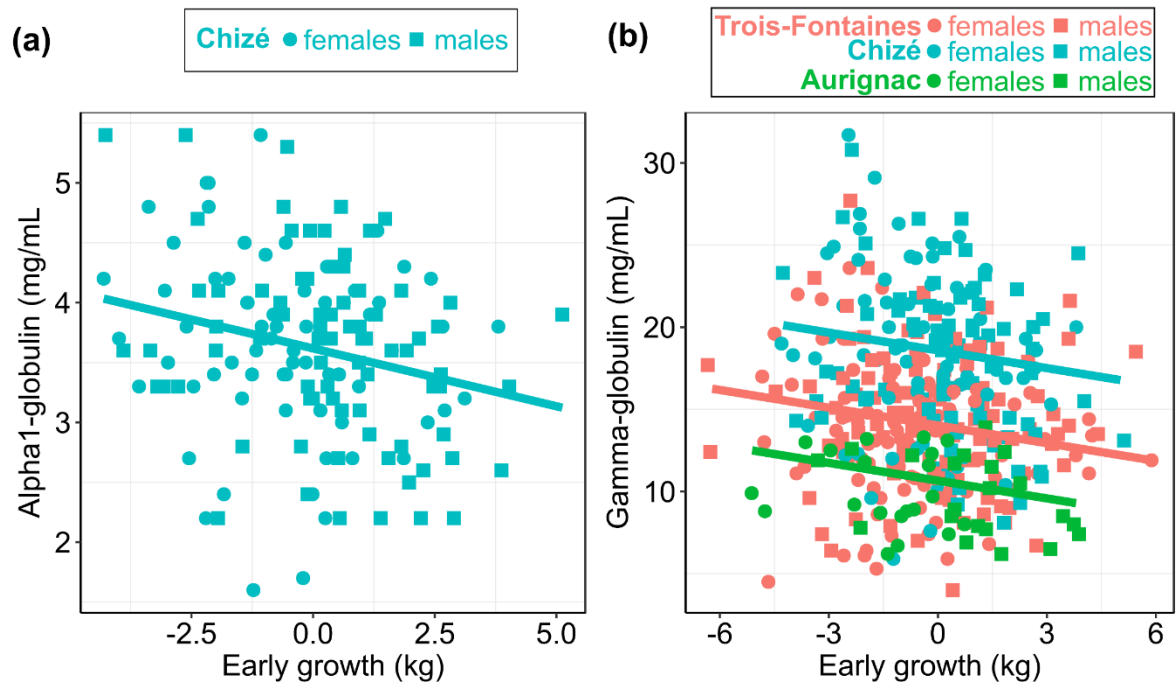
**Fig. 1** Biplots of PCA analyses of the 12 immunity traits measured during the growing (left) and adulthood (right) life stages in female and male roe deer of the two populations above (Chizé and Trois-Fontaines), and in individuals with extremely fast (top 25%) or slow (bottom 25%) growth below. Arrows indicate the contribution of immune traits to each of the first two PCs (i.e. longer and darker arrows denote stronger correlations). Small symbols indicate individual coordinates on the first two PCs. Average PCs scores are reported by the larger symbols. See text for definition of variables.



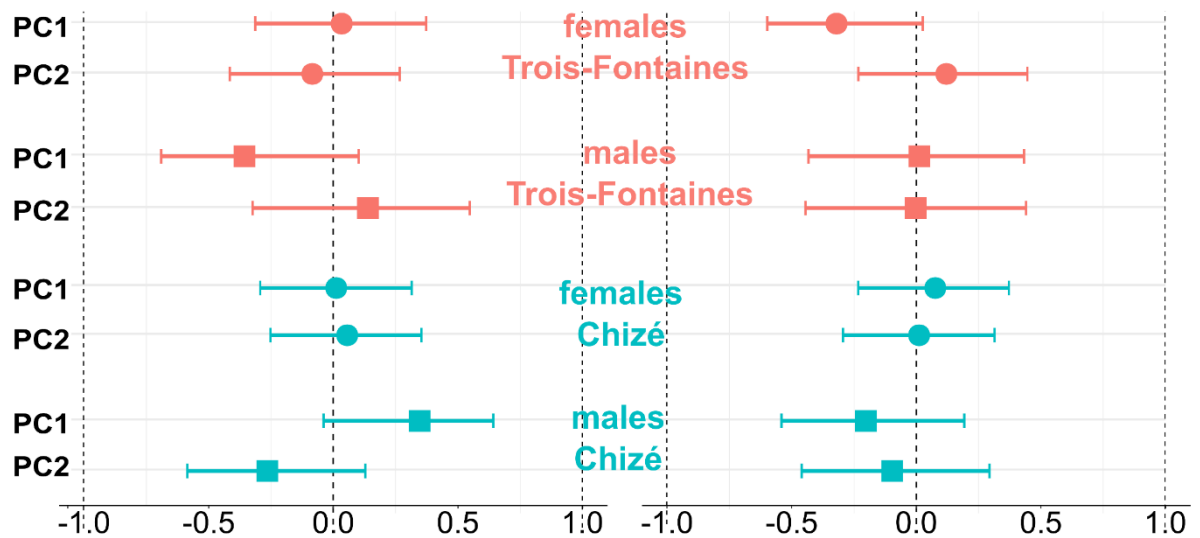
**Fig. 2** Effect size of *early* growth on the two main axes of covariation among immune traits (PC1/PC2) measured in growing roe deer (at 8 months of age) at Chizé and Trois-Fontaines. Effect sizes (symbols) are reported together with associated 95%-confidence interval.



**Fig. 3** Negative effect of *early* growth on two traits of humoral immunity of fawns (8-months old, a-b) in the three populations of Chizé, Aurignac and Trois-Fontaines.

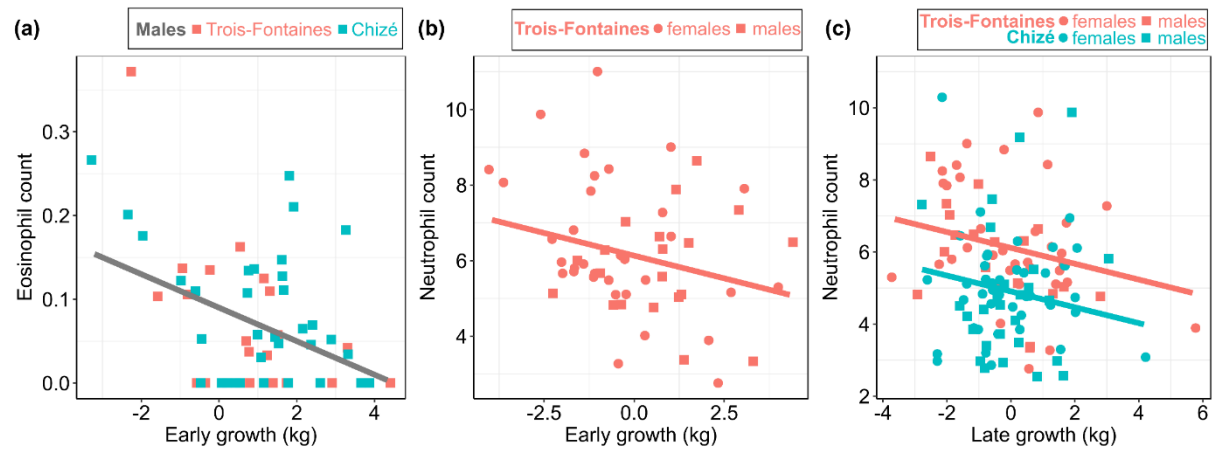


**Fig. 4** Effect size of *early* growth (left) and *late* growth (right) on the two main axes of covariation among immune traits (PC1/PC2) measured in adult roe deer (from 4 to 9 years of age included) at Chizé and Trois-Fontaines. Effect sizes (symbols) are reported together with associated 95%-confidence interval.





**Fig. 5** Negative effect of *early* or *late* growth on two traits of cellular immunity of adult roe deer (from 4 to 9 years of age included) in the two populations of Chizé and Trois-Fontaines.



**Online Resource 1.** Detailed protocol and repeatability of the 12 immune measurements of our study assessed with the rptR package.

#### DETAILED PROTOCOL

In this study, as in two previous studies [1,2], we measured eleven immune traits using haematological parameters and specific assays. These multiple measures are analysed simultaneously because they reflect both the innate and the adaptive responses, which are represented by both humoral and cell-mediated components [3, 4].

Concerning cellular immunity, we measured neutrophil, monocyte, lymphocyte, eosinophil and basophil counts. Neutrophils and monocytes counts reflect acute and chronic inflammatory responses, respectively, and both may increase after infection. Eosinophils are specifically induced by Th2 responses, but may be also present in other contexts like hypersensitivity [4]. Basophils, which are quite rare, play a key role against macroparasites such as ticks. Lymphocytes counts include both T and B cells, the latter being particularly involved in the production of antibodies and thus in adaptive responses [4]. We manually estimated the proportion of the various leucocytes forms by counting the 100 first cells on blood smears with a microscope under 200x magnification (in %). Blood smears are stained with May-Grünwald's (#T863.2, Carl Roth GmbH) and Giemsa (#T862.1, Carl Roth GmbH) solution, a method that has been used before in studies on mammalian immunology [5]. A complete blood count was also performed using an ABC Vet automaton (Horiba Medical, Montpellier, France). It measured the total white blood cells ( $10^3$  cells/mL) by impedance technology, considering parameters for bovine samples, since the size of blood cells is comparable between the two species [6]. With both the proportion of each leucocyte forms (in %) and the total white blood cells measurement ( $10^3$  cells/mL), we obtained the count of each leucocyte ( $10^3$  cells/mL).

Humoral aspects were first assessed using the levels of alpha1-,alpha-2, beta- and gamma-globulins (mg/mL). Alpha1-, alpha2- and beta-globulins fractions including several acute phase proteins (APPs), a group of proteins which concentration changes following external or internal challenges such as trauma, inflammation or infection [7]. We also measured the specific level of haptoglobin (in mg/mL) which belongs to alpha2-globulin fraction. Total protein content (in mg/mL) was first assessed by refractometry followed by automatic agarose gel electrophoresis (HYDRASYS, Sebia, Evry, France) that separates albumin and the 4 fractions of globulins ( $\alpha_1$ ,  $\alpha_2$ ,  $\beta$ , and  $\gamma$ ). Haptoglobin analyses were performed on a Konelab 30i automaton (Fisher Thermo Scientific, Cergy-Pontoise, France) using phase Haptoglobin assay (Tridelta Development LTD, County Kildare, Ireland) chromogenic kit. Finally, we used the level of gamma-globulins (mg/mL) derived from the protein analysis described above as an estimator of total antibodies. Gamma-globulins are indeed essentially constituted of circulating antibodies [8], produced during adaptive response and often used as a measurement of allocation to long-term immunity [9].

Humoral innate immunity was also assessed by the levels of natural antibodies and complement. Natural antibodies are circulating antibodies that are present in the absence of any previous exposure to antigens. Their level is thus independent from the exposure of individuals to infection, but they are correlated to the ability to produce antibodies after a challenge [10]. Their presence is revealed by hemagglutination (HA, titre score), which measures the ability of samples to agglutinate exogenous cells. The complement is a group of proteins that acts through chain reactions and causes the lysis of exogenous cells in the presence of an antigen-antibody complex. They can thus be revealed by their ability to cause hemolysis (HL, titer score) [10]. We used the hemagglutination-hemolysis protocol defined in [10] and reported below, but modified using chicken red blood cells as target cells. The assay

is carried out in 96-well (eight rows by 12 columns) round (U) bottom assay plates. Twenty-five microliters of eight plasma samples are pipetted into columns 1 and 2 of the plate, and 25  $\mu$ l of 0.01M phosphate buffered saline (PBS; Sigma #P3813, St Louis, MO) are added to the columns 2–12. Using a multi-channel pipetter the contents of the column 2 wells are serially diluted (1:2) through column 11. This results in dilutions ranging from 1 to 1/1024 and 25 ml in every well. The column 12 with 25  $\mu$ l of PBS serves as a negative control. For the assay itself, 25  $\mu$ l of a 1% chicken blood cell suspension is added to all wells, effectively halving all plasma dilutions. Each plate is then sealed with Parafilm M (Pechiney Plastic Packaging, Neenah, WI) and covered with a polystyrene plate lid. Plates are gently vortexed for 10 s prior to incubation during which they are floated in a 37°C water bath for 90 min. Upon completion of the incubation, the long axis of each plate is tilted to a 45° angle for 20 min at room temperature in order to enhance visualization of HA. Plates are then scanned (full size image at 300 dpi) using the positive transparency (top-lit) setting of a flatbed scanner. Afterward, plates are kept at room temperature for an additional 70 min and scanned for a second time to record maximum lytic activity. From the digitized images, lysis and agglutination are scored for each sample. Lysis reflects the interaction of complement and NABs, whereas agglutination results from NABs only. Both variables are recorded as the negative log<sub>2</sub> of the last plasma dilution exhibiting each behavior, i.e. column 9 is a score of 9 (see the figure below). Half scores between two titres are recorded when the termination of lysis or agglutination is intermediate or is ambiguous.

Titre = 1 2 3 4 5 6 7 8 9 10 11 Neg.



