

Ecophysiological and ecotoxicological consequences of global changes in terrestrial insects

Julie Engell Dahl Bjørge

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THESE DE DOCTORAT DE

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Par
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Ecophysiological and ecotoxicological consequences of global changes in terrestrial insects

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Title: Eco-physiological and eco-toxicological consequences of global changes for terrestrial insects

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Abstract

Terrestrial insects are affected by many environmental factors that vary temporally and spatially at micro- and macroscales. Climate change is moreover affecting the variations of these factors in several ways, with potential cascading effects for insect individuals, populations and communities. This thesis addresses how certain environmental abiotic factors can affect the physiological ecology of insects, with beetles as model insects. Firstly, I examined how geographic expansion could affect the thermal tolerance of invasive insects. For that purpose, different populations from the beetle *Merizodus soledadinus* invading a sub-Antarctic Island were used. This investigation illustrated a general pattern that most populations were best at coping with either cold or heat, and a few did not excel at recovering from either, compared to other populations of the species. This indicates that there are trade-offs between insects having high tolerance to hot or cold temperatures, and other un-investigated traits.

Further, researches were conducted to elucidate tolerance levels and responses of insects to temperature and insecticide (cyfluthrin) stress, and investigate how thermal tolerance can be affected by pesticides. Different populations of the invasive pest beetle *Alphitobius diaperinus*, i.e. one laboratory-reared population and three populations collected from different farms were used. The investigations revealed that all populations of *A. diaperinus* had a large thermal tolerance breadth. However, farm collected populations had a wider temperature tolerance than the investigated laboratory reared population. It evidences how variable environments experienced by farm-collected populations can increase tolerance as compared with the individuals reared at constant temperature. In addition, pre-treatment with daily heat spikes decreased the recovery time of the insects to subsequent extreme heat exposure (but not cold), whereas pesticide exposure prior to temperature stress increased recovery times. This provided evidence of interactions between insecticide and heat tolerance that has not been shown in adult terrestrial insects before.

Following stress exposure in the form of daily heat spikes, with or without insecticide exposure, no changes in the animals´ metabolome or antioxidant activity was reported. However, heat exposure resulted in increased numbers of offspring, while insecticide treatment had a tendency to reduce these numbers. In other words, the insects demonstrated a high tolerance to pesticide, but at a reproductive cost. Finally, tolerance and responses of *A. diaperinus* to dry conditions was tested. This study showed that the insects were highly desiccation resistant, but there were no changes in metabolite composition that could be associated with active upregulation of osmolytes in response to desiccating conditions. However, after a period in a very dry environment, the insects' reproductive output decreased, indicating that drought could have detrimental effects, even on populations of this desiccation resistant species.

Titre: Conséquences écophysiologiques et écotoxicologiques des changement globaux chez les insects terrestres

Résumé

Les insectes terrestres sont affectés par de nombreux facteurs environnementaux qui varient dans le temps et dans l'espace à macro- et micro-échelles. Le changement climatique affecte par ailleurs la manière dont ces facteurs varient, avec de potentielles conséquences pour les individus, les populations et les communautés d'insectes. Au sein de ce travail de thèse, les effets de facteurs abiotiques sur différents traits biologiques et physiologiques des insectes ont été étudiés, en employant des coléoptères comme modèles biologiques. Dans une première partie, l'impact de l'expansion géographique sur la plasticité thermique a été étudiée, e en se focalisant sur le coléoptère *Merizodus soledadinus* envahissant une île subantarctique. Différentes populations ont été collectées, et ce travail a permis de metter en évidence un schéma général selon lequel la plupart des populations présentaient de fortes aptitudes à tolérer le froid ou le chaud. Ces résultats indiquent qu'il existe des compromis entre ces deux traits avec d'autres caractères biologiques, les insectes.

Au sein de ce travail de thèse, les niveaux de tolérance et les réponses des insectes aux thermiques et chimiques (cyfluthrine) ont été étudiés. L'objectif était de déterminer si les réponses mises en place pour faire face au stress thermique pouvaient affecter la sensibilité au stress chimique et vice versa. Dans ce but, différentes populations du coléoptère ravageur envahissant *Alphitobius diaperinus* ont été employées : une population élevée en conditions contrôlées, et trois populations collectées dans différents élevages avicoles. Toutes les populations d'*A. diaperinus* testées possèdent une large tolérance thermique. Cependant, les populations collectées dans les élevages avicoles présentent une tolérance thermique plus importante que la population élevée en conditions contrôlées. Ces recherches démontrent le rôle des environnements variables comme moteur d'une plasticité thermique accrue. Le prétraitement des populations par exposition quotidienne à des pics de chaleur réduit le temps de récupération des insectes qui sont exposés à une chaleur extrême (mais pas au froid). A l'inverse, l'exposition aux pesticides avant le stress thermique a augmenté les temps de récupération. Ces recherches fournissent des preuves des interactions entre insecticide et tolérance à la chaleur, ce qui n'avait pas été démontré auparavant chez les insectes terrestres adultes.

Suite à une exposition au stress sous forme de pics de chaleur quotidiens, avec ou sans exposition à des insecticides, aucune altérationdu métabolome ou l'activité antioxydante des insectes n'a été observée. Cependant, l'exposition à la chaleur a entraîné une augmentation du nombre de descendants, tandis que le traitement insecticide avait tendance à réduire ce nombre. En d'autres termes, les insectes ont démontré une tolérance élevée aux pesticides, mais cela se traduit par un contre coût en termes de reproduction. Pour finir, la tolérance et les réponses d'*A. diaperinus* aux stress hydriques ont été prises en compte. Cela a permis de mettre en évidence les fortes capacités de cet insecte à faire face à des épisodes de sécheresse, sans que cela n'affecte la composition en métabolites. Cependant, après une période dans un environnement très sec, le succès reproducteur des insectes a ici aussi été diminué.

List of Publications

Engell Dahl, J., Bertrand, M., Pierre, A., Curtit, B., Pillard, C., Tasiemski, A., Convey, P., Renault, D. Thermal tolerance patterns of a carabid beetle sampled along invasion and altitudinal gradients at a sub-Antarctic island. *Journal of Thermal Biology* **86**, 102447 (2019).

Engell Dahl, J., Marti, S.-L., Colinet, H., Wiegand, C., Holmstrup, M., Renault, D. Thermal plasticity and sensitivity to insecticides in populations of an invasive beetle: Cyfluthrin increases vulnerability to extreme temperature. *Chemosphere* **274**, 129905 (2021)

Julie Engell Dahl and David Renault. Effect of heat and pesticide exposure on *Alphitobius diaperinus*. **In preparation** for *Comparative Biochemistry and Physiology C*

Julie Engell Dahl and David Renault. Desiccation in the lesser mealworm *Alphitobius diaperinus*. **In preparation** for *Frontiers in Physiology, Special Issue: Physiological Response to Environmental Stressors in Invertebrates*

Chapter 1

Introduction

Background

Human alterations of Earth have never been more substantial (Fischer-Kowalski, Krausmann, and Pallua 2014; Vitousek et al. 1997). In the context of environmental changes connected to anthropogenic activities, consequences for biodiversity is nowadays one of the main concerns for ecologists (Cardinale et al. 2012; Pereira et al. 2010; Thomas et al. 2004). Numerous authors claim we are at brink of the 6th major extinction event (Barnosky et al. 2011; Chapin et al. 2000). It is estimated that up to 1 000 000 species of plants and animals already face extinction, many within decades unless conservational action is taken (IPBES 2019). In addition to having an intrinsic value as species that have shared the planet for millennia, loss of biodiversity can have detrimental effects on ecosystem function and services, ultimately having potential consequences for human welfare (Reid et al. 2005). Species extinctions are a natural part of life's development on earth, but the current rate of species extinctions is already historically high, the average rate of vertebrate species loss over the last century being estimated to over 100-fold higher than the background rate. The magnitude of this threat can be directly attributed to human activity, mainly through habitat degradation, over exploitation, pollution, climate change or spread of invasive species (Ceballos et al. 2015).

One of the more urgent issues for the society is **climate change**. Human activities, especially transportation, agriculture and industrialization, have resulted in an increase in greenhouse gas emissions over the last century, and emissions continue to grow, driven by economic and population growth (IPCC 2021). Greenhouse gasses trap heat within earth's atmosphere, causing an increase in average global temperature, and, in combination with other anthropogenic drivers, has resulted in a warming of 0.84 ± 0.20 °C from 1880 to 2012 (IPCC 2014). In addition, an increase in frequency of extreme weather events, including heatwaves, with increased duration and/or amplitude can be expected (Beniston et al. 2007). The above mentioned anthropogenic drivers further resulted in a number of climatic changes, such as changes in precipitation, occurrence of extreme temperatures and ocean acidification (Easterling et al. 2000; IPCC 2014). Climatic changes thereby has implications for all organisms, affecting habitat suitability, physiology, life history traits, phenology, species interactions and migrations (Bellard et al. 2012; Bozinovic and Pörtner 2015; Doney et al. 2012; Foden, Mace, and Butchart 2013; Pacifici et al. 2015; Vasseur et al. 2014; Walther et al. 2002). The effects climate change has on the biology and ecology of living organisms can greatly vary among species. Well known examples of the effects of climate change are coral bleaching, including detrimental effects on staghorn coral (*Acropora cervicornis*) populations, and the geographic

expansion of the distribution of processionary moths *Thaumetopoea processionea* and *T. pityocampa* (De Boer and Harvey 2020; Buckley and Csergo 2017; Renema et al. 2016). In order to increase reliability of predictions of the effects of climate change, research that increases our understanding of the mechanisms and impact of environmental stressors affecting population dynamics, and thereby community composition and ecosystem function, is needed (Clark et al. 2001).

In addition to climate change, pollution constitutes another important stress factor of anthropogenic origins. Chemical exposure patterns have changed drastically over the last decades, with large repercussions on animal and plants (Secretariat of the Convention on Biological Diversity 2010). Pollution from agriculture by use of pesticides has intensified over the last decades, as the pressure to increase food production follows human population growth. Because many pesticides (or products of their conversion) can be very persistent in the environment, they are widespread and can also accumulate in organisms (Lushchak et al. 2018). When pesticides are used, they get into contact with a number of non-target organisms (Pimentel 1995), which in turn can change their behaviour or physiology, and contribute to trophic transfers, with potential impact on populations, communities and even ecosystems (Khan and Law 2005; Köhler and Triebskorn 2013; Liess et al. 2021).

The simultaneous exposure to thermal and chemical stress can lead to interactions having synergetic, additive or antagonistic outputs, depending on the type (hot/cold temperature or chemicals with different modes of action or pathways), duration and intensity of the stress (Walker et al. 2012). Such interactions can have adverse effects by leading either to climate-induced toxicant sensitivity or toxicant- induced climate sensitivity (Hooper et al. 2013).

Among animals, **ectotherms** are considered to be more sensitive to climate change, as exterior sources of heat and cold determine their body temperature. Indeed, these organisms cannot regulate their body temperature by use of metabolic activity like endotherms. Ectotherms includes fishes, amphibians, reptiles and invertebrates. Invertebrates are a diverse group of animals, constituting more than 90% of all living species, and includes important agricultural pests, pollinators, parasites, or agents for transmission of infections to humans and other vertebrates. Hence, invertebrates are important for ecosystems as well as humans. The most abundant and diversified terrestrial invertebrates are insects, constituting over 50% of known species living on earth, and almost 24% of the known species on earth are beetles (Ogunkunle, Adewumi, and Adepoju 2019). Insects' development, survival, fitness, and spatial distribution

are all dependent on environmental conditions, particularly temperature and humidity (Angilletta et al. 2002; Bowler et al. 2017; Chown, Sorensen, and Terblanche 2011). In addition, many pesticides (the insecticides) are designed to kill target insect species, but they also effectively influence a number of other insects as the insecticides' modes of action are not species specific (Main et al. 2020). Thus, knowledge of insects' responses to climate change, in addition to insecticides, is of particular interest with recent emergence of reports of insect declines (Wagner et al. 2021). In this thesis, I therefore focus on the effects of climate change and pesticides on insects, and try to uncover interactions between these stressors. The current knowledge in this field will be presented in this introduction.

Thermal performance curve

The performance of insects, in terms of development, longevity, reproduction and activity, depends on the temperature of their environment. The temperature-performance relationship can be modelled by a bell shaped curve based on Jensen's inequality (Jensen 1906), called the "**thermal performance curve**" (this pattern can also apply to other parameters than temperature). Knowledge of an insect thermal performance curve can be conveniently used for predictions of their sensitivity to thermal changes, and thereby predict effects of climate change on their distribution (Deutsch et al. 2008; Huey et al. 2012; Sinclair et al. 2016).

The curve is delimited by a **critical minimum and maximum temperature** (**CT_{min}** and **CT_{max}**) between which the insect can perform the described trait (for example metabolic rate, locomotion speed or growth rate) at an acceptable level (Huey and Stevenson 1979). The range of temperature between CT_{min} to CT_{max} corresponds to the thermal breadth of the animal and is species-specific. In this range, performance reaches a maximum, called **optimal temperature** (**T_{opt}**) (Angilletta, Huey, and Frazier 2010), although the term "Optimal temperature" should be used with care, as different traits can differ in their T_{opt}, meaning we are not necessarily measuring the optimal temperature for the animals as a whole, or

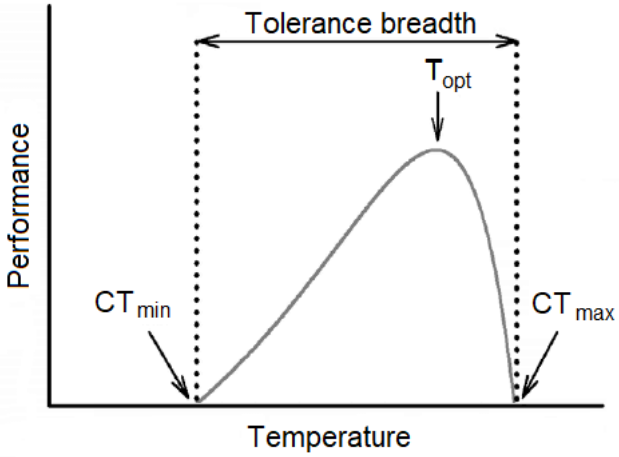


Figure 1 *General shape of a thermal performance curve (grey line) in relation to (body) temperature, edited from Huey and Kingsolver (1989). The optimum temperature (T_{opt}) specifies the temperature at maximum performance of the measured trait, whereas critical thermal minimum (CT_{min}) and maximum (CT_{max}) delimit an organism's thermal tolerance.*

over time. When temperature increases from CT_{min}, an organism's performance gradually increases, reaching its T_{opt} before quickly decreasing to CT_{max}, in accordance with the Boltzmann-Arrhenius relationship. Thus, biochemical and physiological rate performance is expected to be skewed to the left of the relationship. However, it has been shown that performance rates are more symmetrical around T_{opt} when measuring more integrative processes (for example reproductive output) (Knies and Kingsolver 2010). As can be seen from the skewed shape of a typical performance curve, despite tolerating increase in average temperatures, the occasional occurrence of extremely hot periods can have detrimental effects on survival and fitness of insects.