Result = Ly Ly Ag Ag No No No No No No No No

Figure 1: An example of scan . Ly = lysis, Ag = agglutination, No = no lysis or agglutination

To conclude, our main indicators of innate immunity were the neutrophil, monocyte and basophil counts, globulins levels (alpha1-,alpha2-,beta-), haptoglobin and hemolysis, while hemagglutination and gamma- globulins were more reflecting adaptive response. Eosinophil and lymphocyte counts may reflect both aspects. Haematological and biochemical assays were performed at the Biochemical and Endocrinological laboratory, VetAgro-Sup, France, while the hemagglutination-hemolysis assay was performed at the UMR 5558, Villeurbanne, France.

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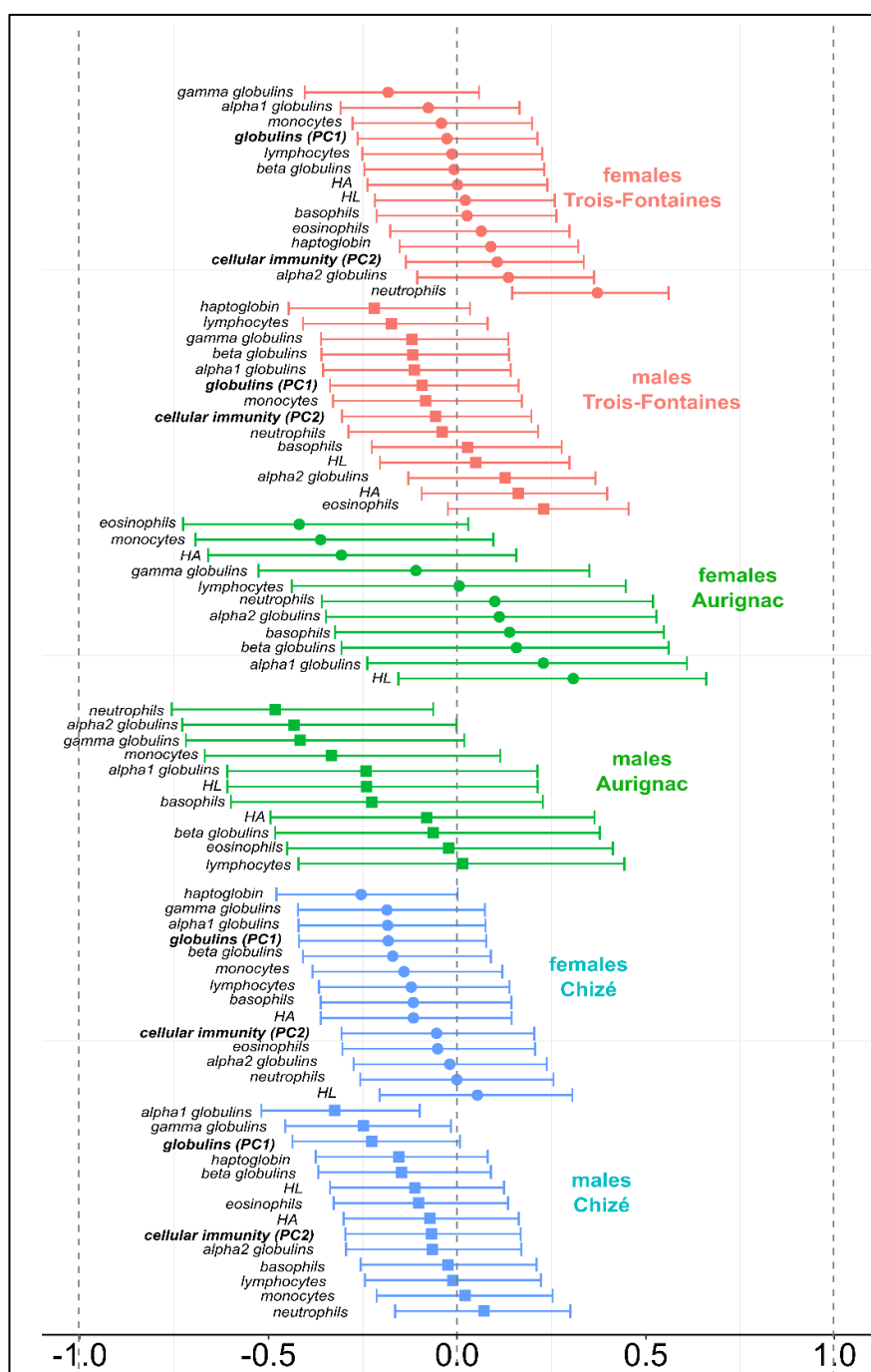
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## REPEATABILITY

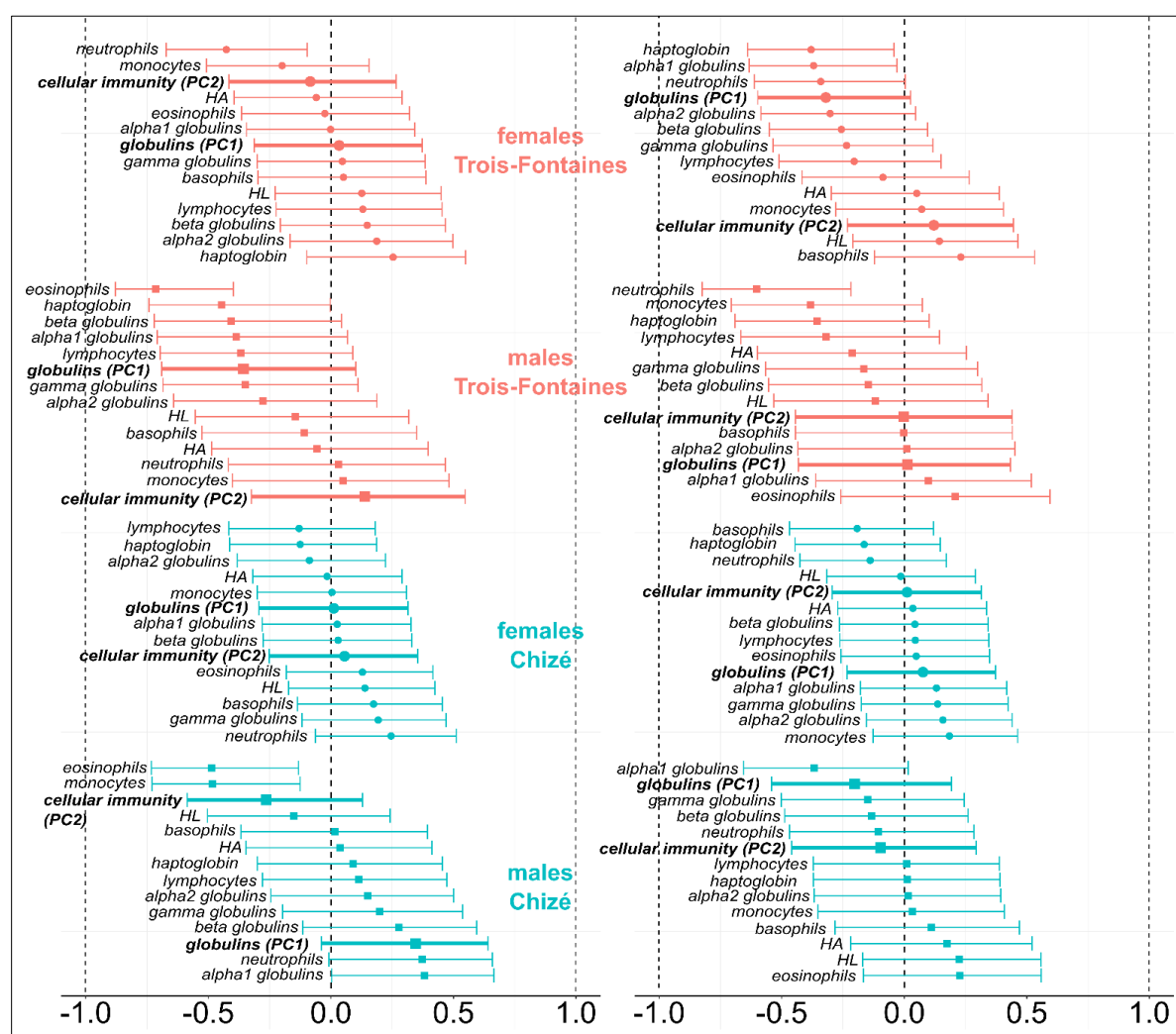
| Immune trait     | R    | SE    | CI          |
|------------------|------|-------|-------------|
| Neutrophil count | 0.41 | 0.072 | [0.26-0.55] |
| Monocyte count   | 0.29 | 0.140 | [0.00-0.55] |
| Basophil count   | 0.22 | 0.154 | [0.00-0.54] |
| Eosinophil count | 0.23 | 0.160 | [0.00-0.55] |
| Lymphocyte count | 0.33 | 0.080 | [0.17-0.48] |
| Hemagglutination | 0.07 | 0.066 | [0.00-0.22] |
| Hemolysis        | 0.42 | 0.093 | [0.24-0.61] |
| Alpha1-gloublin  | 0.03 | 0.057 | [0.00-0.20] |
| Alpha2-globulin  | 0.04 | 0.060 | [0.00-0.19] |
| Haptoglobin      | 0.10 | 0.073 | [0.00-0.26] |
| Beta-globulin    | 0.35 | 0.080 | [0.18-0.50] |
| Gamma-globulin   | 0.46 | 0.070 | [0.31-0.58] |

**Online Resource 2.** Effect size of *early* growth on the immune traits measured in growing (8 months of age) at Chizé, Trois-Fontaines and Aurignac ; and effect size of *early* (left) and *late* growth (right) in adult roe deer at Chizé and Trois-Fontaines Effect sizes (symbols) are reported together with associated 95%-confidence interval.

## GROWING ROE DEER



# ADULT ROE DEER





**Online Resource 3.** Supplementary methods (additional models testing the effect of *early* and *late* growth on roe deer immunity) and model selection table for immune traits of growing and adult roe deer. The best fitting models among the set of candidate models considering the inclusion of ‘early growth’ (EG), and late growth (LG) for adults, sex, population (Pop.), their interaction, and average cohort fawn mass (ACF mass) are reported. The selected model is indicated in grey.

## SUPPLEMENTARY METHODS

We performed complementary analyses by testing the effect of *early* and *late* growth on immunity using linear mixed-effect models. Results of model selection in growing and adult roe deer are reported in Online Resources 7 and 8, respectively. Parameter estimates of the selected models in growing and adult roe deer are reported in Online Resources 5 and 6, respectively. Each immune trait (and the two first PCS measuring their covariation) during the growing period or at adulthood, was analysed as a response variable. When individuals had repeated measures during a given growth period, the median of these individual measures was retained to avoid any pseudo-replication problems (*sensu* Hurlbert 1984). *Early* growth, *late* growth, sex and population, as well as all possible interactions were included as fixed effects in the full model. All models included cohort as a random effect and average cohort fawn mass (calculated in each population from the total number of fawn mass records in a given year) as a fixed effect, to account for cohort variation in immunity that is not directly related to body growth (Gaillard et al. 1997). The different models accounting for the possible effects of *early* and *late* growth were ranked based on the Akaike Information Criterion corrected for small sample size (AICc, Burnham, Anderson 2002) in the R package MuMIn (Barton 2016). We selected the model with the lowest AICc. When the difference in AIC

(denoted  $\Delta AICc$ ) of two competing models was less than 2, we retained the model with the lowest number of parameters by following parsimony rules. In addition, we calculated the AIC weights ( $\omega_i$ ) to measure the relative likelihood of each model to be the best among the set of fitted models.