Traditionally, data describing a thermal performance curve originate from exposing groups of animals to a range of constant temperatures and plotting their average performance as a function of exposure temperature, which is then used to estimate the animal's performance in that trait, at any temperature. This is a useful tool for ecologists, who often use the average temperature in an environment to estimate the animal's performance in that environment. This can be precise in, for example, production facilities or the tropics, where temperatures are fairly constant over time. However, the environmental temperature usually fluctuates daily and seasonally, which can affect the performance of the animals and render the result of a performance function based on research performed with constant temperatures imprecise. As such, fluctuating temperature regimes have received more attention in research in the context of climate change, to make extrapolations to field scenarios more realistic (Colinet et al. 2015; Thompson et al. 2013).

The span of the curve, representing the **thermal breadth** of the animals' thermal tolerance, is limited by the fact that adaptation to a large range of temperatures comes at physiological costs and includes trade-offs, yet temporal variation in environmental conditions are generally the main determinant of tolerance breadth (Angilletta et al. 2002; Huey and Kingsolver 1989). Animals living in temperate areas, experiencing greater temperature variations over a season are generally predicted to withstand greater temperature extremes, as thermal breadth generally increase with latitude (Sunday, Bates, and Dulvy 2012). However, this is more true for marine species than for terrestrial ectotherms, probably due to differences in experienced temperature variations, and particularly terrestrial ectotherms' upper thermal limits vary only little (Sunday, Bates, and Dulvy 2011).

Over time, shifts in the performance function can occur, through acclimation, acclimatization or adaptation. **Acclimation** referring to animals' phenotypic responses (compensatory physiological adjustments) to a factor, often in a laboratory environment, while **acclimatization** is the phenotypic response in an animal, in response to changes (e.g., diurnal or seasonal shifts in temperature and light conditions) in their natural environment. **Adaptation** is the evolutionary process by which genotypes are modified so that the best fitness in a particular habitat becomes favoured by natural selection.

What is stress?

An organism depends on its ability to maintain a fairly constant internal environment, and the scientist Walter Cannon coined the term "**homeostasis**" to describe the range of permissible values of physiological variables to ensure normal body function (Cannon 1929). The concept of **stress** has been developed as a descriptor of threat to homeostasis. However, further research found that different activities, even everyday actions such as ingesting a meal or even just changing posture could be associated with different sets of steady states (Gerra et al. 2001; Patel et al. 2002). This contributed to the development of the "allostasis" concept, where there is no single "ideal" steady state, but several changeable setpoints around which physiological values should be (Goldstein and Kopin 2007). As a result, understanding of stress was further developed into a system concept, where the body has homeostatic setpoints, continually compared to sensory input, and regulating mechanisms responding if a variable is outside the acceptable range of values (Goldstein 1995). Regulation systems can function through negative feedback loops, where changes in censored values result in comparable change in the activity that opposes the monitored variable, thereby counteracting the changes. Often, physiological systems use multiple effectors to control and regulate the monitored biological and physiological variables, constituting the stress response of the organism, which varies depending on, for example, the nature and level of the factor that initiated the response (Goldstein and Kopin 2007). As the internal environment of an organism is so changeable in the long term, regulation to more chronic stress response initiators is under homeorhetic rather than homeostatic regulation, which means that the systems aims to return the dynamic of the internal environment towards its natural trajectory rather than to a particular state (Collier et al. 2019). The factors that can initiate a stress response are called **stressors**, so it is stressors that perturbate the internal system and causes the system to be in a state of stress. As an illustration, "heat stress" is the state of an animal affected by the stressor "heat".

Ecological consequences of climate stress

Increased temperature and thermal fluctuations

To improve predictions of species responses to climate change, thermal fluctuations should be taken into account (Thompson et al. 2013; Vasseur et al. 2014). Predicted increases in the amplitude of thermal fluctuations elevate the risk of detrimental effects of temperature stress on animals, as the risk of experiencing temperatures close to, or even above, their CT max increases (Hansen, Sato, and Ruedy 2012; IPCC 2014; Stillman 2019). Furthermore, studies have found that a period of extreme heat can have a larger impact than a moderate increase in mean daily temperatures (Vasseur et al. 2014).

Theoretically, it has been shown that insects developing in a fluctuating environment live at temperatures lower than that of their thermal optima, which greatly limit the risks of deleterious temperature effects when thermal fluctuations occur at high amplitudes (Sunday et al. 2014). Therefore, the fitness of these insects could be expected to increase with the mean temperature of the environment. Insects developing in environments having relatively constant temperatures should have a thermal optimum close to the environmental temperature, due to the reduced risk of occurrences of detrimental thermal fluctuations; for these organisms, increasing temperature should thus result in steep fitness decline. This is why tropical species have long been considered as being more sensitive to climate change than their temperate relatives (Deutsch et al. 2008). However, recent research has also reported that some temperate insects may also live in thermal niches with temperatures close to their thermal optima, meaning that their fitness and/or current distribution could also be affected by climate change (Johansson, Orizaola, and Nilsson-Örtman 2020).

Finally, changes in humidity can be a direct effect of increased temperatures that should increase desiccation risks for small animals like insects. Moreover, predicted shifts in entire weather systems should reduce rainfall in certain areas (Trenberth 2011). In turn, desiccation can further affect insects' ability to cope with temperature stress owing to the physiological costs incurred by desiccation stress (Kleynhans, Clusella-Trullas, and Terblanche 2014). Meanwhile, positive effects have also been reported when physiological responses to climate change overlap with desiccation (particularly tolerance to freezing temperatures, Sinclair *et al.*, 2013). The latter effect is based on what is called **cross tolerance**, which is when increased tolerance to one stressor induces tolerance to another (Gotcha, Terblanche, and Nyamukondiwa 2018).

Invasive species

One of the currently well-characterized effects of increasing temperatures is the range expansion (movement) of species (Hickling et al. 2006), and displacement of populations to avoid being exposed to unfavourable conditions. In particular, movements of populations towards the north (in the northern hemisphere), or movement uphill, have been reported. This is thought to occur either to mitigate temperature change, or because the warming climate makes a wider area suitable for species that were normally living in habitats at lower altitude or latitude (I. C. Chen et al. 2011; Parmesan and Yohe 2003; Sunday, Bates, and Dulvy 2012). In sum, warming may open new niches for ectotherms. Increase in average temperatures can facilitate the spread of certain species, previously limited in their range by thermal limits (Battisti et al. 2005). Such range changes have been well documented in butterflies. For example, Parmesan et al. (1999) found that over half of 35 non-migratory European butterfly species had shifted their range northward during the 20th century.

In addition, **invasion of species** by human transportation may increase a species' range (Ward and Masters 2007). Insular ecosystems, including (relatively) small islands, where ecological niches can be poorly diversified, are prone to invasion. The high level of endemism and total of few species means island communities often evolved at a low biotic pressure, which also renders them vulnerable to effects of invasive species (Case 1990). Large scale changes in species range can cause new biotic interactions to occur, such as increased competition or predation, which could decrease the ability of vulnerable species to persist (Tylianakis et al. 2008). Furthermore, habitat disturbance by human activity is associated with invasion success (Gonzalez, Lambert, and Ricciardi 2008; Lozon and MacIsaac 1997). By consequence, with the increasing ease of movement of goods and people over the last decades, invasive species are establishing populations in far reaching places of the world. As range-shifting and invasive species adapt to local thermal conditions, it has been shown that thermal ranges are increased at expansion fronts (Hillaert et al. 2015), and several examples of changes in traits that follow a pattern of the residence time of individuals at a locality have been described (Fronhofer and Altermatt 2015; Ochocki and Miller 2017; Renault et al. 2018). Such adaptations could further increase the possible range of an invading species.

Mild winters

Insect development often requires thermal seasonality, with life cycles being completed in the periods where it is most favourable. In many habitats, winter temperatures result in the need for either migration or entering diapause in the form of eggs, larva, or more rarely, as adults in order

to survive (Yang and Rudolf 2010). Diapause is a dynamic state of low metabolic activity, characterized by inactivity, morphogenesis and a pause in reproductive function, regulated by different environmental signals preceding the occurrence of unfavourable conditions (Tauber, Tauber, and Masaki 1986). Diapause can also refer to aestivation, or summer diapause, commonly triggered by arid conditions (Storey and Storey 2012). Animals cannot break diapause immediately, even if favourable conditions appear, and it should be seen more as a process rather than a status (Koštál 2006). During the diapause period, survival is directly linked to environmental temperatures, warmer winter temperatures usually being associated with increased mortality (Marshall and Sinclair 2012; Sobek-Swant et al. 2012; Stuhldreher, Hermann, and Fartmann 2014). A shorter winter has been shown to reduce emergence and increase time to emergence in insects (Bosch and Kemp 2004; Stålhandske, Gotthard, and Leimar 2017). At the same time, it has been shown that the return of favourable temperatures earlier in the season should trigger continued development sooner, which has been observed in several species, and can lead to a de-synchronization of life cycles of interacting species, be it host and parasite or plant and insect, with possible additional detrimental consequences (Yang and Rudolf 2010).

In addition, shorter and milder winters, giving longer and warmer summers, can decrease ectotherms' cold tolerance. For example, exposure to winter (extreme) warm spells reduced cold tolerance in emerald ash borer (*Agrilus planipennis*) even after they had been returned to low temperatures (Sobek-Swant et al. 2012). Furthermore, it has been shown that summer acclimated or acclimatized insects will generally show more cold sensitivity (Block 1990; Košťál and Šimek 1996). Nonetheless, increase in average winter temperatures has been shown to increase the average number of species in terrestrial communities, possibly due to range expansion of many species (Bowler et al. 2017).

Climate stress tolerance strategies

Adaptation or acclimatization to stressful conditions/extreme temperatures include morphological, behavioural, ecological and/or physiological changes. Insects can change morphologically by for example reducing body size or melanism or increase pubescence. Changes in body size has been observed in several species as a response to climate change, but the direction of change varies as well as suggested causes, from changes in food availability to temperature (Gardner et al. 2011; Sheridan and Bickford 2011). In a review of studies on morphological changes associated with recent climate change (temperature or precipitation), only changes in coloration often had a strong association to climate change (MacLean et al.

2019). A very specialized example of morphological adaptation are desert beetles with a body shape specially capable of collecting water droplets from fog or morning dew at the mouth for ingestion (Guadarrama-Cetina et al. 2014; Hamilton and Seely 1976).

Behavioural changes include, for example, habitat selection and changes in activity patterns (Hutchison and Maness 1979). General behaviour to cope with stressful situations include sit-and wait strategy, where the animals reduce activity to an absolute minimum in hopes of surviving until the environment is suitable again (Renault, Hervant, and Vernon 2003; Renault and Coray 2004). This greatly contribute to reduce energy expenditure, water loss and potential exposure to environmental pollution, making it a possible solution to endure a wide range of stressors. Oppositely, an individual might increase activity for foraging or looking for a more suitable habitat (Lorenzo and Lazzari 1998; Yu et al. 2010). When experiencing stress, animals might also burrow into the ground awaiting better conditions (Bayley et al. 2010). Research is still needed to try to develop a universal framework for standardized behavioural tests, in order to determine if a stressor is above a critical level. As behavioural observations are often less stressful and invasive for the experimental animals, recognizing general and easily observed behavioural responses could improve ethical and economical implication of risk assessment of environmental factors, and is therefore of great interest. Recently, methods for large-scale phenotyping of the behavioural responses of insects to thermal stress have been proposed (Laursen et al. 2021).

When animals go through so called ecological changes, it can have detrimental effects on the function of an ecosystem, because for example changes in timing of phenology can cause mismatches with interacting species, with potential detrimental outcomes (Parmesan and Yohe 2003; Yang and Rudolf 2010). Over the last decades, a trend of advancement of phenological traits in the spring and summer have been described for plants and animals, but with a stronger effect in insects than plant, which could lead to a decoupling of plant-insect interactions (Gordo and Sanz 2005).

Tenebrionid beetles can serve as a clear example of insects adapted behaviourally, morphologically, and physiologically to their environment. They can be found naturally in areas like the African dry savanna, where they spend the hot dry season as adults rather than larvae like most other insects (Zachariassen and Einarson 1993). As the name Tenebrionid (also called darkling beetle) indicates, these insects spend their lives in darkness, to avoid the hot and dry surface of their natural habitat. They have an extremely impermeable cuticle which reduces their

transpiratory water loss, in combination with fused elytra which reduces respiratory water loss from abdominal spiracles (Ahearn 1970; Zachariassen 1991). In addition, their metabolic rate is so low that respiratory water loss from breathing is minimized and they can reabsorb almost all water in the gut, producing extremely dry faeces (Zachariassen et al. 1987).

Physiological changes can involve several other responses, such as increased production of certain proteins or changing metabolite composition in the haemolymph, described in more detail below. Some of the more important responses, such as heat shock protein regulation and freeze tolerance, are described below because of their high relevance in the field of thermal tolerance, despite not being included in the work of this thesis.