# MODEL SELECTION FOR GROWING ROE DEER

| Trait            | Model   | df | $\Delta AICc$ | $\omega_i$ |
|------------------|---|----|---------------|------------|
| PC1              | EG + Pop. + (EG $\times$ Pop.)                  | 6  | 0.00          | 0.225      |
|                  | EG + Pop.                                       | 5  | 1.03          | 0.134      |
|                  | EG + Pop. + (EG $\times$ Pop.) + ACFmass        | 7  | 1.94          | 0.085      |
|                  | Null  | 3  | 18.22         | 0.000      |
| Alpha1-globulin  | EG + Pop. + (EG $\times$ Pop.) + ACFmass.       | 9  | 0.00          | 0.470      |
|                  | EG + Pop. + (EG $\times$ Pop.) + Sex + ACFmass  | 10 | 1.99          | 0.174      |
| Alpha2-globulin  | Pop. + Sex + (Sex $\times$ Pop.) + ACFmass      | 9  | 0.00          | 0.155      |
|                  | Pop. + Sex + (Sex $\times$ Pop.)                | 8  | 0.77          | 0.106      |
|                  | Pop.  | 5  | 1.43          | 0.076      |
|                  | EG + pop. + Sex + (Pop. $\times$ Sex) + ACFmass | 10 | 1.47          | 0.074      |
| Haptoglobin      | ACFmass + Pop. + EG                             | 6  | 0.00          | 0.227      |
|                  | ACFmass + Pop.                                  | 5  | 0.17          | 0.209      |
|                  | ACFmass + Pop. + EG + Sex                       | 7  | 1.54          | 0.105      |
|                  | Null  | 3  | 9.33          | 0.002      |
| Beta-globulin    | Pop.  | 5  | 0.00          | 0.105      |
|                  | EG + Pop. + (EG $\times$ Pop.)                  | 8  | 0.06          | 0.102      |
|                  | ACFmass   | 4  | 0.16          | 0.097      |
|                  | EG + Pop.                                       | 6  | 0.49          | 0.082      |
| Gamma-globulin   | EG + Pop. + (EG $\times$ Pop.) + ACFmass        | 9  | 0.00          | 0.171      |
|                  | EG + Pop. + (EG $\times$ Pop.)                  | 8  | 0.18          | 0.156      |
|                  | EG + Pop. + ACFmass                             | 7  | 0.84          | 0.112      |
|                  | EG + Pop.                                       | 6  | 0.88          | 0.110      |
| PC2              | Pop. + ACFmass                                  | 5  | 0.00          | 0.200      |
|                  | Pop.  | 4  | 1.51          | 0.094      |
|                  | Pop. + ACFmass + EG                             | 6  | 1.82          | 0.081      |
|                  | Null  | 3  | 8.94          | 0.002      |
| Neutrophil count | Pop.  | 5  | 0.00          | 0.177      |
|                  | EG + Sex + Pop. + (EG $\times$ Sex)             | 8  | 0.82          | 0.117      |
|                  | EG + Pop.                                       | 6  | 1.51          | 0.082      |
|                  | Pop. + Sex                                      | 6  | 1.70          | 0.075      |
| Monocyte count   | Pop.  | 5  | 0.00          | 0.229      |
|                  | Pop. + ACFmass                                  | 6  | 0.68          | 0.163      |
|                  | EG + Pop.                                       | 6  | 1.66          | 0.099      |
| Basophil count   | Sex   | 4  | 0.00          | 0.240      |
|                  | Sex + ACFmass                                   | 5  | 1.12          | 0.137      |
|                  | Null  | 3  | 1.50          | 0.113      |
|                  | EG + Sex  | 5  | 1.50          | 0.088      |
| Eosinophil count | Null  | 3  | 0.00          | 0.214      |
|                  | Sex   | 4  | 1.03          | 0.128      |
|                  | EG  | 4  | 1.30          | 0.111      |
|                  | ACFmass   | 4  | 1.95          | 0.080      |
| Lymphocyte count | Sex + Pop.                                      | 6  | 0.00          | 0.243      |
|                  | EG + Sex + Pop.                                 | 7  | 1.24          | 0.131      |
|                  | Pop. + Sex + ACFmass                            | 7  | 1.77          | 0.101      |
| Hemagglutination | ACFmass + Pop.                                  | 6  | 0.00          | 0.334      |
|                  | ACFmass + Pop. + Sex                            | 7  | 1.06          | 0.197      |
|                  | ACFmass + Pop. + Sex                            | 7  | 0.00          | 0.281      |
| Hemolysis        | ACFmass + Pop.                                  | 6  | 0.06          | 0.273      |
|                  | EG + ACFmass + Pop.                             | 7  | 1.87          | 0.110      |
|                  | EG + ACFmass + Pop. + Sex                       | 9  | 1.94          | 0.107      |

# MODEL SELECTION FOR ADULT ROE DEER

| Trait            | Model   | df | $\Delta AICc$ | $\omega_i$ |
|------------------|---|----|---------------|------------|
| PC1              | Pop. + Sex + (Sex $\times$ Pop.)  | 6  | 0.00          | 0.167      |
|                  | Pop. + Sex + (Sex $\times$ Pop.) + LG   | 7  | 1.43          | 0.052      |
|                  | Pop. + Sex + (Sex $\times$ Pop.) + ACFmass  | 7  | 2.13          | 0.058      |
|                  | Null  | 3  | 30.92         | 0.000      |
| Alpha1-globulin  | Pop. + Sex + (Sex $\times$ Pop.)  | 6  | 0.00          | 0.071      |
|                  | Pop. + Sex + (Sex $\times$ Pop.) + LG   | 7  | 0.54          | 0.055      |
|                  | Sex   | 4  | 1.43          | 0.035      |
|                  | Null  | 3  | 3.91          | 0.010      |
| Alpha2-globulin  | Null  | 3  | 0.00          | 0.059      |
|                  | Pop.  | 4  | 0.39          | 0.049      |
|                  | ACFmass   | 4  | 0.62          | 0.043      |
| Haptoglobin      | Pop. + Sex + (Sex $\times$ Pop.)  | 6  | 0.00          | 0.193      |
|                  | Pop. + Sex + (Sex $\times$ Pop.) + LG   | 7  | 1.76          | 0.080      |
|                  | Pop. + Sex + (Sex $\times$ Pop.) + EG   | 7  | 1.95          | 0.073      |
|                  | Null  | 3  | 14.4          | 0.000      |
| Beta-globulin    | Pop. + Sex + (Sex $\times$ Pop.)  | 6  | 0.00          | 0.163      |
|                  | Pop. + Sex + (Sex $\times$ Pop.) + LG   | 7  | 1.25          | 0.087      |
|                  | Pop. + Sex + (Sex $\times$ Pop.) + ACFmass  | 7  | 1.71          | 0.069      |
|                  | Null  | 3  | 25.46         | 0.000      |
| Gamma-globulin   | Pop.  | 4  | 0.00          | 0.105      |
|                  | Pop. + EG + (EG $\times$ Pop.)  | 6  | 1.23          | 0.057      |
|                  | Pop. + EG   | 5  | 1.61          | 0.047      |
|                  | Null  | 3  | 62.28         | 0.000      |
| PC2              | Pop. + ACFmass  | 5  | 0.00          | 0.110      |
|                  | Pop.  | 4  | 1.42          | 0.089      |
|                  | Pop. + ACFmass + Sex + (Sex $\times$ Pop.)  | 7  | 1.89          | 0.043      |
|                  | Null  | 3  | 7.62          | 0.002      |
| Neutrophil count | EG + LG + Pop. + Sex + (EG $\times$ Pop.) + (EG $\times$ Sex.)                      | 9  | 0.00          | 0.057      |
|                  | EG + LG + Pop. + (EG $\times$ Pop.)   | 7  | 0.86          | 0.037      |
|                  | EG + LG + Pop. + Sex + (EG $\times$ Pop.) + (EG $\times$ Sex.) + (LG $\times$ Pop.) | 10 | 0.89          | 0.037      |
|                  | Null  | 3  | 22.58         | 0.000      |
| Monocyte count   | LG + Sex + (LG $\times$ Sex.)   | 6  | 0.00          | 0.057      |
|                  | LG + Sex + (LG $\times$ Sex.) + ACFmass   | 7  | 0.67          | 0.040      |
|                  | Null  | 3  | 1.07          | 0.033      |
| Basophil count   | Null  | 3  | 0.00          | 0.123      |
|                  | EG  | 4  | 1.23          | 0.066      |
|                  | Pop.  | 4  | 1.51          | 0.058      |
| Eosinophil count | Sex + EG + (EG $\times$ Sex.)   | 6  | 0.00          | 0.142      |
|                  | Sex + EG + (EG $\times$ Sex.) + Pop.  | 7  | 1.97          | 0.053      |
|                  | Sex + EG + (EG $\times$ Sex.) + LG  | 7  | 2.03          | 0.052      |
|                  | Null  | 3  | 3.22          | 0.028      |
| Lymphocyte count | Pop.  | 4  | 0.00          | 0.114      |
|                  | Pop. + Sex  | 5  | 0.99          | 0.070      |
|                  | Pop. + LG   | 5  | 1.50          | 0.054      |
|                  | Null  | 3  | 20.58         | 0.000      |
| Hemagglutination | Null  | 3  | 0.00          | 0.164      |
|                  | Sex   | 4  | 1.33          | 0.084      |
|                  | LG  | 4  | 2.00          | 0.064      |
| Hemolysis        | Pop.  | 5  | 0.00          | 0.055      |
|                  | Pop. + ACFmass  | 4  | 0.03          | 0.054      |
|                  | Null  | 3  | 0.79          | 0.037      |

**Online Resource 4** Parameter estimates of the selected linear mixed-effect model of immune traits in **growing roe deer** (at 8 months of age). Potential fixed effect are *early growth* (EG), population (Pop.; Chizé (CH), Aurignac (AURI) minus Trois-Fontaines), sex (males (M) minus females), their interaction, and average cohort fawn mass (ACFmass). All models included the cohort of individuals as random effects. Estimates are presented  $\pm$  standard error (SE). Statistical significance: \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .  $R^2_m$  and  $R^2_c$  are the marginal and conditional variance of the model, respectively.

| Immune trait     | Selected model   | Estimate $\pm$ SE                  | t-value      | p   | $R^2_m$     | $R^2_c$     |
|------------------|------------------|------------------------------------|--------------|-----|-------------|-------------|
| <b>PC1</b>       | <b>Intercept</b> | <b>-0.22 <math>\pm</math> 0.38</b> | <b>-0.59</b> | -   |             |             |
|                  | <b>EG</b>        | <b>-0.11 <math>\pm</math> 0.04</b> | <b>-2.55</b> | *   | <b>0.06</b> | <b>0.38</b> |
|                  | <b>Pop. (CH)</b> | <b>0.73 <math>\pm</math> 0.17</b>  | <b>4.30</b>  | *** |             |             |
| Alpha1-globulin  | Intercept        | -0.91 $\pm$ 1.43                   | -0.636       | -   |             |             |
|                  | EG               | -0.01 $\pm$ 0.03                   | -0.385       | -   |             |             |
|                  | Pop. (CH)        | 1.11 $\pm$ 0.33                    | 3.81         | *** |             |             |
|                  | Pop. (AURI)      | -0.05 $\pm$ 0.12                   | -0.47        | -   | 0.08        | 0.31        |
|                  | EG * Pop. (CH)   | -0.11 $\pm$ 0.04                   | -2.90        | **  |             |             |
|                  | EG * Pop. (AURI) | -0.01 $\pm$ 0.05                   | -0.21        | -   |             |             |
|                  | ACFmass          | 0.27 $\pm$ 0.09                    | 3.18         | **  |             |             |
| Alpha2-globulin  | Intercept        | 6.09 $\pm$ 0.33                    | 18.51        | *** |             |             |
|                  | Pop. (CH)        | 0.03 $\pm$ 0.29                    | 0.10         | -   | 0.03        | 0.11        |
|                  | Pop. (AURI)      | -1.49 $\pm$ 0.45                   | -3.33        | *** |             |             |
| Haptoglobin      | Intercept        | 1.83 $\pm$ 0.46                    | 3.95         | *** |             |             |
|                  | ACFmass          | -0.10 $\pm$ 0.03                   | -3.60        | **  | 0.06        | 0.07        |
|                  | Pop. (CH)        | -0.42 $\pm$ 0.11                   | -3.72        | **  |             |             |
| Beta-globulin    | Intercept        | 7.78 $\pm$ 0.59                    | 13.15        | *** |             |             |
|                  | AFCmass          | -0.13 $\pm$ 0.04                   | -3.62        | *** | 0.04        | 0.26        |
| Gamma-globulin   | Intercept        | 14.02 $\pm$ 0.87                   | 16.11        | *** |             |             |
|                  | EG               | -0.36 $\pm$ 0.09                   | -3.63        | *** |             |             |
|                  | Pop. (CH)        | 4.57 $\pm$ 0.43                    | 10.56        | *** | 0.33        | 0.51        |
|                  | Pop. (AURI)      | -3.36 $\pm$ 0.66                   | -5.11        | *** |             |             |
| <b>PC2</b>       | <b>Intercept</b> | <b>0.12 <math>\pm</math> 0.37</b>  | <b>0.56</b>  | -   | <b>0.02</b> | <b>0.52</b> |
|                  | <b>Pop. (CH)</b> | <b>-0.37 <math>\pm</math> 0.12</b> | <b>-3.10</b> | **  |             |             |
| Neutrophil count | Intercept        | 5.80 $\pm$ 0.18                    | 32.02        | *** |             |             |
|                  | Pop. (CH)        | -1.57 $\pm$ 0.23                   | -6.86        | *** | 0.14        | 0.16        |
|                  | Pop (AURI)       | -0.10 $\pm$ 0.34                   | -0.30        | -   |             |             |
| Monocyte count   | Intercept        | 0.32 $\pm$ 0.12                    | 2.76         | *   |             |             |
|                  | Pop. (CH)        | 0.07 $\pm$ 0.03                    | 2.11         | *   | 0.05        | 0.58        |
|                  | Pop. (AURI)      | -0.24 $\pm$ 0.05                   | -4.73        | *** |             |             |

|                  |             |              |       |     |      |      |
|------------------|-------------|--------------|-------|-----|------|------|
| Basophil count   | Intercept   | 0.06 ± 0.02  | 3.85  | -   | 0.00 | 0.16 |
| Eosinophil count | Intercept   | 0.06 ± 0.01  | 6.79  | -   | 0.00 | 0.05 |
| Lymphocyte count | Intercept   | 2.66 ± 0.12  | 16.28 | *** | 0.07 | 0.11 |
|                  | Pop. (CH)   | -0.36 ± 0.12 | -2.98 | **  |      |      |
|                  | Pop. (AURI) | 0.30 ± 0.18  | 1.70  | -   |      |      |
|                  | Sex (M)     | -0.27 ± 0.11 | -2.43 | *   |      |      |
| Hemagglutination | Intercept   | 11.41 ± 2.46 | 4.63  | *** | 0.10 | 0.35 |
|                  | ACFmass     | -0.44 ± 0.15 | -2.97 | **  |      |      |
|                  | Pop. (CH)   | -1.28 ± 0.57 | -2.25 | *   |      |      |
|                  | Pop. (AURI) | -0.49 ± 0.21 | -2.35 | *   |      |      |
| Hemolysis        | Intercept   | 10.88 ± 1.95 | 5.57  | *** | 0.07 | 0.61 |
|                  | ACFmass     | -0.53 ± 0.12 | -4.58 | *** |      |      |
|                  | Pop. (CH)   | -2.15 ± 0.44 | -4.87 | *** |      |      |
|                  | Pop. (AURI) | 0.59 ± 0.16  | 3.73  | *** |      |      |