Heat shock proteins and chaperones

Heat shock proteins (hsps) were first discovered in 1973 after subjecting *Drosophila* cells to heat shock (Tissieres, Mitchell, and Tracy 1973). Because this category of proteins was highly induced by the high temperature treatment, they were given the name **heat shock proteins** (**hsps**). Since then, hsps have been observed in most living organisms and the production of these proteins is elicited by a variety of stress (Parsell and Lindquist 1993). In the process of uncovering their functions, several groups of hsps have been discovered, and they have been grouped in families named after their molecular weights (for example Hsp70 constitutes proteins weighing approximately 70 kilodaltons) (Brocchieri, Conway de Macardio, and Macario 2008; Hoffmann, Sørensen, and Loeschcke 2003). These proteins can protect the cell either by ensuring the function of other proteins in the cell, increasing degradation of dysfunctional proteins and/or inhibiting stress induced apoptosis (cell death) (Parsell and Lindquist 1993; Samali and Cotter 1996). Because they prevent improper protein associations, they are also called protein chaperones. Hsps are both inducible (only expressed following stress), constitutive (always expressed) and forms that are always present and yet can be induced following stress. The main trigger for heat shock protein induction is thought to be the presence of abnormal (denatured or unfolded) proteins, themselves resulting from the stressful conditions (Ananthan, Goldberg, and Voellmy 1986; Lepock, Frey, and Ritchie 1993).

During heat stress, small sections of a denatured protein can bind to a hsp. It is the binding of only a small section of the substrates that allows many hsps to be so versatile, because it means that they are not limited by substrate size. Low levels of ATP are typical in heat stress, and this causes the hsp binding site to be kept closed until it can bind ATP again. This sustained binding keeps proteins from aggregating during heat stress (Vollemy and Boellmann 2007). In addition to preventing aggregation of denatured proteins, hsps can untangle proteins that have already

aggregated. In this process, alternating cycles of hsp binding, protein unfolding and hsp release, converts proteins in a stable misfolded aggregated state to a temporarily unstable state, which can then allow the spontaneous correct folding of individual proteins (Ben-Zvi et al. 2004). Hsps' role in degradation of damaged proteins largely entails contributing in the ubiquitin-proteosome pathway, which is also one of the cells main degradation pathways under normal conditions. When a hsp is bound to an unfolded protein, it can recruit Ubiquitin, which is also a hsp, which functions as the tag, marking substrates for proteasomal degradation.

Apoptosis inhibition is another function of hsps, which not only improves the integrity of cell proteins, but the entire cell. Hsps can either reduce cell damage and thereby prevent the death, or they can inhibit the activation or activity of sensor or effector molecules, and thereby inhibit stress induced apoptosis (Mosser et al. 1997). In fact, several points of function for hsps are being identified in the apoptosis pathways, making them important apoptosis regulators (Lanneau et al. 2008). This is a role of hsps that has been given a lot of attention, because it seems that these protective mechanisms can also stabilize mutant proteins, and thus enables tumour cells to function. Furthermore, the anti-apoptotic system appears to protect these cells from dying, and thus could be major carcinogens.

Because several types of stress result in the induction and activation of a common set of stress proteins, hsp induction can be associated with increased tolerance to subsequent stress conditions. The molecular basis of this cross tolerance is that these proteins remain active and their expression remains elevated. This occurs for a period of time dependent on several factors such as species, cell-type, prior stress exposure (history), interactions between genes and the environment during development and the stress severity (Kültz 2005). This is advantageous for organisms thriving in unstable environments, where they can be exposed to more than one type of stress over a short time period, or even at the same time.

The many beneficial effects of the hsps suggest that it would be advantageous to always have a high level of hsp expression. However, their gene expression is usually not maximized but highly regulated, suggesting a cost related to expression of these proteins, as is the case for many hsps (and other stress responses). When hsps are induced, a large reduction in other protein synthesis has been observed, meaning these proteins are built at the expense of others (Feder et al. 1992). This means that normal cell function is shut down during the stress response, clearly demonstrating that such protein expression cannot persist (Sørensen, Kristensen, and Loeschcke 2003). Furthermore, Krebs and Feder (1997) showed that overexpression of hsp70

reduced growth, development and survival in transgenic *Drosophila melanogaster* larvae, even though it increased protection, and thus survival, during heat stress exposure. Feder and Hofmann (1999) suggested that the underlying cause of these effects could be that hsp production is associated with a significant consumption of cell nutrients and/or too much pressure on the anabolic/catabolic apparatus for proper processing of other important substances. Thus, the regulation of hsps (as many other stress responses) is a consequence of apparent cost/benefit trade-offs, but the mechanisms behind the deleterious effects are unclear. Yancey (2001) illustrated that reduction in cell content of an hsp (hsp70) coincided with increased levels of other chaperones and cryoprotectants, most of which are molecules with a relatively small production cost. This can help explain why the cell can keep increasing survival while downregulating stress proteins (Dahlgaard et al. 1998).

In conclusion, hsps are important in relation to stress resistance, as they ensure the proper function of proteins, contribute to degradation of damaged polypeptides and increase cell survival. These benefits come at a cost, which is why they are carefully up- and downregulated in the cell.

Oxidative stress

Oxygen is an essential substance for all animals, but it is also the cause of many pathological conditions and associated with the natural aging process, due to its biotransformation into **reactive oxygen species (ROS)** (Hermes-Lima and Zenteno-Savın 2002). Additionally, stressful conditions can cause overproduction of ROS, which can damage important cellular molecules, leading to, for example, lipid peroxidation, protein oxidation and DNA damage. This is why mechanisms to limit ROS formation, repair damage and detoxify damage products are of vital essence for all organisms (Fridovich 1998). In response to ROS stress, antioxidant defences include metabolites such as ascorbate and vitamin E, peptides such as glutathione and thioredoxin, and enzymes such as superoxide dismutase (SOD), catalase (CAT), thioredoxin peroxidase and selenium-dependent glutathione peroxidase (Se-GPX) (Felton and Summers 1995; Storey and Storey 2010). It has been shown that animals that experience oxidative stress regularly maintain high constitutive levels of antioxidants, whereas others demonstrate inducible defences (Hermes-Lima and Zenteno-Savın 2002).

Measurements of the total antioxidant capacity (TAC) describe the cumulative action of all antioxidants in an organism, which account for the actions of known (and measurable) and unknown antioxidants, as well as their interactions affecting productivity (Ghiselli et al. 2000). Measuring important antioxidant enzyme activities as well as TAC, Zhang et al. (2015) found

increased antioxidant capacity in the ladybeetle *Propylaea japonica* at high, but not lethal, temperatures. Furthermore, low temperature has been shown to increase antioxidant activity in *Alphitobius diaperinus* (Lalouette et al. 2011). This indicates that thermal stressors lead to oxidative stress in insects, for which antioxidant activity is increased to limit oxidative damage.

Desiccation

There is considerable variation in the level of dehydration that insect species can endure. Studies have shown that the tolerable body water loss is only 20% in the beetle *Callosobruchus maculatus* (Yoder, Christensen, and Keeney 2010), while it can reach 90% in the semiaquatic beetle *Peltodytes muticus* (Arlian and Staiger 1978). Water loss tolerance does not only vary between species, but also between developmental stage, sex, or age (Hadley 1994). It is generally important in stress research to consider all such factors when executing experiments, in order to reduce variance and increase validity of results. As mentioned earlier, some animals have adapted to dry conditions by reducing water loss, for example by having very low cuticular water permeability (Zachariassen 1991) or by absorbing a very large amount of water from faecal material before excretion (O'Donnell and Machin 1991). An alternative solution to tolerating dry conditions and high water loss is the ability developed by some organisms who can absorb water from the surrounding air by accumulation of sugars and polyols in the body fluids, so that it creates hyperosmoticity, causing water to enter through a permeable cuticle. This phenomenon has been described for the springtail *Folsomia candida* (Bayley and Holmstrup 1999), which seems to be the coping strategy for mild water stress for the springtail. When water stress increases, the animal then dehydrates to a state resembling anhydrobiosis (Sjursen, Bayley, and Holmstrup 2001). Tenebrionid larvae can also absorb vapor from the surrounding air, down to 88% relative humidity (L. L. Hansen, Ramløv, and Westh 2004).

Cryptobiosis is a state of unmeasurable respiration in response to physiological stress, which is reversed when conditions become favourable again (Womersley 1981). When this state is caused by desiccation, it is called **anhydrobiosis** (Watanabe et al. 2002). A well described example of anhydrobiosis in insects is that of the midge larvae of the species *Polypedilum vanderplanki*, which lives in temporary pools in Central Africa. When dehydrated, the midge can withstand temperatures from -270 to +100 °C (Sogame and Kikawada 2017). The final process of achieving a state of anhydrobiosis depends on the stabilization of membranes (as does desiccation and cold tolerance in general) and enzymes. LEA (late embryogenesis abundant) proteins have a stabilizing role for other proteins as well as membranes (Chen et al. 2019). They are well described as a plant stress response, but have recently been associated with

desiccation tolerance in animals, for example *P. vanderplanki* (Hand et al. 2011). It is also likely that the drastic reduction of the metabolism of highly dehydrated organisms diminishes the level of oxidative stress. In turn, this could likely increase thermotolerance (Hadley 1994). Polyols, such as glycerol and trehalose, function in a stabilizing role by replacing bound water to help proteins and membranes maintain their structure (Crowe 2002; Thompson 2003).

It is interesting to mention that several protective measures against dehydration overlap with those preventing damage from freezing or low temperature in general. This is particularly true for the upregulation of **compatible osmolytes,** also known as cryoprotecting agents (Benoit et al. 2009). These are molecules that can be accumulated to high concentrations as they do not generally interact with macromolecules in detrimental ways (Yancey 2005). In insects, the most common cryoprotecting agents are polyols in the form of glycerol, sorbitol and mannitol, but several sugars (often trehalose) and amino acids (alanine and proline, for instance) function similarly and are often upregulated in combination to increase the capacity to remain unfrozen (Somme 1999). Cryoprotectants have also been associated with acclimation to high temperatures, suggesting that these compatible osmolytes may provide protection against the injuries caused by both high and low temperatures (Hendrix and Salvucci 1998).. Because several of these solutes have the ability to readily cross a cell membrane, they can decrease osmotic stress and limit water loss of a cell, and some compounds stabilize proteins and cell membranes (Denlinger and Lee 2010).

Other extreme temperature tolerance strategies

Originally, research on cold stress divided insects into two (three) main categories of cold hardiness; freeze tolerant and freeze intolerant (the latter group including both cold-hardy freeze avoiding species and chill sensitive species), where both freeze tolerant and freeze avoiding species have a lower lethal temperature (LLT), and the LLT of both can be equally low (Lee 2010). Freeze avoidant species depend on supercooling to avoid freezing at temperatures below the melting point (i.e., equilibrium freezing point) of their bodily fluids. To achieve supercooling down to temperatures well below zero, animals may use several tools (Sømme 1999). Normally, body fluids do not freeze until at least a few degrees below zero because of the presence of several inorganic and organic solutes (ions, amino acids, proteins, sugars and other dissolved solutes). Thermal hysteresis proteins (THPs), or “anti-freeze proteins”, decrease the haemolymph freezing point further by adhering to the surface of ice crystals beginning to form, preventing them from growing (Storey and Storey 1996). Because this does not simultaneously decrease the melting point of the body fluids, THPs increase the difference between the freezing

point and melting point, which is called thermal hysteresis, which explains the proteins' name. Increasing the concentration of such proteins in the haemolymph, or even epidermal cells to protect from ice invading through the cuticle from the animals' surroundings, maintains and stabilizes the supercooled state (Olsen et al. 1998). This makes them very important for improved cold tolerance in insects, in addition to low molecular weight **cryoprotectant** substances, as described above.

Before water, or a physiological solution, starts freezing, a nucleus around which water can aggregate in an ice-like pattern is required. This is why contact with foreign substances, such as mineral particles or bacteria, increases the chances of ice formation (Lee, Costanzo, and Mugnano 1996). These substances are called ice nucleating agents (INA), and must be removed or biochemically concealed to increase the capacity of supercooling, called the **supercooling point**, the temperature where supercooled insects freeze after gradual cooling (Somme 1999). Clearing the gut is therefore vital for freeze intolerant species in preparing for freezing temperatures. In freeze tolerant species, on the other hand, some molecules acting as INAs function to control the freezing process, to make sure freezing takes place extracellularly, and at a relatively high sub-zero temperature, as freezing occurring inside the cell is generally lethal (Somme 1999). This is an example of how freeze tolerant species can diminish their ability to supercool, and some species can only tolerate freezing if ice formation starts at relatively high temperatures. This is mainly because ice forming at higher temperatures grows slower, and so the critical time to allow for mobilization of cryoprotection is gained and osmotic stress is reduced, a protective measure overlapping in mechanism to that of dehydration tolerance. This is probably because freezing only recruits water molecules, so solutes in the unfrozen haemolymph become increasingly concentrated, causing osmotic flow of water out of cells, effectively dehydrating them until osmotic equilibrium is re-established (Denlinger and Lee 2010).

Metabolomics – finding the players of physiological responses to stress exposure

Metabolites are both substrates and the end product of cellular processes. As such, they represent useful descriptors of phenotypes, and can be measured more easily than genetic or proteomic responses in non-model species. Moreover, changes in gene or protein expression does not always correlate with variation in protein activity while an alteration in metabolites does (Kuile and Westerhoff 2001). Therefore, metabolomic studies can provide molecular-level

compilation of upstream genomic, transcriptomic and proteomic responses of an organism to a given stimulus or change (Kell 2004).

Metabolic profiling, or “fingerprinting”, has the purpose of attaining as much of the total metabolomic information as possible from different systems to compare their metabolome, and thereby infer a relationship between treatment and changes in metabolite composition/concentrations (Tyagi et al. 2010). Since this method quantifies a lot of metabolites at the same time, it generates a dataset with more observed variables than the number of observations, making traditional linear regression methods unsuitable for the analyses (Worley and Powers 2013). The most popular multivariate analysis methods for analysing metabolomics data are principal component analysis (PCA) and partial least squares projection to latent structures (PLS) (Abdi and Williams 2010; Wold, Sjöström, and Eriksson 2001). These methods produce scores that can be seen as good “summaries” of observations, and help to identify biologically relevant spectral features and differentiate groups even when datasets are complex (Worley and Powers 2013). Initial observation of groups’ separation in a PCA followed by a confirmation and more detailed PLS classification have a greater likelihood of producing biologically relevant results. This is because separation only is observed between groups in PCA scores when within-group variation is significantly less than between-group variation in the data, while separation in PLS analysis has a tendency to over-fit models to the data. In addition, efforts have been made to standardise the processing of raw spectral data and analysis interpretation, enabling researchers to generate reliable and comparable models (van den Berg et al. 2006; Goodacre et al. 2007; Kjeldahl and Bro 2010; Lindon et al. 2005; Sysi-Aho et al. 2007; Wongravee et al. 2009).