Parameter estimates of the selected linear mixed-effect model of immune traits in **adult roe deer**. Potential fixed effect are *early* growth (EG), *late* growth (LG), population (Pop.; Chizé (CH) minus Trois-Fontaines), sex (males (M) minus females), their interaction, and average cohort fawn mass (ACFmass). All models included the cohort of individuals as random effects. Estimates are presented ± standard error (SE). Statistical significance: \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001. R<sup>2</sup>m and R<sup>2</sup>c are the marginal and conditional variance of the model, respectively.

| Immune trait    | Selected model             | Estimate ± SE       | t-value      | p   | R <sup>2</sup> m | R <sup>2</sup> c |
|-----------------|----------------------------|---------------------|--------------|-----|------------------|------------------|
| <b>PC1</b>      | <b>Intercept</b>           | <b>-0.37 ± 0.25</b> | <b>-1.47</b> | -   | <b>0.26</b>      | <b>0.26</b>      |
|                 | <b>Pop. (CH)</b>           | <b>0.81 ± 0.34</b>  | <b>2.38</b>  | *   |                  |                  |
|                 | <b>Sex (M)</b>             | <b>-0.38 ± 0.41</b> | <b>-0.92</b> | -   |                  |                  |
|                 | <b>Pop. (CH) * Sex (M)</b> | <b>1.64 ± 0.55</b>  | <b>3.01</b>  | **  |                  |                  |
| Alpha1-globulin | Intercept                  | 3.14 ± 0.08         | 39.99        | *** | 0.04             | 0.12             |
|                 | Sex (M)                    | 0.21 ± 0.10         | 2.23         | *   |                  |                  |
| Alpha2-globulin | Intercept                  | 5.77 ± 0.10         | 2.23         | *   | 0.00             | 0.06             |
| Haptoglobin     | Intercept                  | 0.12 ± 0.12         | 1.06         | -   | 0.15             | 0.15             |
|                 | Pop. (CH)                  | 0.11 ± 0.16         | 0.71         | -   |                  |                  |
|                 | Sex (M)                    | 0.03 ± 0.19         | 0.15         | -   |                  |                  |
|                 | Pop. (CH) * Sex (M)        | 0.60 ± 0.25         | 2.36         | *   |                  |                  |

|                  |                     |                     |              |           |             |             |
|------------------|---------------------|---------------------|--------------|-----------|-------------|-------------|
| Beta-globulin    | Intercept           | 6.78 ± 0.24         | 28.17        | ***       |             |             |
|                  | Pop. (CH)           | 0.65 ± 0.32         | 2.01         | *         | 0.23        | 0.23        |
|                  | Sex (M)             | -0.15 ± 0.40        | -0.38        | -         |             |             |
|                  | Pop. (CH) * Sex (M) | 1.40 ± 0.52         | 2.69         | **        |             |             |
| Gamma-globulin   | Intercept           | 15.19 ± 0.52        | 28.96        | ***       | 0.41        | 0.41        |
|                  | Pop. (CH)           | 6.45 ± 0.70         | 9.24         | ***       |             |             |
| <b>PC2</b>       | <b>Intercept</b>    | <b>0.43 ± 0.20</b>  | <b>2.10</b>  | <b>*</b>  | <b>0.07</b> | <b>0.15</b> |
|                  | <b>Pop. (CH)</b>    | <b>-0.70 ± 0.23</b> | <b>-3.08</b> | <b>**</b> |             |             |
| Neutrophil count | Intercept           | 6.11 ± 0.21         | 29.39        | ***       |             |             |
|                  | EG                  | -0.24 ± 0.11        | -2.11        | *         |             |             |
|                  | Pop. (CH)           | -1.38 ± 0.29        | -4.81        | ***       | 0.22        | 0.22        |
|                  | LG                  | -0.22 ± 0.09        | -2.42        | *         |             |             |
|                  | EG* Pop. (CH)       | 0.52 ± 0.17         | 3.08         | ***       |             |             |
| Monocyte count   | Intercept           | 0.30 ± 0.04         | 6.83         | -         | 0.00        | 0.08        |
| Basophil count   | Intercept           | 0.08 ± 0.01         | 7.86         | -         | 0.00        | 0.06        |
| Eosinophil count | Intercept           | 0.10 ± 0.01         | 8.49         | ***       |             |             |
|                  | EG                  | 0.01 ± 0.01         | 1.14         | -         | 0.08        | 0.08        |
|                  | Sex (M)             | -0.01 ± 0.02        | -0.32        | -         |             |             |
|                  | EG * Sex (M)        | -0.03 ± 0.01        | -2.72        | **        |             |             |
| Lymphocyte count | Intercept           | 2.24 ± 0.10         | 22.24        | ***       | 0.17        | 0.18        |
|                  | Pop. (CH)           | -0.64 ± 0.13        | -4.95        | ***       |             |             |
| Hemagglutination | Intercept           | 4.15 ± 0.13         | 32.64        | -         | 0.00        | 0.06        |
| Hemolysis        | Intercept           | 2.17 ± 0.19         | 11.68        | -         | 0.00        | 0.17        |

# Glucocorticoid level predicts short-term telomere dynamics in wild roe deer

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## Abstract

Because evidence that telomere length is associated with health and mortality in humans and wild birds is accumulating, a large body of research is currently seeking to identify factors that modulate telomere dynamics. We tested the hypothesis that high levels of glucocorticoids in individuals under environmental stress should accelerate telomere loss in two wild populations of roe deer (*Capreolus capreolus*) facing different ecological contexts. Using two consecutive annual sampling sessions, we found that individuals with faster rates of telomere shortening had higher mean fecal glucocorticoid metabolites concentrations, suggesting a functional link between glucocorticoid levels and telomere attrition rate. This relationship was consistent for both sexes and populations. This finding paves the way for further studies of the fitness consequences of exposure to environmental stressors in wild vertebrates.

## Keywords

Aging - *Capreolus capreolus* - Fecal glucocorticoid metabolites - Life-history - Stress

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## 1. Introduction

Telomeres are non-coding and repetitive DNA sequences located at the extremity of eukaryotic linear chromosomes. The shortening of telomere sequences observed in somatic cells over the lifetime of most species is expected to be associated with the aetiology of age-related diseases and an increased risk of mortality (see [1,2] for evidence in humans and birds). Therefore, identifying life history traits and ecological factors modulating telomere dynamics and their consequences for lifespan has become an important challenge [1]. In vertebrates, environmental stressors (e.g. exposure to predators, food shortage) have been suggested to modulate telomere attrition rate [1,3] through stimulation of the hypothalamic-pituitary-adrenal axis, triggering the release of glucocorticoids by the adrenal gland cortex (reviewed in [3]). Chronically elevated glucocorticoids are thought to accelerate telomere shortening through diverse physiological pathways [3].

In the wild, most of our knowledge relies on studies of birds that have investigated the influence of glucocorticoids on telomere length rather than telomere attrition rate. These studies have revealed very diverse patterns, suggesting that the direction of the relationship between telomere length and plasma corticosterone is influenced by both environmental conditions and sex (reviewed in [4]). In mammals, experiments on laboratory rodents suggest that stressful environments can be responsible for shorter telomeres (e.g. [5]). However, our current understanding of the link between physiological markers of stress and telomere dynamics remains extremely limited, especially in wild mammals. In this study, we tested whether individuals with high glucocorticoid levels had shorter telomeres and a faster year-to-year telomere attrition rate in two free-ranging populations of roe deer (*Capreolus capreolus*) living in markedly different environments.

## 2. Methods

### (a) Study population and laboratory assays

We studied two populations of roe deer living in enclosed forests. Trois-Fontaines forest (TF - 1,360 ha), located in north-eastern France (48°43'N, 4°55'E), has rich soils and provides high quality habitat for roe deer. In contrast, Chizé forest (CH - 2,614 ha), located in western France (46°05'N, 0°25'W), has a low productivity due to poor soils and frequent summer droughts, providing a less suitable habitat than TF [6]. For 40 years, 10-12 days of capture have been organized each year between December and March as part of a long-term Capture-Mark-Recapture program [6]. Upon capture, individuals are sexed, weighed ( $\pm 50$ g) and a basic clinical examination is performed.

In 2016 and 2017, we collected blood samples (up to 1mL/kg) from the jugular vein. Buffy coat fractions containing leukocytes were isolated after centrifugation and immediately frozen at -80°C. Relative telomere length (hereafter RTL) was measured by quantitative PCR as previously described for these populations (see [7] and supplementary material for details on DNA extraction and RTL measurements).

Baseline glucocorticoid levels at the time of capture were assessed by measuring fecal glucocorticoid metabolites (hereafter FGMs). Feces were collected rectally and frozen immediately at -80°C until assayed (except in 2016 at TF when samples were stored at -20°C). FGMs were extracted following a methanol-based procedure and assayed using a group-specific 11-oxoetiocholanolone enzyme immunoassay as previously described and validated for roe deer [8,9]. Briefly,  $500 \pm 5$  mg of each homogenized fecal sample was vortexed for 30 min with 5 ml of 80% methanol before being centrifuged (15 min at 2500 g). FGMs were then quantified in an aliquot of the supernatant diluted 1:10 with assay buffer. Measurements were carried out in duplicate (intra- and inter-assay coefficients of variation were <10% and

<15%, respectively) and results expressed as nanograms per gram wet feces. One male from the TF population had an extremely low (and unexplained) FGM level in 2017 (8 ng/g, see figure S1) and was removed from the analyses. We assessed the average level of stress across both years by computing the mean FGM value over 2016 and 2017 measures. Individual concentrations of FGM in 2016 and 2017 were correlated ( $r = 0.42$ ,  $p = 0.02$ ,  $n = 30$ , figure S2), with a repeatability of 0.22, which is within the range generally observed for FGM [10].

#### (b) Statistical analyses

We performed a set of analyses seeking to understand whether both RTL (in 2016 and 2017) and the change in RTL between years (computed as the difference in RTL between 2016 and 2017 for a given individual) could be explained by the stress level experienced during that period. We analyzed the influence of mean FGM (log-transformed) and FGM levels measured in 2016 and 2017 (log-transformed) separately (see Table S1 for sample sizes). For each FGM variable entered as an independent covariate, we ran a set of linear models that also included a possible additive or interactive effect of sex, population and log-transformed body mass (using body mass measured in 2016, 2017 or mean body mass for models with FGM in 2016, FGM in 2017 and mean FGM, respectively) on RTL or year-to-year change in RTL. Finally, we also included age as a covariate to control for possible age-effects on telomere length [7] (see Tables S2 to S6 for the full set of models). We repeated the analyses without individuals sampled in their first year of life (i.e. at eight months, Tables S3 and S5) because telomere attrition may be much faster in early life compared to adulthood in wild mammals [11]. All results were qualitatively unchanged (Table S3 and S5). We repeated the analyses including cohort (2003 to 2015) as a random effect (Table S6) to control for non-independence among animals born in the same year using linear mixed effect models (using the R package lme4). In

all cases, results were qualitatively unchanged (Table S6). Our results were also not impacted by removing animals captured at 8 months old (Table S3), or by inter-individual differences in the number of days between the 2016 and 2017 capture sessions (mean number of days  $\pm$  SE:  $368.8 \pm 2.26$ ; Table S7). Model selection was based on the Akaike Information Criterion (AIC) and we retained the model with the lowest AIC except when the difference in AIC ( $\Delta$ AIC) between two competing models was less than 2, in which case we retained the simplest model [12].

### 3. Results

Within years, we detected no association between FGMs and RTL (Table S2,  $\beta \pm \text{SE} = -0.04 \pm 0.07$ ,  $n = 38$ ;  $\beta = -0.11 \pm 0.08$  in 2017,  $n = 32$ , for 2016 and 2017, respectively; figures S3 and S4). Between 2016 and 2017, 51.2% (22 out of 43 individuals) showed a decrease in telomere length (figure S5). Our model selection procedure revealed that high average FGM levels across 2016 and 2017 were associated with a stronger decrease in RTL between these two consecutive years (Table S4,  $\beta = -0.23 \pm 0.11$ ,  $n = 30$ , figure 1a). Likewise, FGM levels of roe deer captured in 2017 were also negatively associated with the change in RTL ( $\beta = -0.20 \pm 0.09$ ,  $n = 32$ , figure 1b). Population, sex and body mass had no detectable effect on telomere dynamics. The level of FGMs measured in 2016 had no effect on the year-to-year change in RTL (the constant model was selected, Table S4 and Table S5, figure 1c).