The covariance (or correlation) matrix of principal components in a PCA can be split into a scale part (eigenvalues, the variance explained by the principal components) and a direction part (eigenvectors, coefficient of projection). By multiplying the eigenvector by the square root of the eigenvalue, the coefficient is “loaded” by the amount of variance, giving what is called the loadings, representing the covariance between the original variables and the principal components. Therefore, it is usually beneficial to use loadings or eigenvectors to complement PCA scores, as they represent a measure of the association between variables on which the PCA is based. Unfortunately, researchers and other sources (including some R packages) use these two terms interchangeably, causing confusion, although different meanings of “loadings” generally lead to the same interpretations of the components (Abdi and Williams 2010). How easily loadings can be interpreted is directly affected by the number of variables and the scaling

method used (if any). Variables whose loadings are placed close together (away from the origin) in a loadings plot, can be assumed to be correlated. Moreover, variables with loadings in a given position in a loading plot contribute greatly to observations whose scores are found in a similar position in a scores plot (van den Berg et al. 2006). In addition, for PCA and validated PLS scores, quantitative measures must be applied to reliably infer significant separations between classes within a scores plot. One simple method of inferring class membership is through 95% confidence ellipses calculated from scores, which then completes an analysis of metabolomics data (Worley, Halouska, and Powers 2013).

Metabolites that have been observed to fluctuate in various organisms after exposure to an environmental stressor can serve as valuable bioindicators of environmental stress. (Lankadurai, Nagato, and Simpson 2013). As is often the case, the fruit fly *Drosophila melanogaster* has been used in several studies to investigate their metabolic responses to environmental stressors (Colinet et al. 2012; Feala et al. 2007; Kostal et al. 2011; Overgaard et al. 2007; Pedersen et al. 2008; Vesala et al. 2012). For example, Colinet et al. (2012) showed that cold acclimation resulted in increased concentrations of the sugars sucrose, fructose and trehalose, which could be potential biomarkers of cold acclimation. In addition, Colinet et al. (2012) found a decrease in metabolic intermediates glycerate, citrate, fumarate and malate, which suggests a mechanism for the flies energy saving strategy when cold acclimated. In such ways, metabolomics can be used to unravel the biochemistry of thermal tolerance.

Pesticides

Pesticides are substances meant to control harmful organisms (pests), the -cide ending deriving from the Latin word “cida” meaning “killer”, although not all pesticides have lethal outcomes. Pesticides include a wide range of chemicals such as herbicides, fungicides and insecticides, but the focus in this mini-review will be on **insecticides**, that is, pesticides with the aim of controlling insect pests for crop protection and reduction of (vectors of) harmful diseases. These substances are in widespread use in agriculture, forestry, gardens, homes and offices, with the bulk of insecticides being used in agriculture (Walker et al. 2012). The use of pesticides to maintain food production and prices is of absolute importance, as crop yields would be expected to decrease substantially if farmers stopped using pesticides, resulting in a comparable rise in food prices (Rickard 2010). Despite pesticide applications being limited in their geographical span and duration, the substances used can find their way to far reaching places in the environment by several routes. For example, wind can carry chemicals far from their place of origin, and lead to contamination of different terrestrial and aquatic habitats (Dubus, Hollis, and

Brown 2000). Pesticides can enter water bodies through surface run-off, seeping into groundwater (leaching), or be transported by erosion, which is why they can be widely found around the globe (Vryzas 2018; van der Werf 1996). A major concern with respect to climate change is that changes in precipitation or occurrence of extreme weather events, such as storms and flooding, will remobilise toxic substances in soils and sediments, and increase pesticide dispersion by erosion, run-off and leaching (Presley et al. 2006; Whitehead et al. 2009). However, warmer temperatures can also increase the rate of biotransformation and degradation of pesticides in the environment, thereby reducing the concentration of pollutants in the environment, making future concentrations of bioavailable pesticides difficult to predict (Op de Beeck et al. 2017).

Measuring toxicity

The toxicity of an insecticide to an organism is often described as the dose required to kill 50% of a test population of a species in a certain period of time, called the **LD_{50}** (**lethal dose 50**). When the dose is difficult to determine, the concentration in the surroundings (for example in water for aquatic species) that kills 50% of a test population, called **LC_{50}** (**lethal concentration 50**) (Walker et al. 2012) is used. In other instances, these indicators can be adjusted to fit the specific situation; for example, using time to reach 50% mortality at a certain concentration (LT_{50}), or measuring other outcomes than mortality, such as knockdown (for example knockdown time 50 (KT_{50})) or fecundity (effect concentration 50, EC_{50}) which can be more relevant if, for example, the insecticide under consideration does not cause acute lethality at relevant concentrations (Yu 2015).

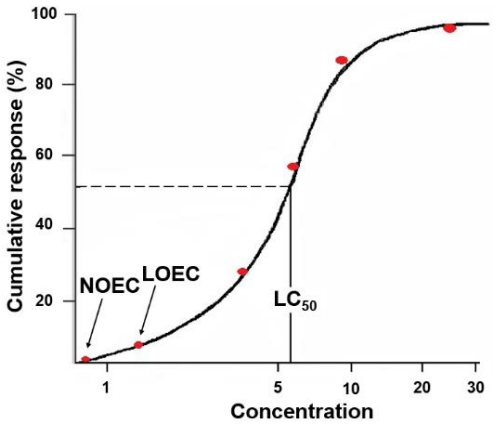


Figure 2 Hypothetical toxicity test results, NOEC being determined when LOEC is known. NOEC= no observed effect concentration. LOEC= lowest observed effect concentration. LC_{50} = lethal concentration, killing 50% of the a test population in the given time period.

Severe consequences of pollution on populations may be the result of sublethal effects, making them highly relevant to describe, as they can be expected to occur earlier, and at lower doses than lethality (see for review on sublethal effects on pesticides: Desneux, Decourtye, and Delpuech 2007). For this reason, establishing the highest dose or concentration that has no observed effect (NOED/NOEC) has increased in popularity (Walker et al. 2012). However, since the NOEC values depended strongly on experimental design, using a small effect concentration (for example EC10) as a substitute for NOEC is being increasingly popular (Chapman and Caldwell 1996). When performing these investigations, a dose-response curve is often the

starting point of the study, as illustrated in ***Figure 2***. Such toxicity data can then be compared to measured or predicted environmental concentrations in order to assess the risk of detrimental effects

When bioassays are performed, topical application (applying a solution (often in acetone) directly on the cuticle or skin of the animal) is commonly used to know the dose administered to each individual insect. However, a more realistic setup for field extrapolations is to expose insects to insecticides deposited on the surface on which they spend their experimental time (Walker et al. 2012). In addition, researchers may observe sublethal effects such as effects on mobility, which can be conveniently measured as the number of line crossings in a marked dish or time spent motionless. Modern development of video monitoring may improve precision and replicability of such research, and allows researchers to look at several behaviours and over longer time (Wallingford et al. 2017).

Types of insecticides

The most common insecticides are the organic compounds organophosphates, carbamates, organochlorides (chlorinated Hydrocarbons), pyrethroids and nicotinoids, and these will be the focus of this mini review. Other pesticides include growth disrupters (hormones) that target insect specific processes such as moulting (Retnakaran et al. 2003).

Organochlorides are a relatively large group of neurotoxic insecticides including for example DDT, aldrin, dieldrin and endosulfan. Most of these substances are highly lipophilic (they have low water solubility), very toxic to vertebrates and can be very persistent in the environment (endosulfan being a more water-soluble exception). Due to their general cause for environmental concern, these pesticides have been banned for most purposes today, but residues from their extensive use in the 1900s are still widespread in the environment, albeit in declining concentrations (Weber et al. 2010).

Organophosphates (OPs) include examples such as; chlorpyrifos, Pirimiphos-methyl and malathion. These insecticides are less persistent than organochlorides, but they are generally lipophilic. These substances inhibit the enzyme acetylcholinesterase (AChE), responsible for “resetting” the neurotransmitter acetylcholine at neuromuscular junctions and brain synapses, making these pesticides extremely neurotoxic, some even being categorized as chemical warfare agents (Casida and Durkin 2013; Upadhyay et al. 2011).

Organocarbamates include carbaryl and propoxur, and also inhibit AChE. Like OPs, they are not very persistent, but present acute toxicity hazard, also to mammals (Lushchak et al. 2018).

Some carbamates (and other pesticides) induce oxidative stress by enhancing levels of **reactive oxygen species** (ROS – molecules that are easily converted into radicals (contains at least one unpaired electron, making them harmfully reactive)) leading to neuronal damage (Fitsanakis et al. 2002; Stefan et al. 1995).

Pyrethroids are classified as type 1 (such as bifenthrin or permethrin) or 2 (such as fenvalerate, cypermethrin and cyfluthrin), based on whether they have a cyano-group in their chemical structure (type 2) or not (type 1) (Baskar and Murthy 2018). The chemical difference can affect their toxicity and interaction with other stressors; type 2 pyrethroids are generally more potent (Wadleigh et al. 1991). Based on the natural insecticide pyrethrin produced in chrysanthemum flower heads, these substances have very low water solubility and generally are readily biodegradable, but shows some persistence where it binds to soil or sediments. Pyrethroids are especially popular because they pose very little hazard to mammals, but can have detrimental effects on fish and other non-target invertebrates. Like DDT, these insecticides interact with sodium (Na^+) channels in nerve membranes, usually by prolonging their opening necessary to respond to nerve stimulus. They cause uncontrolled discharges of the nerves, seen as tremors or twitches, and can cause paralysis, when the toxin is at the active site (Casida and Durkin 2013; Palmquist, Salatas, and Fairbrother 2012).

Neonicotinoids such as imidacloprid, cause irreversible blockage of nicotinic acetylcholine receptors found in the central nervous system of insects, making them non-toxic to vertebrates (Matsuda et al. 2001). These systemic insecticides can be absorbed in plants, making them also present in pollen and nectar. These substances cause adverse effects on non-target insect pollinators, which has led to the recent ban of several types of neonicotinoid insecticides in the EU (Ihara and Matsuda 2018).

Pathways of uptake and excretion

As mentioned, a large proportion of the insecticides used each year are dispersed in the environment by, for example, runoff, leaching and aerial drift (Vryzas 2018). Insecticides can affect insects when ingested (through the alimentary canal/gut), inhaled (through trachea) or by penetrating the body surface upon contact; many synthetic insecticides can penetrate animals by all these pathways. Ingestion of insecticides or their metabolites can also be indirect, when plants or animals having already been contaminated by the substance are eaten by another animal. When substances move up trophic levels this way, it often leads to biomagnification, as a predator will eat many of its prey (Franke et al. 1994). Further passage of chemicals through membranes depends on (molecular) size, fat solubility, concentration and resemblance with

endogenous molecules. Passage takes place through passive diffusion through the phospholipids that make up most of the cell membranes, through water and ion channels, active transport through channels or endocytosis, but in toxicology, passive diffusion is most important (Walker et al. 2012).

Organisms can neutralize or minimize the deleterious effects of insecticides through **biotransformation** and excretion or deposition in less susceptible tissue (fat depots, skeleton) or in intracellular organelles (Lushchak et al. 2018; Wang et al. 2019). Biotransformation covers all processes whereby a compound is transformed chemically within a living organism. By biotransformation, both toxic and nontoxic metabolites can be synthetized. These metabolites can be activated or detoxified, often in the endoplasmic reticulum of cells, where mixed function oxidase enzymes (cytochrome P450) and other enzymes work primarily to make lipophilic compounds more hydrophilic in order to excrete the compound efficiently (Buhler and Williams 1988). Therefore, a response to insecticide exposure in many insects is the increased production of such enzymes, meaning (pesticide) stress is sometimes manifested in augmented antioxidant activity (Brogdon and McAllister 1998).

Ecological consequences of pesticides in the environment

The selection pressure exerted by prolonged exposure to pesticides can gradually eliminate non-resistant insects and reduce the genetic diversity of populations (Medina, Correa, and Barata 2007). Lower genetic variability will make insects even more fragile in the face of other stressors such as the events accompanying global warming and extreme weather (Moe et al. 2013). Increased climate variation can in turn accelerate responses to selection pressures, contributing to the evolution of pesticide resistance (Maino, Umina, and Hoffmann 2018). Details on interactions between pesticide and temperature exposure in insects is described below. Elimination of non-resistant insects contribute selecting pesticide resistant strains. This has been shown for several species, such as agricultural pests like the lesser mealworm *Alphitobius diaperinus* (Hamm et al. 2006; Renault and Colinet 2021) or the mosquito disease vectors *Anopheles gambiae* and *Aedes aegypti* (Martinez-Torres et al. 1998; Rodriguez et al. 2001). High insecticide resistance can be associated with target site modification, resulting from random mutations or selection of variants of target sites, rendering pesticides less effective on selected strains (Hawkins et al. 2018). Pesticide resistance can also involve metabolic resistance, comprising changes in enzyme and detoxification pathway expression, cytochrome P450 (CYP) being the most frequently involved enzyme family, but others, such as glutathione

S-transferases (GSTs) and cholinesterases have also been observed in this context (David et al. 2013; Hemingway and Ranson 2000; Valles and Yu 1996).

Exposure to pesticides could impair the ability of insects to adapt to new environmental conditions, because the energy allocation in pesticide resistance (detoxification mechanisms, repair of damages) can come at the expense of other defence mechanisms (Calow 1991). Furthermore, movement to avoid pesticide exposure, or increased foraging to meet increased energy demand due to detoxification and repair of damage caused by exposure to toxic substances, can increase the probability of encounters between (new) species (Congdon et al., 2001). Pesticides can also influence biotic interactions by changing how predator- prey interactions unfold. It has been shown that pesticides can change anti-predator traits such as avoiding detection or escaping after detection, for example by making the damselfly larvae *Enallagma cyathigerum* more active, but slower after insecticide exposure, resulting in increased predation mortality (Janssens and Stoks 2012). When looking at interactions between the prey, mosquito larvae *Culex pipiens*, and one of its predators, the damselfly larvae *Ischnura elegans*, predation was reduced when the tests were performed with a pesticide sensitive damselfly strain (Tran et al. 2016). Despite mosquito larvae being more active, they also moved into areas of the water column less frequented by the damselfly larvae (more towards the surface and in the water column), thereby counteracting the effects of the changed behaviour, meaning there was no observed effect on predation due to effects on the prey behaviour (Tran et al. 2016).

Pesticide and temperature interactions in insects

Encountering sub-optimal temperatures in combination with pesticide exposure is a likely threat to insect fitness in the currently changing environment. Reviews by Holmstrup et al. (2010), Hooper et al. (2013), Moe et al. (2013) and Noyes and Lema (2015) synthetizing the existing knowledge of the effects of climate change combined with environmental chemicals showed that despite considerable progress in our understanding of how temperature affects pesticide tolerance in ectotherms, interactions between temperature stress and pesticides is an understudied field. In particular, the role of fluctuating rather than constant thermal regimes urgently requires attention.

The interactions pesticides x stressful temperature result either in what is described as the effect of climatic changes on the toxicity of pesticides or “climate-induced toxicant sensitivity”, or the effect of exposure to pesticides on organisms vulnerability to climatic changes or “toxicant-induced climate change sensitivity” (Hooper et al. 2013; Noyes and Lema 2015). So far, it has often remained difficult to determine which of the two effects are observed, especially in

simultaneous exposure studies. The current knowledge in relation to temperature and pesticide interactions in insects is described in the following section.

Effect of temperature on pesticide sensitivity in insects

Temperature can affect chemical toxicity by influencing uptake rates, transformation processes and excretion rates (Buchwalter, Jenkins, and Curtis 2003; Lydy, Belden, and Ternes 1999). In addition, resource allocation and physiological imbalance provoked by thermal stress can further decrease resistance to other stressors (Gordon 2003). It is suggested that the effect of temperature on pesticide toxicity is mainly derived from effects inflicted to the metabolism (in turn affecting absorption and elimination of pesticides). Yet, the mechanisms are still poorly investigated, and when knowledge exists, it is mainly restricted to aquatic species (Camp and Buchwalter 2016; Noyes et al. 2009; Schiedek et al. 2007). Furthermore, the outcome of the temperature x pesticide interaction highly depends on the substance under investigation, the timing of exposure (sensitivity of life stage), as well as whether increase in metabolism dominantly increases degradation or bio-activation (Buès, Boudinhon, and Toubon 2003; Harwood, You, and Lydy 2009; Noyes et al. 2009).

Lydy, Belden, and Ternes (1999) found increase in acute toxicity of methyl parathion and chlorpyrifos with increasing (constant) temperatures in the midge *Chironomus dilutus*, due to increased uptake and probably increased metabolic activation rate of the substances. These results represent the expected effect of increased temperature on the toxicity of any organophosphate, as their activated form has far greater effect on acetylcholinesterase compared to the parent form, and activation rate increases with temperature. Conversely, type 1 and some type 2 pyrethroids are typically less toxic at higher temperatures in insects, as illustrated for the midge *C. dilutes* exposed to permethrin and lambda-cyhalothrin (Harwood, You, and Lydy 2009). The authors attributed this modification in toxicity to increased nerve sensitivity combined with an increased accumulation of parent compound at lower temperatures. The same pattern was illustrated for four moth larvae *Spodoptera littoralis* (cotton leafworm) strains, where organophosphorus and carbamate insecticides demonstrated increased toxicity when temperatures increased from 20 to 35 °C after insecticide treatment (topical application), whereas pyrethroids were more toxic at 20 °C than 35 °C after insecticide treatment (Riskallah 1984). When LC_{50} of 10 pyrethroids topically applied to adult male German cockroaches (*Blattella germanica*) was determined at three different temperatures, it was found that eight of them (Type 1: d-phenothrin, resmethrin, and permethrin, and type 2: fluvalinate, fenvalerate, esfenvalerate, tralomethrin, and X-cyhalothrin,) were more toxic with decreasing temperatures,

while cyfluthrin toxicity increased with temperature and cypermethrin toxicity was similar at all temperatures (Wadleigh et al. 1991). Increasing toxicity of organophosphates and decreased toxicity of pyrethroids with increasing temperature was subsequently found in several insect species (Brown 1987; Grafius 1986; Toth and Sparks 1988; Valles et al. 1998).

Using **constant temperatures and simultaneous temperature and pesticide exposure** as the studies above, more recent studies have also found increased toxicity of the organophosphate chlorpyrifos at higher temperatures, and similar findings were reported for the neonicotinoid imidacloprid (Camp and Buchwalter 2016; Dinh Van et al. 2014). Interestingly, neonicotinoids (imidacloprid and thiamethoxam) have recently been shown to be more toxic to Honey bees at lower temperatures as compared with the bees kept at 35 °C as in their hive (Saleem, Huang, and Milbrath 2020). Additionally, Op de Beeck, Verheyen, and Stoks (2018) observed increased sensitivity to chlorpyrifos at lower constant temperature, probably because they used several pulses of pesticide additions to the water, and the pesticide accumulated more at the lower temperature. This shows the importance of considering several environmental factors in research to improve risk assessment. In this respect, **using varying temperatures** rather than constant ones is more realistic for turning experimental results into environmental scenarios (Colinet et al. 2015).

Verheyen, Delnat, and Stoks (2019) showed that an increase in constant temperature had a significantly smaller effect on pesticide toxicity to the larvae of the damselfly *Ischnura elegans*, compared to an increase in temperature variation (resulting in a comparable change in average temperature). Increased toxicity with increasing temperature variation, but not average temperature, was also shown in aquatic larvae exposed to chlorpyrifos by Delnat et al. (2019) and Verheyen and Stoks (2019). To compare the effect of constant temperature to a fluctuating thermal regime (with the same average) on the midge *C. dilutus*, Willming, Qin, and Maul (2013) exposed the animals for more than a week to the pyrethroid bifenthrin, and showed that toxicity of the insecticide increased (lower LC_{50}) at varying temperature compared to the constant thermal regime. These results indicate that temperature peaks have a larger impact on pesticide toxicity than average temperature, and underline the importance of increasing the number of studies on temperature effect on pesticide toxicity in insect species using temperature peaks and fluctuations

As in all studies described above, temperature stress usually occurs simultaneously to chemical stress in the performed experiments. In a recent study, it has been shown that the chronology

of the exposure can have a strong effect on how much a heat spike increases pesticide toxicity (Meng, Delnat, and Stoks 2020b). The same research team also found that *C. pipiens* larvae were less sensitive to chlorpyrifos (mortality decreased) after they were exposed to heat spike (Meng, Delnat, and Stoks 2020a), and this could have been due to survival selection in the heat spike treatment, or to the existence of cross-tolerance. A study exposing mosquito larvae (*Anopheles stephensi* and *Aedes aegypti*) to the carbamate propoxur, after exposure to sublethal high temperatures, showed that the insects subsequently had increased tolerance to the pesticide (Patil, Lole, and Deobagkar 1996). Another study on the effect of a heat wave on subsequent pesticide exposure on *Ischnura elegans* damselfly larvae showed that survival decreased following the heat wave, although it did not appear to be connected to accumulation of stress damage or energy depletion during the heat wave as hypothesised, but to an increase in AChE inhibition (Arambourou and Stoks 2015). However, a similar experiment on the *Coenagrion puella* damselfly larvae that was also food deprived showed immediate negative effects of the heat wave, and there was only a significant decrease in survival in chlorpyrifos exposed larvae that had previously been exposed to the heat wave (Dinh, Janssens, and Stoks 2016). Again, these results underline the importance of multi-stress research, as inclusion of a new factor can change the outcome of the investigation.

Research covering a wider range of (especially terrestrial) species and life stages are also needed. Differences in outcomes in different life stages when looking at pesticide toxicity and temperature tolerance was well illustrated by Delnat et al. (2019), clearly showing that the *Culex pipiens* mosquito larvae more often showed a change in sensitivity to temperature or pesticides when combining them, than the adults of the same species.

Pesticide effect on temperature sensitivity in insects

Effects of pesticides on temperature sensitivity is even less documented than that of temperature on pesticides. A few of the studies mentioned above also showed a decrease in temperature tolerance as a consequence of pesticide exposure. Specifically, Meng, Delnat, and Stoks (2020b) showed a reduction in CT_{max} after exposing *C. pipiens* to a heat spike and then to chlorpyrifos, or to chlorpyrifos before the heat spike, the former resulting in the largest reduction in heat tolerance. Delnat et al. (2019) also showed a decrease in temperature tolerance of *C. pipiens* larvae and male adults following chlorpyrifos exposure. However, females' temperature tolerance was not affected, possibly because exposure to the used pesticide concentration was observed to be less toxic to females than males. The authors also speculate whether the decrease in heat tolerance observed was due to increased oxygen demand and decreased

oxygen supply caused by multiple stressors, leading to a metabolic mismatch at a lower temperature; the occurrence of such mismatch has been shown to often limit thermal tolerance in arthropods (Delnat et al. 2019; Verberk et al. 2016). Op de Beeck, Verheyen, and Stoks (2018) found that heat tolerance was reduced after chlorpyrifos exposure in *I. elegans*, but not in the closely related, but comparably small and fast paced damselfly species *Ischnura pumilio*, further underlining the importance of considering species, or their specific traits, when predicting outcomes of stress exposures. Op de Beeck, Verheyen, and Stoks (2017) showed that the reduction in CT_{max} after pesticide exposure occurred in *I. elegans* larvae from different latitudes and temperature regimes (constant 20 vs 24 °C), but the reduction was larger in animals kept at lower temperatures, again because of higher accumulation of pesticide in the water at lower temperature.

The research presented above indicate that global warming can be expected mainly to affect pesticide tolerance by influencing environmental degradation as well as animal uptake and elimination, and not through resource depletion (unless food supply is scarce). Furthermore, it seems that pesticide exposure can reduce heat tolerance; the underlying mechanisms are very poorly investigated, and the ultimate increase in oxygen consumption when coping or recently having coped with pesticide exposure could be one of the main drivers of this observed reduction in heat tolerance. For specialist species, who generally live close to their tolerance thermal threshold, this interaction could prove to be all the more harmful (Deutsch et al. 2008).

Effects in insects of freezing temperatures on pesticides and vice versa have not been described, which could be because it is less relevant to investigate, as pesticide applications usually occur at times of warmer temperatures (plant growth season). However, many pesticides persist in the environment, making winter exposure studies important. Another possible explanation is the bias towards publication of effect studies, as for example a study testing effects of two pesticides (abamectin and carbendazim) on freeze tolerance in an earthworm showed no interaction (Bindesbøl et al. 2009). In conclusion, especially effects of pesticides on thermal tolerance are scarcely documented, and more effect studies should be published for ecologists to get a realistic idea of effects of these interactions, for extreme cold and hot weather scenarios, in the future.

This thesis

Aims and hypotheses

The aim of chapter 2 was to investigate if I could uncover an association between altitudinal location of populations of an alien species, and their temperature tolerance. In addition, I studied if there was a pattern of temperature tolerance following the time of colonization of different populations. I hypothesized that animals from increasing altitude would have higher cold tolerance, and that populations showing particularly strong tolerance to cold would be less tolerant to heat and vice versa. Finally, we hypothesized that we would find a wider temperature tolerance range in individuals collected closer to the invasion front, as a part of a dispersal syndrome.

Chapter 3 aims to first describe the tolerance of *Alphitobius diaperinus* to sudden exposure to extreme heat or cold in terms of ability to recovery, and how long recovery from different exposures would take. In addition, I investigated how exposure to pesticide and daily heat peaks (separately and simultaneously) could affect the animals' subsequent tolerance to extreme temperatures. Lastly, the ability of different populations to withstand pesticide exposure in combination with daily heat peaks, and effects of treatment on the animals' metabolome, antioxidant activity and reproduction was investigated.

In Chapter 4 I describe the desiccation resistance in *Alphitobius diaperinus*, and my aim was to uncover how exposure to dry conditions can affect the biology of this beetle in terms of longevity, activity, water content, metabolic profiles and fecundity. I hypothesized that animals in dry conditions would have shorter longevity. I further expected the animals to lose water over time, but at a very low rate, and I expected the animals to upregulate sugars or other metabolites in order to resist desiccation. I expected to observe a reduction in activity of the animals kept in dry conditions, and a decrease in fecundity following exposure to a dry period.

Biological models

Merizodus soledadinus

As mentioned in the introduction, changes in traits that follow a pattern of the residence time of individuals at a locality have been described for several species, and changes in thermal tolerance of animals have also been found to follow dispersion. However, few examples describing such phenomenon's have used alien species. However, Laparie et al. (2013) showed that the beetle species *Merizodus soledadinus*, introduced to the sub-Antarctic Kerguelen Islands from the Falkland Islands in 1913 (Jeannel 1940), displayed a consistent relationship between residence time of populations (the invasion gradient) and morphology. Now the

question rose whether this species' temperature tolerance also differed along the invasion gradient, which is the main gap of knowledge covered in chapter one of this thesis. The species is well suited for such a question as it has colonized several locations in the time since its introduction, more so in the recent two decades, and it is spreading in a geographical area that sees relatively little human activity.

Alphitobius diaperinus

In order to investigate my remaining research questions, I used the tenebrionid beetle, *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae). This insect is a pest in many poultry houses worldwide and is an especially fitting model for investigations on pesticide effects on the individuals, as we know that several populations are resistant to insecticides. Knowledge about the biology of *A. diaperinus* is of great economic importance, as it serves as a potential reservoir for avian pathogens (Casas, Harein, and Pomeroy 1972; Dinev 2013; McAllister et al. 1995) and can cause physical damage to poultry house walls (Turner 1986). As failures to control infestations occur due to resistance to pesticides in *A. diaperinus* populations (Hamm et al. 2006; Lambkin and Rice 2009; Renault and Colinet 2021), the importance of knowing responses of this species to environmental changes and stress increases, to predict its future distribution as well as improving pest control efficiency (Wolf et al. 2015).