### 4. Discussion

In this study, we did not observe any within-year association between relative telomere length and FGM level. However, we found that high FGM levels in two consecutive years which presumably reflect repeated exposure to stressful conditions predicted the rate of telomere attrition in both roe deer populations.

The relationship between glucocorticoid level and telomere dynamics was not sex-specific, which contrasts with a previous study performed in common terns (*Sterna hirundo*) [13]. In this species, the relationship between baseline corticosterone levels and telomere length (measured in two consecutive years) was found in males only [13]. In terns, males allocate three times more effort to chick feeding than females [14], which may explain these sex-specific responses of telomere attrition. In roe deer, males grow antlers each year and actively defend territories, while female reproductive effort includes both gestation and lactation [15]. A broadly similar global reproductive effort in males and females of this weakly dimorphic species might explain the absence of sex differences we report. Moreover, neither glucocorticoid levels nor the slope of the relationship between telomere dynamics and glucocorticoid levels differed between populations. This contrasts with a previous comparison of three colonies of thick-billed murre (*Uria lomvia*) [16], but could be explained by adaptive down-regulation of the stress response in Chizé, the roe deer population facing harsh environmental conditions (see [17] for similar observations in black-tailed deer, *Odocoileus hemionus sitkensis*). However, we cannot rule out that the absence of sex and population effects in our analyses might be due to low statistical power.

Overall, our findings suggest that the level of stress influence telomere dynamics, even over a short time scale. These among-individual differences in telomere attrition rate could generate substantial individual differences in telomere length in late adulthood and might ultimately be associated with different mortality risk [1].

**Ethics.** The protocol of capture and blood sampling of roe deer under the authority of the Office National de la Chasse et de la Faune Sauvage (ONCFS) was approved by the Director of Food, Agriculture and Forest (Prefectoral order 2009–14 from Paris). The land manager of both sites, the Office National des Forêts (ONF), permitted the study of the populations (Partnership Convention ONCFS-ONF dated 2005-12-23). All experiments were performed in accordance with guidelines and regulations of the Ethical Committee of Lyon 1 University (project DR2014-09, June 5, 2014).

**Data accessibility.** Data available will be downloaded on dryad once the manuscript accepted.

**Authors' contributions.** JFL & EGF conceived and designed the study. JFL, BR, LC, JMG, AJM, HV, FD, JD, SP, MP, EGF performed fieldwork. BR, CR and LC extracted DNA. RP, JC, BR ran FGM assays. HF, RW, SLU and DN ran telomere assays. JFL performed the statistical analysis, wrote the first draft of the paper and then received input from all other co-authors.

**Competing interests.** The authors declare no competing interests.

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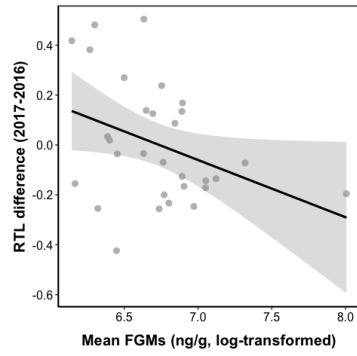
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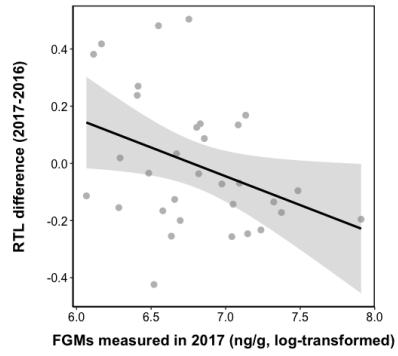
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**Figure 1:** Relationship between the individual-level difference in RTL between 2017 and 2016 and the individual mean FGM level across both years (a), the FGM level measured in 2017 (b) or 2016 (c).

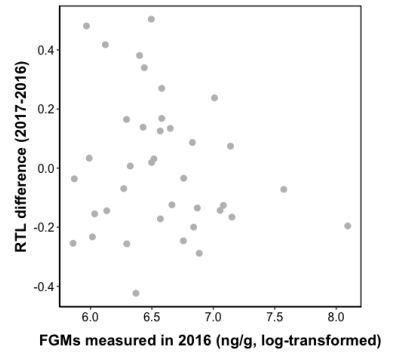
(a)



(b)



(c)



## SUPPLEMENTARY MATERIAL

# Glucocorticoid level predicts short-term telomere dynamics in wild roe deer

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## 1/ Supplementary tables

**Table S1: Sex- and population-specific sample size used in the analyses. (a) Number of individuals with data on relative telomere length (RTL) in 2016 and 2017, (b) Number of individuals with data on both faecal glucocorticoid metabolites (FGMs) in 2016 and RTL in both 2016 and 2017, (c) Number of individuals with data on both FGMs in 2017 and RTL in both 2016 and 2017, (d) Number of individuals with data on both FGMs in 2016 and 2017 and RTL in both 2016 and 2017. The age range of individuals from a given subset is in brackets.**

| (a) RTL (2016 and 2017) |               |                |                | (b) FGMs (2016)  |               |                |                |
|-------------------------|---------------|----------------|----------------|------------------|---------------|----------------|----------------|
|                         | Males         | Females        | Both sexes     |                  | Males         | Females        | Both sexes     |
| Trois-Fontaines         | 12 (age: 1-9) | 13 (age: 1-13) | 25 (age: 1-13) | Trois-Fontaines  | 11 (age: 1-9) | 9 (age: 1-11)  | 20 (age: 1-11) |
| Chizé                   | 6 (age: 1-7)  | 12 (age: 2-13) | 18 (age: 1-13) | Chizé            | 6 (age: 1-7)  | 12 (age: 2-13) | 18 (age: 1-13) |
| Both populations        | 18 (age: 1-9) | 25 (age: 1-13) | 43 (age: 1-13) | Both populations | 17 (age: 1-9) | 21 (age: 1-13) | 38 (age: 1-13) |

| (c) FGMs (2017)  |               |                |                | (d) FGMs (2016 and 2017) |               |                |                |
|------------------|---------------|----------------|----------------|--------------------------|---------------|----------------|----------------|
|                  | Males         | Females        | Both sexes     |                          | Males         | Females        | Both sexes     |
| Trois-Fontaines  | 8 (age: 2-8)  | 6 (age: 2-14)  | 14 (age: 2-14) | Trois-Fontaines          | 8 (age: 2-8)  | 4 (age: 2-9)   | 12 (age: 2-9)  |
| Chizé            | 6 (age: 2-8)  | 12 (age: 3-14) | 18 (age: 2-14) | Chizé                    | 6 (age: 2-8)  | 12 (age: 2-14) | 18 (age: 2-14) |
| Both populations | 14 (age: 2-8) | 18 (age: 2-14) | 32 (age: 2-14) | Both populations         | 14 (age: 2-8) | 16 (age: 2-14) | 30 (age: 2-14) |

**Table S2: AIC summary of the different linear models testing the relationship between relative telomere length measured in 2016 and the concentration of faecal glucocorticoid metabolites (FGMs) measured in 2016 (a) and between relative telomere length measured in 2017 and the concentration of faecal glucocorticoid metabolites (FGMs) measured in 2017 (b). The selected model is displayed with dark grey shading, k is the number of parameters in the model,  $\Delta$ AIC is the difference in AIC between the candidate model and the selected model. The AIC weight (AICw) was calculated to measure the relative likelihood of each model to be the best among the set of fitted models. To avoid fitting over-parameterized models, we did not consider any three-way interactions. We only considered a possible interaction between FGMs and Population to test whether the stress effect on RTL dynamic differed between high quality (i.e. Trois-Fontaines) and low quality (i.e. Chizé) populations and between FGMs and Sex to test whether the stress effect on RTL dynamic were different between males and females.**

|                        | (a) RTL (2016) vs FGMs (2016) - n=38 |        |              |      | (b) RTL (2017) vs FGMs (2017) - n=32 |              |      |
|------------------------|--------------------------------------|--------|--------------|------|--------------------------------------|--------------|------|
|                        | k                                    | AIC    | $\Delta$ AIC | AICw | AIC                                  | $\Delta$ AIC | AICw |
| Constant               | 2                                    | -14.57 | 0.00         | 0.18 | -11.51                               | 0.00         | 0.12 |
| FGM                    | 3                                    | -12.95 | 1.62         | 0.08 | -11.44                               | 0.06         | 0.11 |
| Population             | 3                                    | -14.24 | 0.32         | 0.15 | -10.67                               | 0.84         | 0.08 |
| Sex                    | 3                                    | -12.57 | 2.00         | 0.07 | -10.94                               | 0.57         | 0.09 |
| Mass                   | 3                                    | -12.59 | 1.98         | 0.07 | -10.19                               | 1.31         | 0.06 |
| Age                    | 3                                    | -13.04 | 1.53         | 0.08 | -9.52                                | 1.98         | 0.04 |
| FGM + Population       | 4                                    | -12.36 | 2.21         | 0.06 | -10.49                               | 1.02         | 0.07 |
| FGM * Population       | 5                                    | -10.63 | 3.94         | 0.02 | -8.49                                | 3.02         | 0.03 |
| FGM + Sex              | 4                                    | -10.96 | 3.61         | 0.03 | -10.31                               | 1.20         | 0.06 |
| FGM * Sex              | 5                                    | -9.20  | 5.37         | 0.01 | -8.37                                | 3.14         | 0.02 |
| FGM + Mass             | 4                                    | -11.04 | 3.53         | 0.03 | -10.50                               | 1.01         | 0.07 |
| FGM + Age              | 4                                    | -11.29 | 3.28         | 0.03 | -9.58                                | 1.92         | 0.04 |
| Population + Age       | 4                                    | -12.38 | 2.19         | 0.06 | -8.92                                | 2.58         | 0.03 |
| Sex + Age              | 4                                    | -11.08 | 3.49         | 0.03 | -9.33                                | 2.18         | 0.04 |
| Mass + Age             | 4                                    | -11.05 | 3.52         | 0.03 | -8.21                                | 3.29         | 0.02 |
| FGM + Population + Age | 5                                    | -10.47 | 4.10         | 0.02 | -9.03                                | 2.47         | 0.03 |
| FGM * Population + Age | 6                                    | -8.70  | 5.87         | 0.01 | -7.08                                | 4.42         | 0.01 |
| FGM + Sex + Age        | 5                                    | -9.37  | 5.20         | 0.01 | -8.87                                | 2.63         | 0.03 |
| FGM * Sex + Age        | 6                                    | -7.59  | 6.98         | 0.01 | -6.89                                | 4.61         | 0.01 |
| FGM + Mass + Age       | 5                                    | -9.30  | 5.27         | 0.01 | -8.66                                | 2.84         | 0.03 |

24 **Table S3: AIC summary of the different linear models testing the relationship between relative telomere length measured in 2016 and the concentration**  
25 **of faecal glucocorticoid metabolites (FGMs) measured in 2016 (a) and between relative telomere length measured in 2017 and the concentration of faecal**  
26 **glucocorticoid metabolites (FGMs) measured in 2017 (b). Compared to Table S2, individuals captured at 8 months of age in 2016 are removed from this**  
27 **analysis.**  
28

|                        | (a) RTL (2016) vs FGMs (2016) - n=25 |       |      |      | (b) RTL (2017) vs FGMs (2017) - n=26 |       |      |
|------------------------|--------------------------------------|-------|------|------|--------------------------------------|-------|------|
|                        | k                                    | AIC   | ΔAIC | AICw | AIC                                  | ΔAIC  | AICw |
| Constant               | 2                                    | -7.41 | 0.00 | 0.17 | -12.88                               | 1.19  | 0.06 |
| FGM                    | 3                                    | -5.53 | 1.89 | 0.07 | -11.70                               | 2.37  | 0.03 |
| Population             | 3                                    | -6.43 | 0.98 | 0.10 | -11.99                               | 2.08  | 0.04 |
| Sex                    | 3                                    | -5.99 | 1.42 | 0.08 | -10.88                               | 3.19  | 0.02 |
| Mass                   | 3                                    | -6.52 | 0.89 | 0.11 | -12.35                               | 1.72  | 0.05 |
| Age                    | 3                                    | -5.60 | 1.82 | 0.07 | -14.04                               | 0.03  | 0.11 |
| FGM + Population       | 4                                    | -4.88 | 2.54 | 0.05 | -11.30                               | 2.77  | 0.03 |
| FGM * Population       | 5                                    | -3.49 | 3.92 | 0.02 | -9.37                                | 4.70  | 0.01 |
| FGM + Sex              | 4                                    | -4.07 | 3.35 | 0.03 | -9.71                                | 4.36  | 0.01 |
| FGM * Sex              | 5                                    | -2.59 | 4.82 | 0.02 | -8.47                                | 5.60  | 0.01 |
| FGM + Mass             | 4                                    | -5.38 | 2.03 | 0.06 | -11.46                               | 2.61  | 0.03 |
| FGM + Age              | 4                                    | -3.68 | 3.73 | 0.03 | -12.70                               | 1.37  | 0.06 |
| Population + Age       | 4                                    | -4.59 | 2.82 | 0.04 | -14.07                               | 0.00  | 0.11 |
| Sex + Age              | 4                                    | -4.06 | 3.35 | 0.03 | -12.63                               | 1.44  | 0.05 |
| Mass + Age             | 4                                    | -4.83 | 2.58 | 0.05 | -14.62                               | -0.55 | 0.15 |
| FGM + Population + Age | 5                                    | -2.97 | 4.44 | 0.02 | -13.30                               | 0.77  | 0.08 |
| FGM * Population + Age | 6                                    | -1.59 | 5.83 | 0.01 | -12.04                               | 2.03  | 0.04 |
| FGM + Sex + Age        | 5                                    | -2.12 | 5.29 | 0.01 | -11.05                               | 3.02  | 0.02 |
| FGM * Sex + Age        | 6                                    | -0.65 | 6.76 | 0.01 | -9.15                                | 4.92  | 0.01 |
| FGM + Mass + Age       | 5                                    | -3.63 | 3.78 | 0.03 | -13.59                               | 0.48  | 0.09 |

29  
30

31 **Table S4: AIC summary of the different linear models testing the relationship between changes in relative telomere length (2017-2016) and the**  
32 **concentration of faecal glucocorticoid metabolites (FGMs) measured in 2016 (a), 2017 (b) or across both years (c). The selected model is displayed with**  
33 **dark grey shading, k is the number of parameters in the model,  $\Delta$ AIC is the difference in AIC between the candidate model and the selected model. The**  
34 **AIC weight (AICw) was calculated to measure the relative likelihood of each model to be the best among the set of fitted models. To avoid fitting over-**  
35 **parameterized models, we did not consider any three-way interactions. We only considered a possible interaction between FGMs and Population to test**  
36 **whether the stress effect on RTL dynamic differed between high quality (i.e. Trois-Fontaines) and low-quality (i.e. Chizé) populations and between FGMs**  
37 **and Sex to test whether the stress effect on RTL dynamic were different between males and females.**

|                        |   | (a) FGMs (2016) - n=38 |              |      | (b) FGMs (2017) - n=32 |              |      | (c) Mean FGMs (2016-2017) - n =30 |              |      |
|------------------------|---|------------------------|--------------|------|------------------------|--------------|------|-----------------------------------|--------------|------|
|                        | k | AIC                    | $\Delta$ AIC | AICw | AIC                    | $\Delta$ AIC | AICw | AIC                               | $\Delta$ AIC | AICw |
| Constant               | 2 | -1.11                  | 0.00         | 0.12 | 0.86                   | 2.64         | 0.05 | 2.60                              | 2.26         | 0.05 |
| FGM                    | 3 | -0.38                  | 0.73         | 0.08 | -1.78                  | 0.00         | 0.19 | 0.34                              | 0.00         | 0.16 |
| Population             | 3 | 0.89                   | 2.00         | 0.04 | 2.81                   | 4.59         | 0.02 | 4.60                              | 4.26         | 0.02 |
| Sex                    | 3 | -0.39                  | 0.72         | 0.08 | 1.90                   | 3.68         | 0.03 | 3.91                              | 3.57         | 0.03 |
| Mass                   | 3 | 0.09                   | 1.20         | 0.06 | 2.49                   | 4.27         | 0.02 | 4.05                              | 3.70         | 0.03 |
| Age                    | 4 | 1.55                   | 2.66         | 0.03 | 0.07                   | 1.86         | 0.07 | 2.11                              | 1.77         | 0.07 |
| FGM + Population       | 5 | 3.10                   | 4.21         | 0.01 | 2.06                   | 3.84         | 0.03 | 4.11                              | 3.77         | 0.02 |
| FGM * Population       | 4 | 0.72                   | 1.83         | 0.05 | -0.09                  | 1.69         | 0.08 | 2.28                              | 1.94         | 0.06 |
| FGM + Sex              | 5 | 1.75                   | 2.86         | 0.03 | 1.70                   | 3.49         | 0.03 | 3.25                              | 2.91         | 0.04 |
| FGM * Sex              | 4 | 0.31                   | 1.42         | 0.06 | 0.11                   | 1.89         | 0.07 | 1.60                              | 1.26         | 0.09 |
| FGM + Mass             | 5 | 1.98                   | 3.09         | 0.02 | 1.71                   | 3.49         | 0.03 | 3.39                              | 3.05         | 0.04 |
| FGM + Age              | 4 | 0.30                   | 1.41         | 0.06 | -0.87                  | 0.91         | 0.12 | 0.83                              | 0.49         | 0.13 |
| Population + Age       | 4 | 2.03                   | 3.14         | 0.02 | 4.48                   | 6.27         | 0.01 | 5.95                              | 5.61         | 0.01 |
| Sex + Age              | 4 | -0.24                  | 0.87         | 0.07 | 2.77                   | 4.55         | 0.02 | 4.53                              | 4.18         | 0.02 |
| Mass + Age             | 4 | 0.74                   | 1.85         | 0.05 | 4.48                   | 6.26         | 0.01 | 5.73                              | 5.39         | 0.01 |
| FGM + Population + Age | 5 | 2.30                   | 3.41         | 0.02 | 1.13                   | 2.91         | 0.04 | 2.83                              | 2.49         | 0.05 |
| FGM * Population + Age | 6 | 3.61                   | 4.72         | 0.01 | 3.07                   | 4.85         | 0.02 | 4.82                              | 4.48         | 0.02 |
| FGM + Sex + Age        | 5 | 0.38                   | 1.49         | 0.06 | 0.14                   | 1.93         | 0.07 | 2.28                              | 1.94         | 0.06 |
| FGM * Sex + Age        | 6 | 1.43                   | 2.54         | 0.03 | 1.70                   | 3.48         | 0.03 | 3.93                              | 3.59         | 0.03 |
| FGM + Mass + Age       | 5 | -0.74                  | 0.37         | 0.10 | 1.00                   | 2.78         | 0.05 | 1.80                              | 1.45         | 0.08 |

38

39

40 **Table S5: AIC summary of the different linear models testing the relationship between changes in relative telomere length (2017-2016) and the**  
41 **concentration of fecal glucocorticoid metabolites (FGMs) measured in 2016 (a), 2017 (b) or across both years (c). Compared to table S4, individuals captured**  
42 **at 8 months of age in 2016 are removed from this analysis.**  
43

|                        | k | (a) FGMs (2016) - n=25 |      |      | (b) FGMs (2017) - n=20 |      |      | (c) Mean FGMs (2016-2017) - n=19 |      |      |
|------------------------|---|------------------------|------|------|------------------------|------|------|----------------------------------|------|------|
|                        |   | AIC                    | ΔAIC | AICw | AIC                    | ΔAIC | AICw | AIC                              | ΔAIC | AICw |
| Constant               | 2 | 5.22                   | 1.94 | 0.06 | 5.84                   | 2.81 | 0.04 | 6.55                             | 2.51 | 0.05 |
| FGM                    | 3 | 4.72                   | 1.44 | 0.08 | 3.03                   | 0.00 | 0.18 | 4.05                             | 0.00 | 0.16 |
| Population             | 3 | 7.09                   | 3.81 | 0.02 | 7.68                   | 4.65 | 0.02 | 8.18                             | 4.14 | 0.02 |
| Sex                    | 3 | 5.15                   | 1.87 | 0.06 | 6.24                   | 3.20 | 0.04 | 7.15                             | 3.11 | 0.03 |
| Mass                   | 3 | 7.09                   | 3.81 | 0.02 | 7.50                   | 4.47 | 0.02 | 8.29                             | 4.25 | 0.02 |
| Age                    | 3 | 5.83                   | 2.55 | 0.05 | 7.56                   | 4.53 | 0.02 | 7.87                             | 3.82 | 0.02 |
| FGM + Population       | 4 | 6.71                   | 3.43 | 0.03 | 4.62                   | 1.59 | 0.08 | 5.99                             | 1.94 | 0.06 |
| FGM * Population       | 5 | 8.51                   | 5.23 | 0.01 | 6.16                   | 3.13 | 0.04 | 7.65                             | 3.60 | 0.03 |
| FGM + Sex              | 4 | 4.88                   | 1.60 | 0.07 | 3.73                   | 0.70 | 0.13 | 4.74                             | 0.70 | 0.11 |
| FGM * Sex              | 5 | 6.85                   | 3.57 | 0.03 | 5.72                   | 2.69 | 0.05 | 6.42                             | 2.37 | 0.05 |
| FGM + Mass             | 4 | 6.56                   | 3.28 | 0.03 | 4.55                   | 1.51 | 0.08 | 6.00                             | 1.96 | 0.06 |
| FGM + Age              | 4 | 4.58                   | 1.30 | 0.09 | 4.87                   | 1.84 | 0.07 | 5.31                             | 1.27 | 0.09 |
| Population + Age       | 4 | 7.73                   | 4.45 | 0.02 | 9.45                   | 6.42 | 0.01 | 9.44                             | 5.39 | 0.01 |
| Sex + Age              | 4 | 4.49                   | 1.21 | 0.09 | 6.91                   | 3.88 | 0.03 | 7.21                             | 3.16 | 0.03 |
| Mass + Age             | 4 | 7.57                   | 4.29 | 0.02 | 9.18                   | 6.15 | 0.01 | 9.30                             | 5.25 | 0.01 |
| FGM + Population + Age | 5 | 6.54                   | 3.26 | 0.03 | 6.52                   | 3.49 | 0.03 | 7.22                             | 3.18 | 0.03 |
| FGM * Population + Age | 6 | 8.30                   | 5.02 | 0.01 | 7.59                   | 4.56 | 0.02 | 8.59                             | 4.55 | 0.02 |
| FGM + Sex + Age        | 5 | 3.28                   | 0.00 | 0.17 | 4.79                   | 1.76 | 0.08 | 4.73                             | 0.69 | 0.11 |
| FGM * Sex + Age        | 6 | 5.24                   | 1.96 | 0.06 | 6.75                   | 3.72 | 0.03 | 6.72                             | 2.68 | 0.04 |
| FGM + Mass + Age       | 5 | 6.49                   | 3.20 | 0.03 | 6.34                   | 3.31 | 0.03 | 7.31                             | 3.27 | 0.03 |

44



45 **Table S6: AIC summary of the different linear mixed effect models testing the relationship between changes in relative telomere length (2017-**  
46 **2016) and the concentration of fecal glucocorticoid metabolites (FGMs) measured in 2016 (a), 2017 (b) or across both years (c). Compared to**  
47 **Table S4, the cohort is fitted as a random effect.**  
48

|                        | (a) FGMs (2016) - <i>n</i> =38 |      |      |      | (b) FGMs (2017) - <i>n</i> =32 |      |      | (c) Mean FGMs (2016-2017) - <i>n</i> =30 |      |      |
|------------------------|--------------------------------|------|------|------|--------------------------------|------|------|--|------|------|
|                        | k                              | AIC  | ΔAIC | AICw | AIC                            | ΔAIC | AICw | AIC                                      | ΔAIC | AICw |
| Constant               | 3                              | 0.89 | 0.00 | 0.11 | 2.86                           | 2.64 | 0.05 | 4.60                                     | 2.26 | 0.05 |
| FGM                    | 4                              | 1.62 | 0.73 | 0.08 | 0.22                           | 0.00 | 0.19 | 2.34                                     | 0.00 | 0.16 |
| Population             | 4                              | 2.89 | 2.00 | 0.04 | 4.81                           | 4.59 | 0.02 | 6.60                                     | 4.26 | 0.02 |
| Sex                    | 4                              | 1.61 | 0.72 | 0.08 | 3.90                           | 3.68 | 0.03 | 5.91                                     | 3.57 | 0.03 |
| Mass                   | 4                              | 2.22 | 1.33 | 0.06 | 4.85                           | 4.63 | 0.02 | 6.35                                     | 4.01 | 0.02 |
| Age                    | 4                              | 2.09 | 1.20 | 0.06 | 4.49                           | 4.27 | 0.02 | 6.05                                     | 3.70 | 0.03 |
| FGM + Population       | 5                              | 3.55 | 2.66 | 0.03 | 2.07                           | 1.86 | 0.08 | 4.11                                     | 1.77 | 0.07 |
| FGM * Population       | 6                              | 5.10 | 4.21 | 0.01 | 4.06                           | 3.84 | 0.03 | 6.11                                     | 3.77 | 0.02 |
| FGM + Sex              | 5                              | 2.72 | 1.83 | 0.04 | 1.91                           | 1.69 | 0.08 | 4.28                                     | 1.94 | 0.06 |
| FGM * Sex              | 6                              | 3.75 | 2.86 | 0.03 | 3.70                           | 3.49 | 0.03 | 5.25                                     | 2.91 | 0.04 |
| FGM + Mass             | 5                              | 2.31 | 1.42 | 0.05 | 2.11                           | 1.89 | 0.07 | 3.60                                     | 1.26 | 0.09 |
| FGM + Age              | 5                              | 2.30 | 1.41 | 0.06 | 1.13                           | 0.91 | 0.12 | 2.83                                     | 0.49 | 0.13 |
| Population + Age       | 5                              | 4.03 | 3.14 | 0.02 | 6.48                           | 6.27 | 0.01 | 7.95                                     | 5.61 | 0.01 |
| Sex + Age              | 5                              | 1.76 | 0.87 | 0.07 | 4.77                           | 4.55 | 0.02 | 6.53                                     | 4.18 | 0.02 |
| Mass + Age             | 5                              | 2.74 | 1.85 | 0.04 | 6.48                           | 6.26 | 0.01 | 6.93                                     | 4.58 | 0.02 |
| FGM + Population + Age | 6                              | 4.30 | 3.41 | 0.02 | 3.13                           | 2.91 | 0.04 | 4.83                                     | 2.49 | 0.05 |
| FGM * Population + Age | 7                              | 5.61 | 4.72 | 0.01 | 5.07                           | 4.85 | 0.02 | 6.82                                     | 4.48 | 0.02 |
| FGM + Sex + Age        | 6                              | 2.38 | 1.49 | 0.05 | 2.14                           | 1.93 | 0.07 | 4.28                                     | 1.94 | 0.06 |
| FGM * Sex + Age        | 7                              | 3.43 | 2.54 | 0.03 | 3.70                           | 3.48 | 0.03 | 5.93                                     | 3.59 | 0.03 |
| FGM + Mass + Age       | 6                              | 1.26 | 0.37 | 0.09 | 3.00                           | 2.78 | 0.05 | 3.80                                     | 1.45 | 0.08 |