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Chapter 2

Investigating how thermal traits change
along an invasion gradient or with altitude in Kerguelen

Thermal tolerance patterns of a carabid beetle sampled along invasion and altitudinal gradients at a sub-Antarctic island

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Abstract

The extent to which an insect can cope with changes in its thermal environment is an important predictor of its geographical distribution and thermal tolerance. During range expansion, invasive insect populations are often characterized by rapid niche shifts reflected by the colonization of a wide range of habitats distinct from those of their native regions. Yet, the potential changes of thermal tolerance of range expanding species have been poorly examined so far. Here, we investigated the cold and heat tolerance of different populations of the carabid beetle *Merizodus soledadinus* invading the Kerguelen Islands. In this archipelago, the geographic expansion of *M. soledadinus* has sorted populations according to residence time and altitudinal, and seashore – inland gradients, thus allowing to examine if invasion process (dispersal syndrome) and colonization of altitudinal habitats could be associated with shifts in thermal sensitivity of the insects. We compared the recovery ability of adult *M. soledadinus* after they were exposed to cold (-5 to -8 °C) or heat (36 to 38 °C) temperatures for 15 min. We found that thermal niche of *M. soledadinus* was not expanded along the invasion gradient. While cold tolerance tended to increase for insects sampled at higher altitudes in one transect, this finding was not generalizable to the second altitudinal gradient. Potential sources of the observed differences in thermal tolerance are discussed, especially in relation to an apparent pattern that most populations are best at coping with either cold or heat, and a few do not excel at recovering from either, compared to other populations of the species. This leads us to believe there are tradeoffs between having comparatively high tolerance to both hot and cold and other un-investigated traits.

Keywords:

Biological invasions, climate change, temperature tolerance, heat, cold, chill coma recovery, invasion gradient, altitudinal gradient, *Merizodus soledadinus*

1 Introduction

The ectothermic nature of insects implies that all aspects of their life are shaped by temperature and its variation. As a result, thermal tolerance (the degree of tolerance of an individual to thermal extremes), in parallel with the plasticity of this trait (the capacity to adjust thermal tolerance through acclimatization, are important predictors of the geographic distribution and sensitivity to global warming of organisms (Andersen et al., 2015; Buckley and Huey, 2016; García-Robledo et al., 2016; Hoffmann et al., 2013). In addition, considering variation in temperature tolerance between populations within species can be used as an indicator of potential abundance and distribution in response to climate change (Sorte et al., 2011). Indeed, thermal tolerance, and the underlying physiological mechanisms permitting adaptation to temperature changes and extremes, represent important traits under selection (Angilletta et al., 2002), particularly for range-shifting and invasive species.

Cold and warm thermal extremes determine the temperature tolerance range over which the performance of an insect can be measured, the so-called thermal performance curve. Using mathematical models, Hillaert et al. (2015) concluded that thermal performance curves of invasive insect populations matched local thermal conditions. Most importantly, they reported that the thermal ranges of individuals were increased at the expansion front, thus sorting the population according to the value of this trait along the invasion gradient; an invasion gradient refers here to increased residence time of the individuals among different colonized localities. However, despite the numerous examples of rapid changes in individual traits along an invasion gradient (Fronhofer and Altermatt, 2015; Laparie et al., 2013; Ochocki and Miller, 2017), few authors have examined potential changes in thermal tolerance in range expanding alien species (Renault et al. 2018).

Thermal biology of insects may vary greatly among ecotypes (individual x habitat characteristics) (Diamond et al., 2017; Sinclair et al., 2012; Tougeron et al., 2016), as the level of thermal tolerance is not only dependent on environmental temperature exposures but is also influenced by the capacity of individuals to tolerate other environmental factors (Gotcha et al., 2018; Renault et al., 2015) and by the quality of available trophic resources

(Colinet et al., 2013). At cellular and molecular levels, a certain uniformity of stress responses can be observed in organisms facing different abiotic stressors (Sulmon et al., 2015), also showing that activating responses against one stress can also enhance the ability to cope with other stress (cross-tolerance; Sinclair et al., 2013; Everatt et al., 2014).

The carabid beetle, *Merizodus soledadinus*, is an invasive insect in the sub-Antarctic Kerguelen Islands in the Indian Ocean. It was introduced from the Falkland Islands to Port-Couvreux (Kerguelen Islands) in 1913 (Arnaud and Beurois, 1996; Jeannel, 1940), where it has now colonized several locations, creating populations with different times of origin, from 1913 to 2015 (Figure 1), and phenotypes (Laparie et al., 2013, 2010; Lebouvier et al., 2011). During its continuing range expansion in the Kerguelen Islands, populations of *M. soledadinus* have occupied a variety of habitats, ranging from the seashore to herbfields and bare soil (Renault et al., 2015), whose native species richness and environmental conditions vary markedly. The species has also increased its altitudinal range: while no individuals were observed above 110 meters above sea level (m a.s.l.) in 1997 (Chevrier et al., 1997), they can now be found up to 400 m a.s.l. ("Subantarctic fauna and flora database, Program IPEV 136 'SUBANTECO'). This recent colonisation of higher altitude habitats has likely been favoured by the clear warming trends documented at the Kerguelen Islands (Lebouvier et al., 2011).

Few studies have examined the temperature sensitivity and thermal tolerance of *M. soledadinus* (Block and Sømme, 1983; Lalouette et al., 2012; Laparie and Renault, 2016; Ottesen, 1990). Lalouette et al. (2012) examined the thermal sensitivity of adult *M. soledadinus* sampled at the research station (Port-aux-Français) on the Kerguelen Islands. They concluded that the insect has moderate cold tolerance (in the context of the austral origin of the species), with critical thermal minimum (CT_{min}) around -6.5 °C. Conversely, heat tolerance of the insect greatly exceeded any temperature recorded at the Kerguelen Islands, with a CT_{max} of about 37 °C.

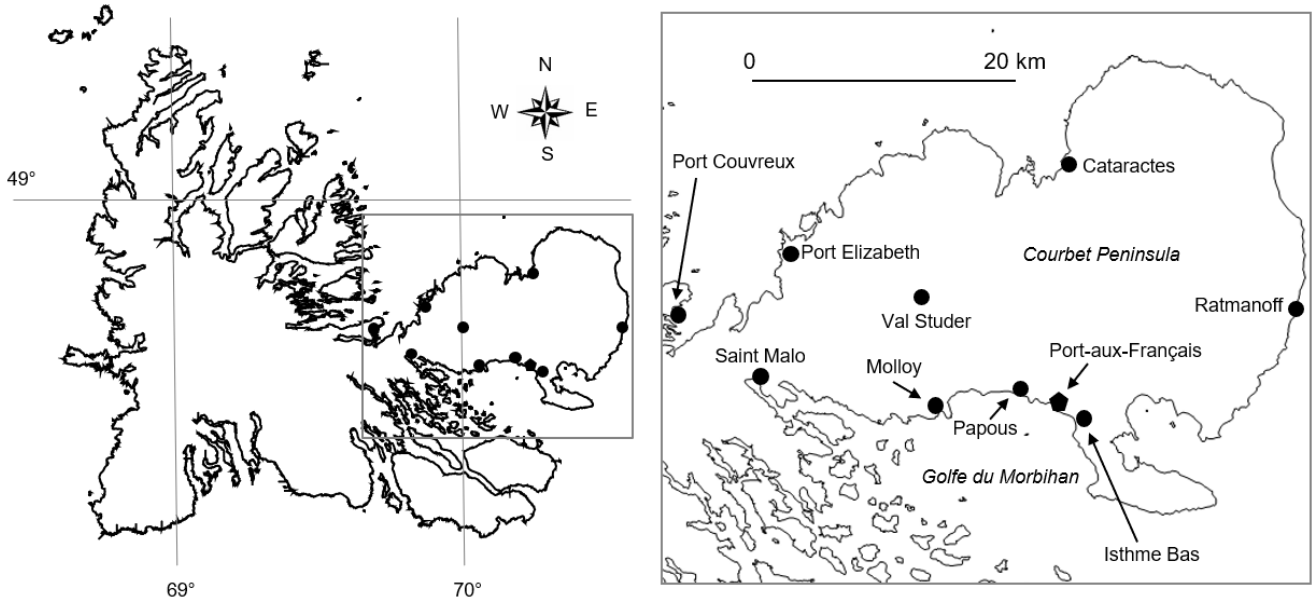


Figure 3 Map of the Kerguelen Islands indicating the localities where populations of the carabid beetle Merizodus soledadinus were sampled. Port Couvreux (introduction site of M. soledadinus in 1913), Port Elizabeth (colonized by M. soledadinus in 1939-1983), Cataractes (colonized in 1983 – 1990), Saint Malo (colonized in 1991-1995), (Anse de) Papous (colonized in 1995-2000), Isthme Bas (colonized in 2000-2005), Molloy (colonized in 1995-2005), Val Studer (colonized in 2014-2016) and Ratmanoff (colonized in 2016)

The aim of this study was to compare temperature tolerance among populations of the carabid beetle, *Merizodus soledadinus* (Guérin-Ménevile, 1832), sampled along invasion (time of colonisation) and altitudinal gradients at the French sub-Antarctic Kerguelen Islands. We assumed that temperature average and variation were similar across non-altitudinal sampling locations, and that temperature average decreases by 0.65 °C with each 100 m elevation (Ahrens, 2009; Dillon et al. 2006). We predicted that this temperature difference along a small altitudinal transect should result in the collection of more cold tolerant insects when sampling at higher altitudes. Furthermore, we collected individuals along a transect inland from the coast, in an area where the elevation was low, to investigate if there was a separate effect of distance to the sea. To compare temperature tolerance among insect populations, we used the time to recover after cold or heat exposure (Hoffmann et al., 2003). By investigating responses to both thermal extremes in populations of *M. soledadinus*, we hypothesised that a trade-off may be found in certain populations, tolerant to heat stress and more sensitive to cold stress, and *vice versa*, while some populations may show reasonable performance at both extremes, without excelling under any conditions. This expectation is typical of species distributed across environmental gradients (Addo-Bediako et al., 2000). More specifically, we hypothesized, first, that the

spatial sorting of populations of *M. soledadinus* would result in different levels of thermal tolerance, with individuals from the invasion front exhibiting a higher temperature tolerance range as a part of a dispersal syndrome. Second, we hypothesized that the colonization of higher altitude habitats has been favoured by global warming, and has resulted in the selection of the most cold-tolerant individuals, that then further acclimatised and/or adapted to cooler habitats.

2 Methods

2. 1 Insect collection

Adults of *M. soledadinus* were hand-collected from nine distinct locations in the eastern part of the Kerguelen Islands: Cataractes, Isthme Bas, Molloy, (Anse de) Papous, Port Couvreux, Port Elizabeth, Ratmanoff, Saint Malo and Val Studer (Figure 1). A brief habitat description was made at each site before insects were collected. Insect collection was carried out using aspirators, and insects were directly transferred into plastic containers (L: 17.5 cm, W: 11.5 cm, H: 7 cm), with a piece of paper tissue (approx. 20cm x 20cm) provided for shelter. The insects were then returned to the research station at Port-aux-Français. They were supplied with water in a small tube (approx. 3 mL) with cotton wool, and placed for 16-36 h in a cabinet at a constant 4 °C before commencement of experiments. This was considered sufficient for the insects to recover from the transport phase, but not too long for them to acclimate to the laboratory conditions.

2. 1. 1 Invasion gradient

Six of the distinct collection locations for *M*. *soledadinus* populations were characterized by different duration of residence since colonisation: Port Couvreux (introduced in 1913), Port Elizabeth (colonized between 1939 and 1983), Cataractes (colonized between 1983 and 1990), Isthme Bas (colonized between 2000 and 2005), Val Studer (colonized between 2014 and 2016) and Ratmanoff (colonized in 2016). There is no evidence that *M. soledadinus* has been introduced to the Kerguelen Islands on several distinct occasions, and therefore we consider that all contemporary populations of *M. soledadinus* have originated from the population of Port Couvreux. For ease of overview of the residence times of the different populations, each location name was given a number corresponding to their minimum

residence time (years) at the time of collection of the insects (2018): Port Couvreux 105, Port Elizabeth 35, Cataractes 28, Isthme Bas 13, Val Studer 2, and Ratmanoff 2.

2. 1. 2 Altitudinal and lowland gradients

At Saint Malo and Molloy, insects were collected along an altitudinal gradient up to 250 m above sea level (a.s.l.). At Saint Malo, three populations were collected from different elevations (0-5 m, 95-110 m and 190-210 m a.s.l.) and given the corresponding names: Saint Malo 0, Saint Malo 100 and Saint Malo 200. At Molloy, four populations were collected at three different elevations (0-5 m, 110-130 m and 245-250 m a.s.l.), with two distinct habitats being sampled at around 125 m a.s.l., one having less vegetation (Molloy 125) than the other (Molloy 125 Vegetation).

At Papous, a horizontal seashore – inland transect was completed, with sampling at different distances from the coast (approximately 2-15 m, 185-215 m, 385-415 m and 785-815 m): Papous 0, Papous 200, Papous 400 and Papous 800 respectively.

2. 2 Measurement of the duration of recovery from cold and heat exposures

Empty Petri dishes lined with paper were placed into an incubator. After approximately 10 min, five individuals were added into each of 4-5 Petri dishes, and covered with lids; the cabinet was closed and a timer started. After 15 min exposure to a given temperature, the dishes were removed from the incubator, rapidly transferred into a walk-in chamber at 9 ± 1.5 °C, and a new timer was started. The number of insects in coma, and number of recovered insects (walking ability restored) were subsequently noted every minute, over 30 min (The recovery period was monitored for up to 120 min in a preliminary test, and no change in the number of recovered beetles was observed as compared with 30 min). Any individual that had not recovered after 30 min was considered dead. This procedure was repeated for each population and temperature treatment. All populations were exposed to -6, -7 or 37 °C for 15 min. The duration of exposure of 15 min was selected after having performed preliminary tests, with insects being exposed at these three temperatures for different durations (from 5 to 120 min; data not shown). Depending on the number of insects available for each population, the remaining adults were exposed to up to eight distinct temperatures (-5, -6, -7, -8, 35, 36, 37 and 38 °C) for 15 min; this additional experiment ensured that we investigated temperatures that were close to the thermal limits of the species, and that any difference in temperature tolerance would be identified.

To test for the effect of prolonged duration of exposure on the subsequent recovery, further assays conducted at -6 °C were completed with duration of exposure of the adult *M. soledadinus* of 1 or 2 h in addition to the 15 min (0.25 h) treatment for the populations of the altitudinal (Molloy, St Malo) and inland-shore (Papous) gradients. During the experiments, each insect was only used once (*i.e.* exposed to one temperature and duration combination on a single occasion).