49

**Table S7: Parameter estimates of the best model describing the change in RTL as a function of the mean concentration in fecal glucocorticoid metabolites (FGMs),  $n=32$  (A) or concentration in fecal glucocorticoid metabolites (FGMs) measured in 2017,  $n=30$  (B) with the number of days between the two capture events entered as a covariate.**

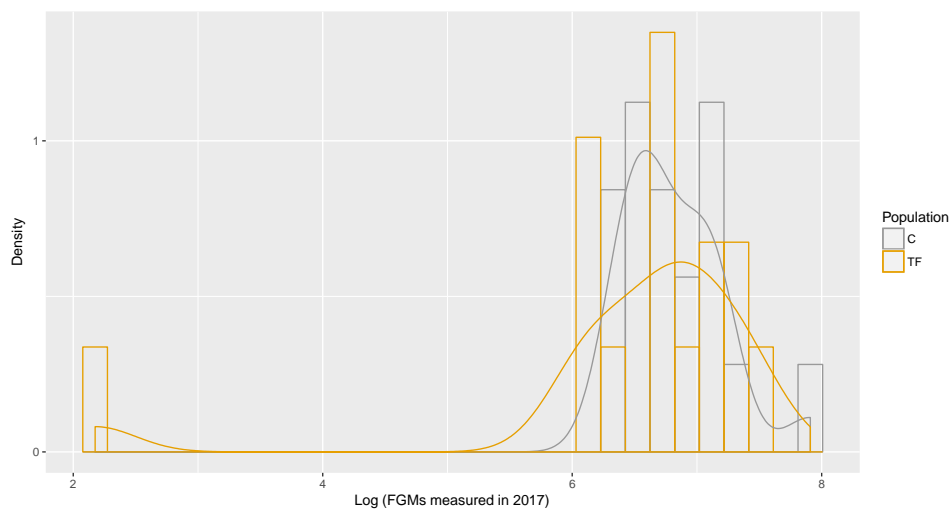
| <b>(A)</b>                      | Estimate | SE   | t     | <i>p</i> |
|---------------------------------|----------|------|-------|----------|
| Intercept                       | -0.64    | 1.21 | -0.53 | 0.60     |
| FGMs (2017)                     | -0.24    | 0.09 | -2.61 | 0.01     |
| Number of days between captures | 0.01     | 0.00 | 1.92  | 0.07     |

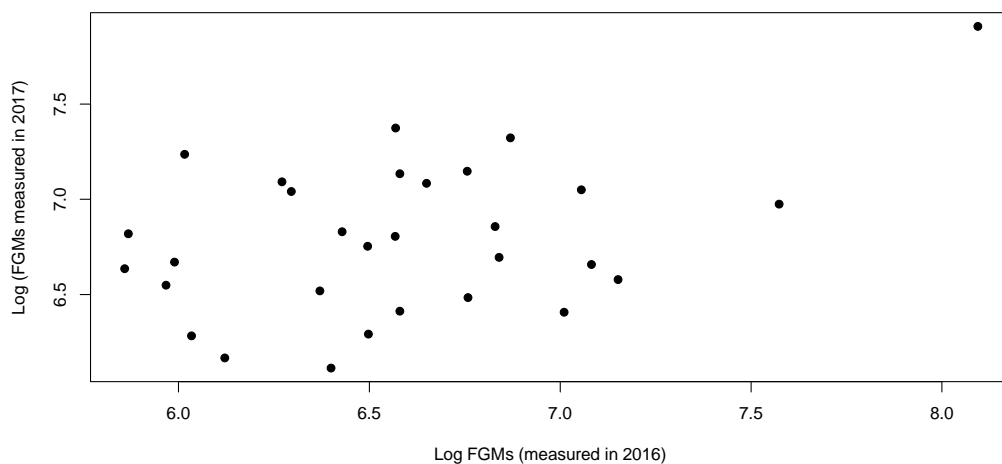
| <b>(B)</b>                      | Estimate | SE   | t     | <i>p</i> |
|---------------------------------|----------|------|-------|----------|
| Intercept                       | -0.12    | 1.44 | -0.08 | 0.94     |
| Mean FGMs                       | -0.24    | 0.11 | -2.15 | 0.04     |
| Number of days between captures | 0.00     | 0.00 | 1.35  | 0.19     |

## 2/ Supplementary figures

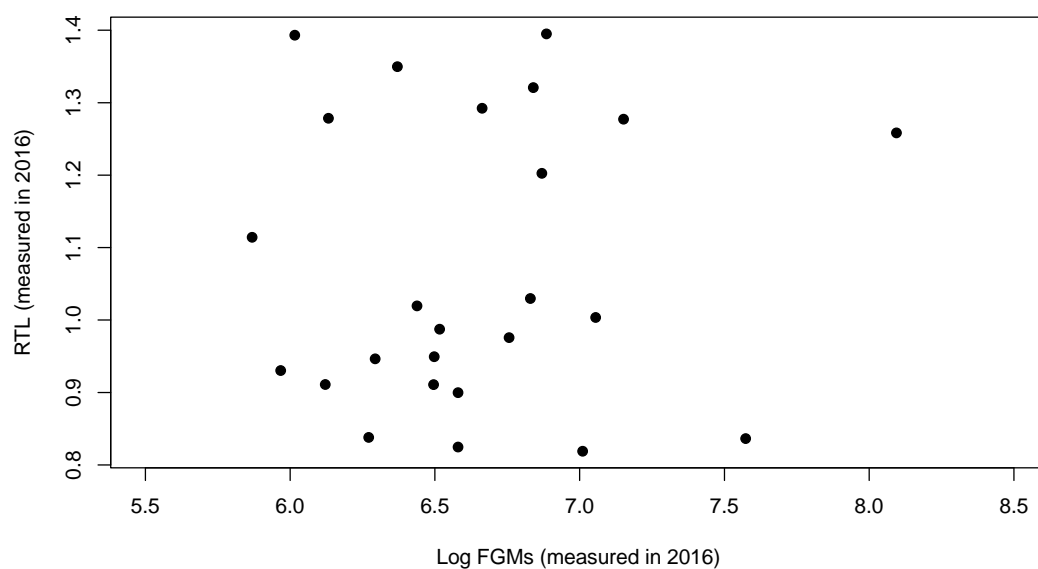
**Figure S1:** Bar plot and density function of fecal glucocorticoid metabolite (FGM) level (ng/g, log-transformed) measured in 2017. One individual had a particularly and unexplained low level of FGM and was thus removed from our analyses (see methods).



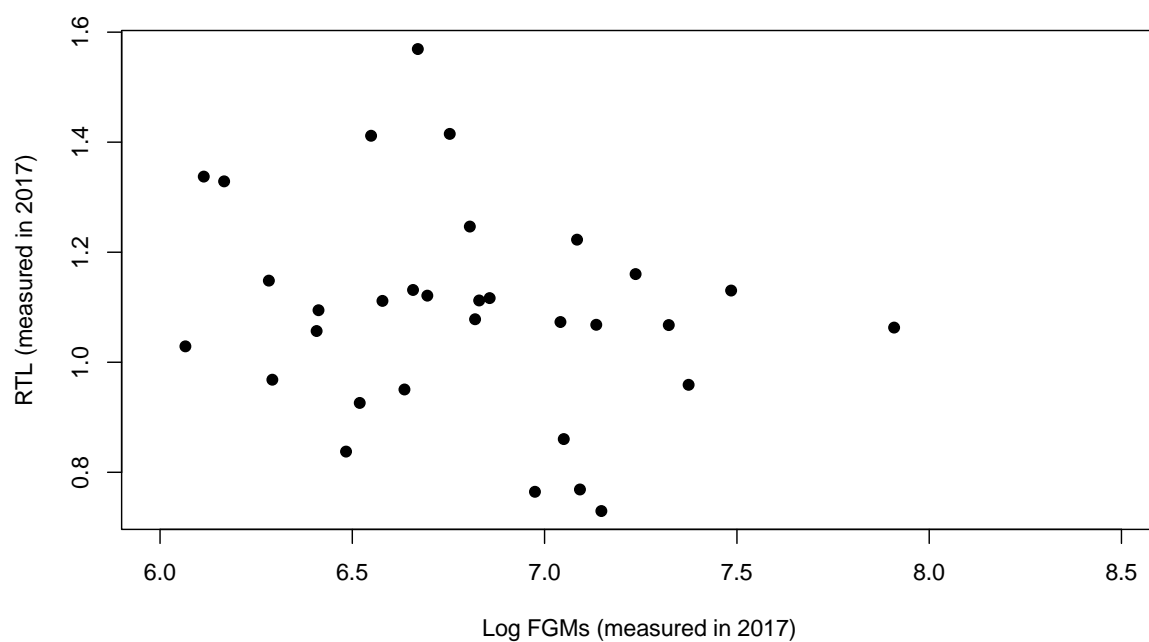
**Figure S2:** Relationship between the concentration of fecal glucocorticoid metabolites (FGMs) measured in 2017 (ng/g, log-transformed) and the FGM concentration measured in 2016 (ng/g, log-transformed). Individual FGM concentrations in 2016 and 2017 were correlated ( $r = 0.42$ ,  $p = 0.02$ ,  $n = 30$ ) with a repeatability of 0.22. This relationship was highly influenced by a male in Chizé which had the highest level of FGMs in the dataset in both 2016 and 2017 (without this individual:  $r = 0.18$ ,  $p = 0.34$ ,  $n = 29$ , repeatability of 0.32).



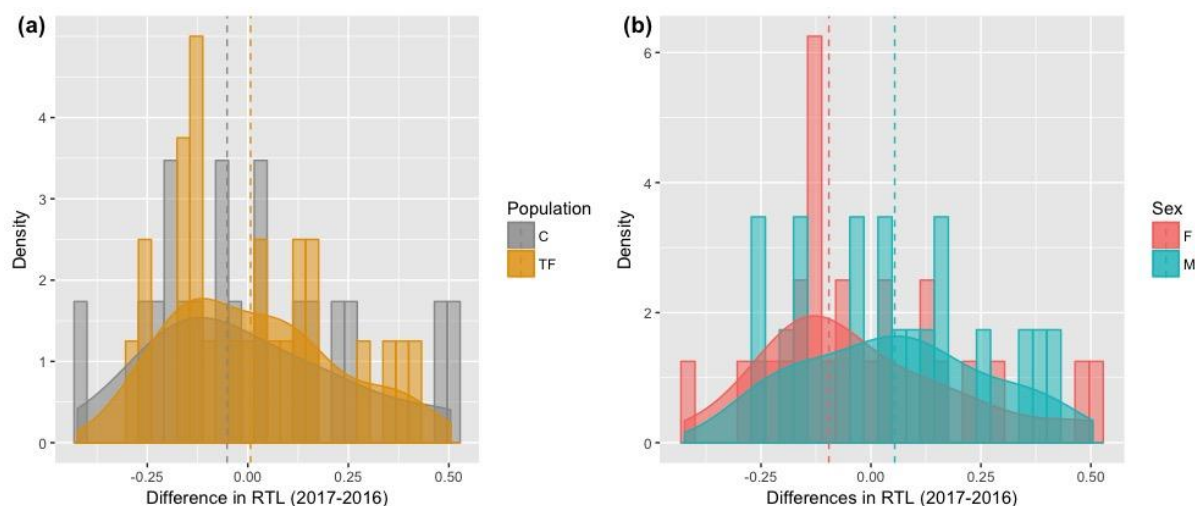
**Figure S3:** Relationship between RTL (measured in 2016) and the concentration in fecal glucocorticoid metabolites (FGMs) measured in 2016 (ng/g, log-transformed).



**Figure S4:** Relationship between RTL (measured in 2017) and the concentration in fecal glucocorticoid metabolites (FGMs) measured in 2017 (ng/g, log-transformed).