2. 3 Statistical analysis

All curves were generated based on Kaplan Meier estimates computed in R version 3.5.2, using the Survminer package (Kassambara and Kosinski, 2018; R Core Team, 2018). The curves were estimated based on the time of recovery of 20-25 individuals for each population. Pairwise comparisons with adjusted P-values (Benjamini & Hochberg) were performed *post-hoc*, with a significance value of 0.05.

3 Results

3. 1 Recovery of adult *M. soledadinus* sampled along an invasion gradient transect

The recovery dynamics of 20 adult *M. soledadinus* from six different populations after exposure to -7 °C or 37 °C for 15 min are presented in Figure 2A, B. There were significant differences in recovery dynamics and temperature tolerance among insects from the different populations (-7 °C: $P<0.0001$; 37 °C: $P=0.0018$), but these differences were not related to residence times of the different populations. The populations from Isthme Bas 13 and Cataractes 28 were more sensitive to cold than individuals from the four other populations (Val Studer 2, Port Elizabeth 35, Ratmanoff 2 and Port Couvreux 105), whose levels of cold tolerance were similar (Figure 2A).

Adults of *M. soledadinus* from Ratmanoff 2 and Val Studer 2 were significantly more sensitive to heat exposure (*i.e.* insects exhibited more limited ability to recover after 15 min exposure at 37 °C) compared to those collected from Cataractes 28 (Figure 2B) (Ratmanoff:Cataractes, $P=0.002$; Val Studer:Cataractes, $P=0.047$). At the end of the recovery period, the proportion of insects from Ratmanoff 2 that recovered was relatively low after heat shock (*ca.* 20% recovery after 30 min), whereas *ca.* 60% of the insects from Port Elizabeth 35 and Port Couvreux 105 recovered, and *ca.* 42% from Isthme Bas 13 and Val Studer 2 (Figure 2B).

The recovery dynamics of adult *M. soledadinus* that were exposed to other temperature conditions (-5, -6, 35, or 36 °C) are presented in Supp Figure 1.

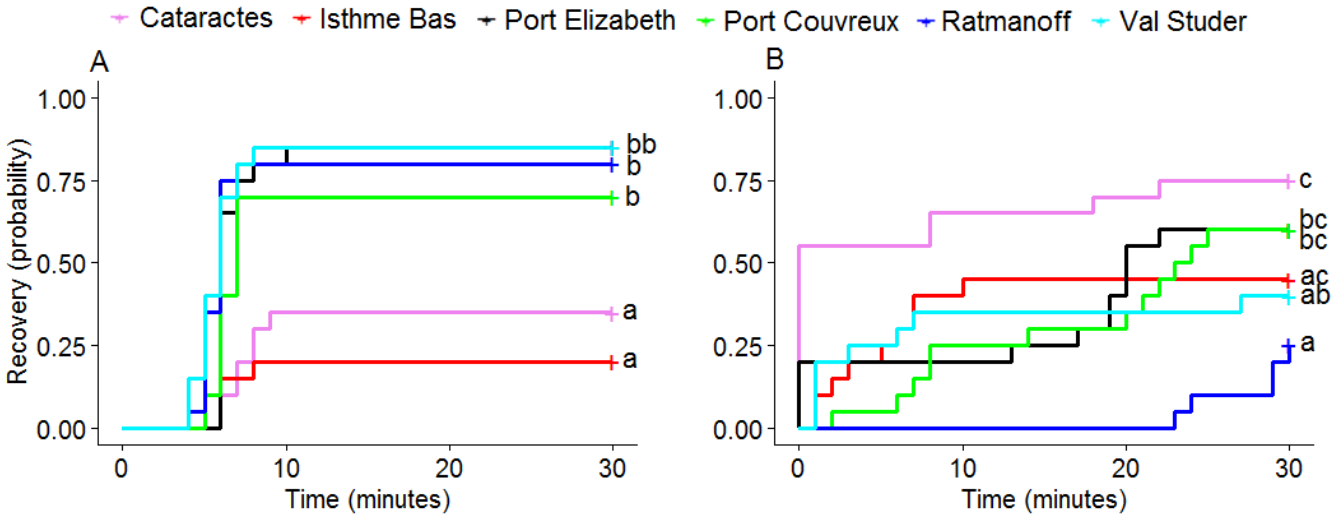


Figure 2 Recovery curves of adult Merizodus soledadinus from six different populations sampled along an invasion gradient at the Kerguelen Islands after they were exposed to A) -7 °C for 15 minutes or B) 37 °C for 15 minutes. Recovery curves were estimated using the Kaplan Meier method; vertical tick-marks represent insects that did not recover at the end of the 30 min recovery period ("right censored data"). Distinct letters indicate different recovery dynamics among the populations (log rank test with adjusted P-values (Benjamini & Hochberg) for pairwise comparisons.

3. 2 Recovery of adult *M. soledadinus* sampled along an altitudinal gradient

The ability to recover from cold or heat exposure in insects sampled along an altitudinal gradient at Saint Malo and Molloy is presented in Figures 3 and 4, respectively. All populations collected from Saint Malo exhibited a similar ability to recover from cold or heat exposure (Figure 3A, B) (-7 °C: P=0.056; 37 °C: P=0.17). A trend was observed in the cold treatment recovery curves (Figure 3A) where, the higher altitude the population was collected from, the more individuals were able to recover.

As shown in Figure 4A, B, there were no significant differences in the ability to recover from extreme low or high temperature exposure in any of the Molloy populations (-7 °C: P=0.13 (4A; 37 °C: P=0.063). The recovery dynamics of the individuals from Saint Malo that were exposed to other temperature conditions (-5, -6, 36 or 38 °C) are presented in Supp Figures 2. The recovery dynamics of the individuals from Molloy that were exposed to other temperature conditions (-5, -6, -8, 35, 36 or 38 °C) are presented in Supp Figures 3.

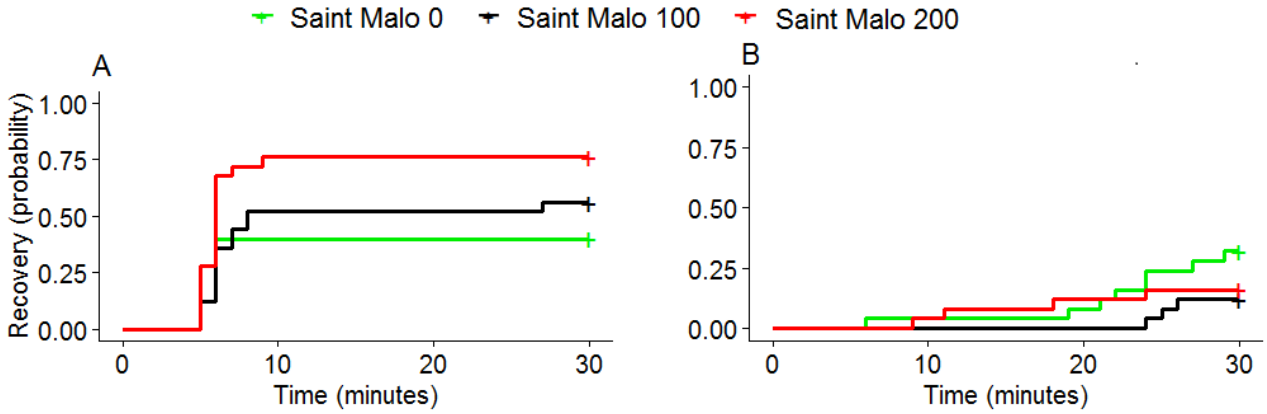


Figure 3 Recovery curves of adult Merizodus soledadinus from three different populations sampled along an altitudinal gradient at the Kerguelen Islands after the insects were exposed to A) -7 °C for 15 minutes (The recovery curves were not significantly different, P = 0.056). B) 37 °C for 15 minutes (The recovery curves were not significantly different, P = 0.17). Recovery curves were estimated using the Kaplan Meier method; vertical tick-marks represent insects that did not recover at the end of the 30 min recovery period ("right censored data").

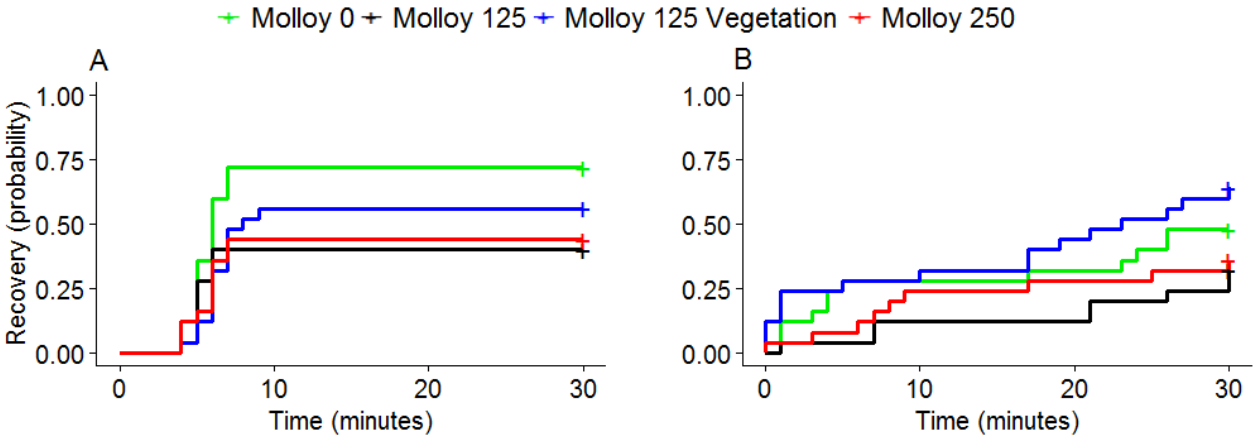


Figure 4 Recovery curves of adult Merizodus soledadinus from four different populations sampled along an altitudinal gradient at the Kerguelen Islands after they were exposed to A) -7 °C for 15 minutes (The recovery curves were not significantly different, P = 0.13). B) 37 °C for 15 minutes (The recovery curves were not significantly different, P = 0.063). Recovery curves were estimated using the Kaplan Meier method; vertical tick-marks represent insects that did not recover at the end of the 30 min recovery period ("right censored data").

3. 3 Duration of recovery of adult *M. soledadinus* exposed for different time periods

The recovery times of 25 individuals from the Molloy populations after they were exposed to -6 °C for 0.25 h, 1 h or 2 h are shown in Figure 5. For populations sampled at Molloy 0 (Figure 5A), Molloy 125 (Figure 5B) and Molloy 125 Vegetation (Figure 5C), the recovery dynamics of the insects exposed to cold for the three durations did not differ significantly (0 m: P=0.17; 125 m rocky habitat: P=0.076; 125 m vegetation: P=0.19). However, there was a significant difference between recovery curves from the Molloy 250 population after 1 h exposure as compared with those exposed for 0.25 h at -6 °C (P=0.002). This latter population was not exposed to -6 °C for 2 h, due to lack of available individuals. No significant difference was observed among the recovery curves when the insects from the altitudinal transect at St Malo and the seashore-inland transect at Papous were exposed to - 6 °C for different durations (Supp Figures 4 and 5) (0 m [Supp Fig. 4A]: P=0.29; 100 m [Supp Fig. 4B]: P=0.08; 200 m [Supp Fig. 4C]: P=0.56; 0 m [Supp Fig. 5A]: P=0.2; 200 m [Supp Fig. 5B]: P=0.48; 400 m [Supp Fig. 5C]: P=0.24; 800 m [Supp Fig. 5D]: P=0.44).

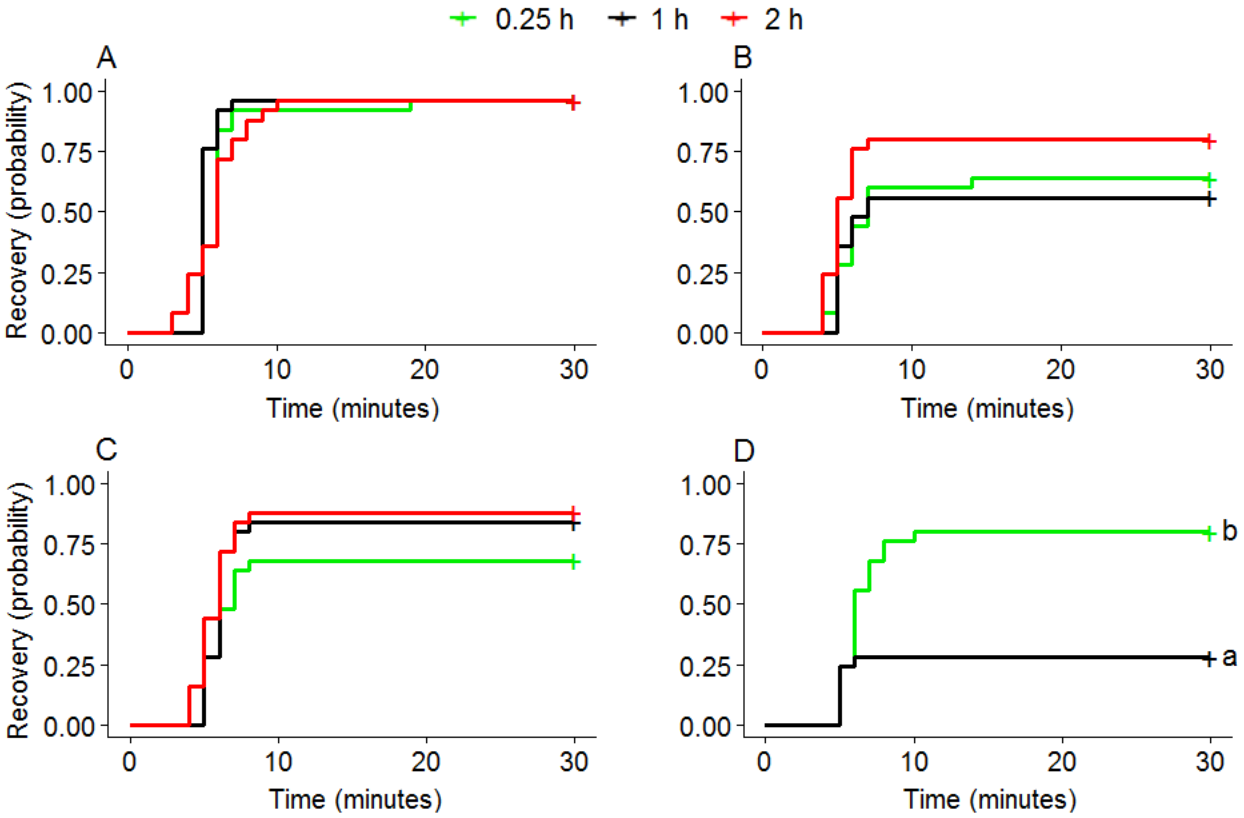


Figure 5 Recovery curves of adult Merizodus soledadinus from four populations sampled along an altitudinal gradient at Molloy (Kerguelen Islands) after they were exposed for increasing periods (0.25 h, 1 h, 2 h) to -6 °C A) Individuals sampled at 0 m a.s.l.; the recovery curves were not significantly different (P = 0.17). B) Individuals sampled at 125 m a.s.l. in a rocky habitat; again, no significant difference between the recovery curves (P = 0.076). C) Individuals sampled at 125 m a.s.l in a vegetated habitat; again, no significant difference between the recovery curves (P = 0.19). D) Individuals sampled at 250 m a.s.l.; distinct letters indicate significant differences between recovery curves (log rank test with Benjamini & Hochberg adjusted P-values for pairwise comparisons). Recovery curves were estimated using the Kaplan Meier method; vertical tick-marks represent insects that did not recover at the end of the 30 min recovery period ("right censored data").