**Figure S5:** Bar plot and density function of the differences in qRTL. (a) Data split according to the population (C=Chizé; TF=Trois-Fontaines) – (b) Data split according to sex (F=Female; M=Male). Dash lines correspond to the median value for each group.



### 3/ Supplementary methods for telomere assays

During the 2016 and 2017 field seasons, blood samples were collected from the jugular vein of known-age individuals. Within 30 min of sampling, whole blood was spun at 3000 g for 10 min and the plasma layer drawn off and replaced by the same quantity of 0.9% w/v NaCl solution and spun again. The intermediate buffy coat layer, comprising mainly leukocytes was collected into a 1.5-mL Eppendorf tube and immediately frozen at  $-80^{\circ}\text{C}$  in a portable freezer (Telstar SF 8025) until further use. The full protocol for DNA extraction and RTL measurement is provided below.

#### 3.1 DNA extraction protocol

Genomic DNA was extracted from white blood cells using Macherey-Nagel NucleoSpin® Blood QuickPure kit (Catalogue number 740569) at the CNRS, Lyon (France). All downstream

processing of samples (e.g. quality control (QC), telomere measurement) was then carried out at the University of Edinburgh, Scotland (UK). DNA yield and purity was quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington DE, USA) and DNA integrity was assessed by running 200 ng total DNA on a 0.5% agarose gel and DNA bands scored on a scale of 1-5 by visual examination. Samples passed QC with a DNA yield of  $\geq 20$  ng/ $\mu$ l, an acceptable purity absorption range of 1.7 - 2.0 for the 260/280 nm ratio and  $> 1.8$  for the 260/230 nm ratio, and a DNA integrity score of either 1 or 2 [1].

### *1. Lyse blood samples*

Pipette 25 $\mu$ L Proteinase K and up to 150 $\mu$ L blood (leucocytes) + 50 $\mu$ L PBS into 1.5mL microcentrifuge tubes. Incubate at room temperature for 1 min. Add 200 $\mu$ L Lysis Buffer BQ1 to the samples and vortex the mixture vigorously (10-20s). Incubate samples at 70°C for 10 min.

### *2. Adjust DNA binding conditions*

Vortex again the mixture and centrifuge few seconds at 11 000 g. Add 200 $\mu$ L ethanol (96-100%) to each samples, vortex again. Centrifuge again few seconds at 11 000 g.

### *3. Bind DNA*

Add the samples to the NucleoSpin Blood QuickPure Columns placed in a collection tubes and centrifuge 1 min at 11 000g. If the samples are not drawn through the matrix completely, repeat the centrifugation at higher g-force (up to 15 000g). Discard collection tube with flow-through.

### *4. Wash & dry silica membrane*

Place the Nucleospin blood quickpure column into a new collection tube (2mL) and add 350 $\mu$ L Buffer BQ2. Centrifuge 3 min at 11 000. If the samples are not drawn through the matrix completely, repeat the centrifugation at higher g-force (up to 15 000g). Discard collection tube with flow-through.

### *5. Elute highly pure DNA*

Place the NucleoSpin blood quickpure column in a 1.5mL microcentrifuge tube and add 50 $\mu$ L prewarmed Buffer BE (70°C). Dispense buffer directly onto the silica membrane. Incubate at room temperature for 1min. Centrifuge 1 min at 11 000g.

### 3.2 Relative leukocyte telomere length measurement

We measured relative leukocyte telomere length (RTL) using a real-time quantitative PCR method (qPCR; [2] which has previously been optimized and validated in sheep and cattle [1]. This method measures the total amount of telomeric sequence present in a DNA sample, relative to the amount of a non-variable copy number reference gene (beta-2-microglobulin (B2M)). For telomere reactions we used the following HPLC purified primers, Tel 1b (5'-CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT-3') and Tel 2b (5'-GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT-3') (from [3]). For B2M reactions, primers were supplied by Primer Design (Catalogue number: HK-SY-Sh-900, Southampton, UK).

Using an automated liquid handling robot (Freedom Evo-2 150; Tecan) we were able to load both the DNA samples and qPCR master mix in 384 well plates; allowing us to run both telomeric and B2M reactions in separate wells but on a single plate. A separate master mix for each primer set was prepared containing 5µl LightCycler 480SYBR Green I Master Mix (Cat # 04887352001, Roche, West Sussex, UK), 0.5µl B2M (300nm) primer or 0.6µl each tel primer (900nm), and 1 ng of sample DNA per individual PCR reaction. DNA was amplified in 10µl reactions. Each plate included a non-treated control (water ; NTC) for each amplicon, a calibrator sample (1ng) on each row to account for plate to plate variation and robot pipetting error, as well as a five step 1:4 serial dilution starting at 20ng/µl to visually inspect the qPCR curves. The calibrator sample is DNA that has been extracted from a large quantity of blood obtained from a single wild roe deer. In this case, the calibrator was extracted using the Qiagen DNeasy Blood and Tissue kit (Cat# 69581. Manchester. UK), pooled and quality controlled in the same way as our DNA samples of interest. All samples, calibrators and NTC's were run in triplicate and all qPCR performed using a Roche LC480 instrument using the following reaction protocol: 10min at 95°C (enzyme activation), followed by 50 cycles of 15s at 95°C (denaturation) and 30s at 58°C (primer annealing), then 30s at 72°C (signal acquisition). Melting curve protocol was 1 min at 95°C, followed by 30s at 58°C, then 0.11°C/s to 95°C followed by 10s at 40°C.

We used the LinRegPCR software package (version 2016.0; [4] to correct for baseline fluorescence, set a window of linearity for each amplicon group and to calculate well-specific reaction efficiencies and Cq values. A constant fluorescence threshold was set within the window of linearity for each amplicon group, calculated using the average Cq across all three

plates. The threshold values used were 0.304 and 0.394, and the average efficiency across all plates were 1.89 and 1.89 for the B2M and telomere amplicon groups, respectively. Samples were excluded from further analysis if the coefficient of variation (CV) across triplicate Cq values for either amplicon was > 5%, or if at least one of their triplicate reactions had an efficiency that was 5% higher or lower than the mean efficiency across all wells on that plate for the respective amplicon. Overall, thirteen samples failed quality control at either the DNA extraction or qPCR stage and were excluded from the study.

We calculated relative telomere length (RTL) for each sample following [5] as follows:

$$RTL = (E_{TEL}^{(CqTEL[Calibrator] - CqTEL[Sample])}) / (E_{B2M}^{(CqB2M[Calibrator] - CqB2M[Sample])})$$

Where  $E_{TEL}$  and  $E_{B2M}$  are the mean reaction efficiencies for the respective amplicon group across all samples on a given plate;  $CqTEL[Calibrator]$  and  $CqB2M[Calibrator]$  are the average Cqs for the relevant amplicon across all calibrator samples on the plate; and  $CqTEL[Sample]$  and  $CqB2M[Sample]$  are the average of the triplicate Cqs for the sample for each amplicon.

## References

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# Abstract

Over the last century, extreme climatic events and landscape modifications due to anthropogenic activities have dramatically increased. These rapid and unpredictable changes in the environment are new selective pressures for wild animals, and can threaten their survival and affect population dynamics. Understanding how wild animals cope with these perturbations and their consequences on life history traits and population dynamics is a necessary step to evaluate their evolutionary potential in a changing world. An important mechanism for adaptation to environmental changes is the stress response, which consist of a suite of physiological and behavioural responses that aim to maximise immediate survival. One of the main physiological pathways of the stress response is the hypothalamic-pituitary-adrenal axis, which triggers the secretion of glucocorticoid hormones. Because these hormones drive the mobilisation and distribution of energy, they underlie trade-offs between key functions such as immunity and reproduction. However, not all individuals respond in the same way to stressful situations, resulting in large among-individual differences. In addition, the natural environment not only offers a great deal of variability in stressors, but also means of responding to stress (e.g. the presence of shelters). In turn, the diversity of stress patterns depends on a complex combination of environmental variables and individual factors. The general aim of my thesis was to understand the determinants of the great among-individual differences in baseline stress levels, and to identify some consequences in a long-lived mammal, the European roe deer. The study analysed several measures of stress and their links with environmental and individual characteristics, and combined observation in three wild population and experimentation in captive roe deer.

Using an integrative approach, I found that proxies of stress level (N:L ratio and faecal cortisol metabolites [FCMs]) were moderately repeatable and weakly associated with behavioural

response to capture. In two populations, stress levels were higher during years of poor resources quality. I also found that FCMs of roe deer increased with proximity to human infrastructures, but this effect was buffered by behavioural adjustments such as using forest as refuge and adopting nocturnal activity patterns.

Secondly, I showed that environmental and behavioural characteristics were already determinant of baseline stress levels since the first weeks of life. Salivary cortisol was higher in fawns when they were closer to anthropogenic structures and exhibited proactive behaviour. Although fawn phenotype may also be shaped by maternal stress, the small data set of 30 mother-fawns pairs did not enable to show such a relationship.

Finally, I showed that the co-variation between immunity and FCMs, depended on individual and environmental characteristics. In a poor environment, high FCMs were related to a low immunity, both for innate and adaptive arms. This effect was exacerbated in individuals with low body condition, old individuals, and those with low food availability. Using experimental approach on captive roe deer, I showed close relationships between changes in FCMs and immunity, positive for adaptive arm, but negative for the innate arm.

Overall, my results highlight the importance of considering both individual (behaviour, age, body condition) and environmental (proxies of anthropogenic disturbance, presence of refuges in the environment, food resources availability) characteristics to better understand the among-individual variations of baseline stress levels and their consequences on fitness related traits (immunity). While adding complexity, such an integrative approach is crucial in reconciling conflicting results found in the literature, and paving the way for future studies on the evolutionary potential of wild populations in a context of increasing environmental disturbances.

# Résumé

Durant le siècle dernier, les événements climatiques extrêmes et les modifications du paysage dues aux activités humaines ont grandement augmenté. Ces changements rapides et imprévisibles représentent de nouvelles pressions de sélection pour la faune sauvage, et peuvent menacer leur survie. Comprendre comment les animaux sauvages font face à ces perturbations et leurs conséquences sur les traits d'histoire de vie et la dynamique des populations est une étape nécessaire pour évaluer leur potentiel évolutif dans un monde changeant. Un mécanisme important permettant de s'adapter aux changements environnementaux est la réponse de stress, qui consiste en une suite de réponses physiologiques et comportementales ayant pour but de maximiser la survie immédiate. Un axe physiologique majeur de cette réponse est l'axe hypothalamo-hypophyso-surrénalien, qui déclenche la sécrétion d'hormones glucocorticoïdes. Parce que ces hormones régulent la mobilisation et la distribution de l'énergie, elles sous-tendent des compromis évolutifs entre des fonctions clés telles que l'immunité et la reproduction. Cependant, tous les individus ne répondent pas de la même manière aux situations de stress, menant à de grandes différences inter-individuelles. De plus, l'environnement naturel offre également des moyens de répondre au stress (e.g. présence de refuge). La diversité des patterns de stress dépend donc d'une interaction complexe entre variables environnementales et individuelles. L'objectif général de ma thèse était de comprendre les déterminants des différences inter-individuelles des niveaux de stress basal, et d'identifier des conséquences chez un mammifère longévif, le chevreuil Européen. Cette étude a analysé différentes mesures de stress et leurs liens avec les caractéristiques environnementales et individuelles, en combinant l'observation de trois populations sauvages et l'expérimentation sur des chevreuils captifs.

J'ai trouvé que des indicateurs du niveau de stress (ratio N:L et métabolites fécaux des glucocorticoïdes [FCMs]) étaient répétables et associé à la réponse comportementale à la capture. Les niveaux de stress étaient également plus élevés durant les années de faible disponibilité en ressources. J'ai aussi trouvé que les FCMs des chevreuils augmentaient avec la proximité avec l'Homme, mais que cet effet était tamponné par des ajustements comportementaux tels que l'utilisation des forêts et l'adoption d'une activité nocturne.

Deuxièmement, j'ai montré que les caractéristiques environnementales et individuelles étaient des déterminants du niveau de stress basal dès les premières semaines de vie. Le cortisol salivaire des faons était plus élevé quand ils étaient plus proche des structures anthropiques et présentaient un comportement actif face à la capture.

Enfin, j'ai montré que la co-variation entre immunité et FCMs dépendaient des caractéristiques environnementales et individuelles. Dans un environnement pauvre, des valeurs élevées de FCMs étaient liées à une faible immunité. Cet effet était exacerbé chez les individus en mauvaise condition physique, vieux, ou ayant un faible accès à la nourriture. Une approche expérimentale m'a permis de montrer des relations étroites entre changement de FCMs et d'immunité, positifs pour la branche adaptative, mais négatifs pour la branche innée.

Globalement, mes résultats montrent l'importance de considérer à la fois caractéristiques individuelles (comportement, âge, masse corporelle) et environnementales (activités humaines et disponibilité en ressources) pour mieux comprendre les différences inter-individuelles des niveaux de stress basal et leur conséquences sur des traits lié à la valeur sélective (immunité). Bien qu'ajoutant de la complexité, une telle approche est cruciale pour réconcilier les résultats contradictoires trouvés dans la littérature, et pour ouvrir la porte à de futures études sur le potentiel évolutif des populations sauvages dans un contexte de perturbations environnementales.