3. 4 Duration of recovery of adult *M. soledadinus* sampled along a seashore-inland transect

Populations collected from Papous were exposed to -7 °C (Figure 6A) and 37 °C (Figure 6B) for 15 min. Insects from the Papous 800 population were characterized by a significantly higher recovery after exposure to -7 °C for 15 min than those from Papous 200 (Figure 6A) (P=0.02). There were no significant differences among the recovery curves from different heat exposures (Figure 6B) (P=0.19). The recovery dynamics of adult *M. soledadinus* that

were exposed to other temperature conditions (-5, -6, -8, 35, 36 or 38 °C) are presented in Supp Figure 6.

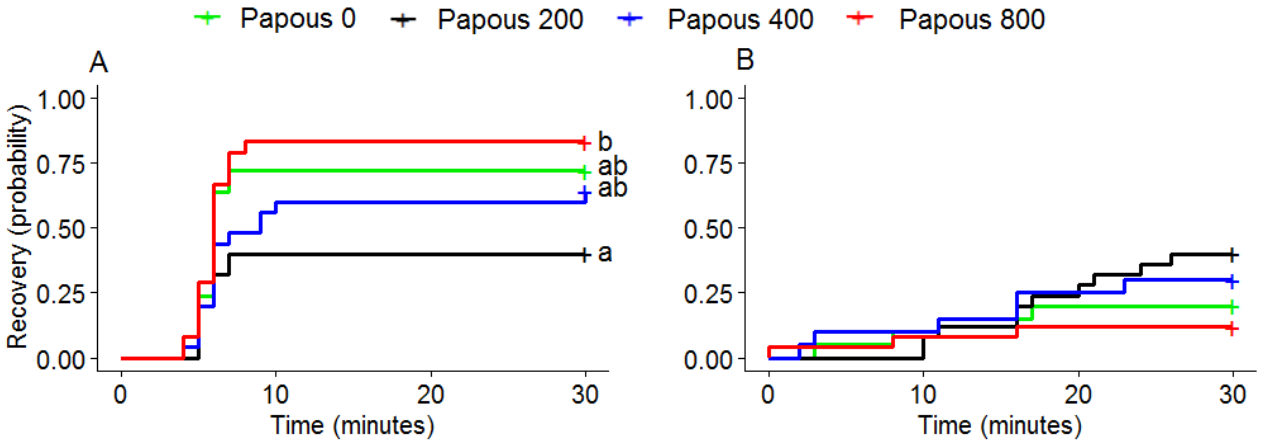


Figure 6 Recovery curves of adult Merizodus soledadinus from four different populations sampled along an inland - seashore transect at the Kerguelen Islands after they were exposed to A) -7 °C for 15 minutes. Distinct letters indicate different recovery dynamics among the populations (log rank test with adjusted P-values (Benjamini & Hochberg) for pairwise comparisons. B) 37 °C for 15 minutes (The recovery curves were not significantly different, P = 0.19). Curves were estimated using the Kaplan Meier method; vertical tick-marks represent insects that did not recover at the end of the 30 min recovery period ("right censored data").

4 Discussion

In the present study, we examined the thermal sensitivity (to cold and heat) of the carabid beetle *M. soledadinus*, which is invasive to the Kerguelen sub-Antarctic Islands. In the Kerguelen archipelago, the progressive range expansion of *M. soledadinus* has generated invasion, residence time, altitudinal, and seashore – inland gradients, allowing examination of whether the invasion process (dispersal syndrome) and colonisation of higher altitude and inland locations are associated with shifts in thermal sensitivity.

4. 1 Thermal sensitivity of *M. soledadinus* adults sampled along an invasion gradient

A large range of habitats have been colonized by *M. soledadinus* in the Kerguelen Islands, and it has been hypothesized that this invasion success is facilitated by the trophic and physiological plasticity of the insect (Hidalgo et al., 2013; Laparie et al., 2012). In this study, measurement of the thermal sensitivity of adult *M. soledadinus* revealed two main recovering groups after cold exposure, and a wide range in ability to recover after heat exposure, among populations. The differences in thermal tolerance among geographically distinct populations have likely been shaped by the different biotic and abiotic filters

encountered by the insects during range expansion, and by genetic drift, as small insect groups are likely to have split off and established new colonies when *M. soledadinus* spread across the island.

We infer that the degree of stress resistance, including thermal tolerance, of the insects has increased along the invasion gradient, as this would enhance the probability of successfully reaching new habitats in heterogeneous environments. Consistent with this, individuals sampled at the invasion front (Val Studer 2 and Ratmanoff 2) exhibited high ability to recover from cold exposure, even though their cold tolerance levels were similar to those of individuals from the longer-established populations at Port Elizabeth 35 and Port Couvreux 105. The colder winter conditions that were typical until late 1970s may have shaped the thermal sensitivity of the populations from habitats that have been colonized longer, in turn explaining the comparatively high recovery abilities in individuals from the populations of Port Couvreux 105 and Port Elizabeth 35. The mean minimum winter temperature was -5.9 °C over the years 1951-69, with frequent occurrence of temperatures lower than – 7 °C. Since then, climatic conditions have progressively changed at the Kerguelen Islands (see also Lebouvier et al. 2011), and mean minimum temperatures increased to -4.6 °C from 1970 to 2017, and even higher (-3.6 °C) in the period 1990-2004 coinciding with when the geographic expansion of *M. soledadinus* greatly increased (Météo France data, https://donneespubliques.meteofrance.fr/, records from Port-aux-Français, Kerguelen Islands).

Since the 1970s, the progressively warming climate across the sub-Antarctic islands (Berthier et al., 2009; Frenot et al., 1993) may have facilitated the geographic expansion of *M. soledadinus* in the Kerguelen Islands. Selection for cold tolerance may have become less intense, at least at low altitudes, while other environmental factors, such as salinity in coastal habitats and desiccation resistance (see for instance Hidalgo et al. (2013), Ouisse et al. (2016) and Yerushalmi et al. (2016)) may have driven the physiological performance of the range-expanding populations of *M. soledadinus*. It is noteworthy that both the well-established population of Cataractes 28, and Isthme Bas 13 are relatively sensitive to cold compared to the other populations, and are thus not following a pattern of increased cold tolerance along the invasion gradient. In parallel, individuals from these two populations are equally or more resistant to heat exposure when compared to individuals from Port Couvreux 105 and Port Elizabeth 35. Studies that have investigated temperature acclimation

under field conditions provide evidence that enhanced thermal tolerance could lead to clear benefits for individuals, but also costs in the case of exposure to high temperatures (Chidawanyika and Terblanche, 2010; Kristensen et al., 2007; Loeschcke and Hoffmann, 2007; Sørensen et al., 2013). In addition, Dewitt et al. (1998) and Auld et al. (2010) described how fitness costs can be associated with the level of adaptive phenotypic plasticity for a certain trait. If such costs apply to the thermal tolerance of *M. soledadinus* in the Kerguelen Islands (the higher the plasticity of thermal tolerance, the higher the fitness cost), this would explain the observation that many populations showing somewhat higher ability to recover from cold also have lower ability to recover from heat, and *vice versa*. However, no such trade-off between cold and heat tolerance was found in a winter acclimatized Danish *Drosophila subobscura* population (Givskov Sørensen et al., 2015), and the absence of this trade-off in populations of *M. soledadinus* from Port Couvreux 105 and Port Elizabeth 35 suggests that additional variables shape the thermal breadth of these insects.

4. 2 Adult *M. soledadinus* sampled along an altitudinal gradient

The colonization of higher altitude habitats may have been favoured by recent climate change. However, the climatic conditions encountered at moderate elevations at the Kerguelen Islands may remain sub-optimal for adult *M. soledadinus*. We thus hypothesized that populations from higher altitudes would be more cold tolerant than those collected closer to sea level. Although no statistically significant difference was detected among the recovery curves of the different populations sampled along the altitudinal gradient at Saint Malo, the curves fall in sequence from the highest location (Saint Malo 200) to the location closest to sea level (Saint Malo 0) in terms of recovery ability from cold exposure. In bumble bees, the critical thermal minimum was lower and the insects recovered at lower ambient temperatures when they were sampled at high altitudes (Oyen et al. 2016), as also reported in the copper butterfly *Lycaena tityrus* (Karl et al. 2008); however, these two studies compared the thermal tolerance of specimens collected from low to high altitudes on a larger altitudinal gradient than in the present work.

A different recovery pattern was observed for the individuals collected from the second altitudinal transect (Molloy), with fewer individuals from Molloy 250 and Molloy 125 recovering from exposure to either high or low temperature compared with those collected

at Molloy 0 and Molloy 125 vegetation. These findings suggest that habitat quality influenced temperature tolerance in these populations as distinct from elevation *per se*. The locations where Molloy 0 and Molloy 125 Vegetation were collected had a larger proportion of vegetation, and can therefore also be expected to give access to more nutrient-rich and varied habitats. Thermal tolerance physiology can be influenced by the quality of trophic resources as reported by Colinet et al. (2013), who found that sugar-enriched diets increased chill coma recovery ability in the fruit fly *Drosophila melanogaster.* In other studies, low diet quality had no effect on the critical thermal maximum of the chrysomelid *Cephaloleia belti* (Garcia-Robledo et al., 2018), while the feeding status (fed *versus* starved insects) of the fly *Ceratitis capitata* (Nyamukondiwa and Terblanche, 2009) affected their thermal tolerance, and starvation lowered the supercooling point - and increased cold tolerance - in the beetle *Alphitobius diaperinus* (Salin et al., 2000). The overall quality of the environment can contribute to temperature tolerance in insects, either directly, or by eliminating some level of multi-stress exposure occurring when factors such as starvation or dehydration coincide. Finally, vegetation cover makes ground temperature more similar to air temperature than in rocky habitats where insects may be more exposed to heat stress because of high level of radiation (Buckley et al. 2013).

4. 3 Adult *M. soledadinus* sampled along a seashore-inland transect and exposed for different time periods

The seashore-inland transect was established in order to identify any effects of the distance to the sea on the thermal tolerance of adult *M. soledadinus*. The distance to the coast did not systematically affect temperature tolerance: the four populations sampled at Papous had very similar recovery curves after exposure to high temperature, but the pattern of the graphs could suggest a trade-off with cold tolerance. As the different populations sampled at Papous were physically closer to each other compared to the other studied populations, and the ground between them apparently without obstruction, it is possible that movement of individuals between populations at Papous 0, Papous 200 and Papous 400 was sufficient to obscure any differences between samples collected from the different locations. Moreover, while landscape composition has been reported to affect the thermal tolerance of parasitic wasps (Tougeron et al. 2016), micro-scale characteristics of the sampled habitats may have remained under oceanic influence, and thus under salinity gradients (Herbst 2001) that constrain the thermal tolerance of adult *M. soledadinus*. Similarly, Warren et al. (2018)

reported that the thermal tolerance of ants from coastal areas was lower as compared with the specimens collected further inland. It is thus possible that insects from Papous 800 were sufficiently well separated from the other populations and far away for the coast to lead to different recovery patterns after extreme cold exposure.

As differences in thermal tolerance sometimes become apparent when animals are exposed to high stress levels, it is interesting to note that increasing the duration of exposure to -6 °C did not have a significant effect on the recovery dynamics of all but one of the tested populations. In insects, chill coma results from the progressive depolarization muscle resting membrane potential (MacMillan et al., 2012; Andersen et al. 2015). Our results suggest that the 15 min exposure at cold temperature was long enough for altering the membrane potential over the critical threshold associated with the onset of chill coma, as the recovery dynamics and number of recovered beetles did not change significantly with increasing exposure duration. The exception to this generalisation was provided by the population from the highest elevation at Molloy (Molloy 250), which showed a significant reduction in tolerance to exposure to -6 °C when the duration was increased from 15 min to 1 h.

5 Conclusions

In this study, carabid beetles sampled from populations at their invasion front in the Kerguelen Islands were characterized by a high level of cold tolerance, but this was also the case for populations with the longest residence times. Conversely, two populations with intermediate residence times exhibited lower cold tolerance, confirming that the expression of this trait is not systematically enhanced along the invasion gradient. A pattern indicating a trade-off between cold and heat tolerance was observed for many of the tested populations collected along the invasion gradient. Temperature tolerance of insects from the two altitudinal transects was not systematically enhanced with elevation, illustrating that other factors in addition to environmental temperature, including trophic resources, determine the temperature tolerance of the animals. Similarly, the thermal tolerance varied among insects sampled from the horizontal seashore-inland transect. In future studies, the influence of quality and quantity of available trophic resources, humidity, and biotic interactions on the thermal tolerance of adult *M. soledadinus* should be investigated in order to better characterise the drivers of this trait along environmental gradients. Finally, the similar recovery curves of the insects exposed to longer duration of cold exposure suggest that any

damage inflicted by cold exposure in the treatment used here occurs within the first 15 minutes of exposure in this species.

Acknowledgements

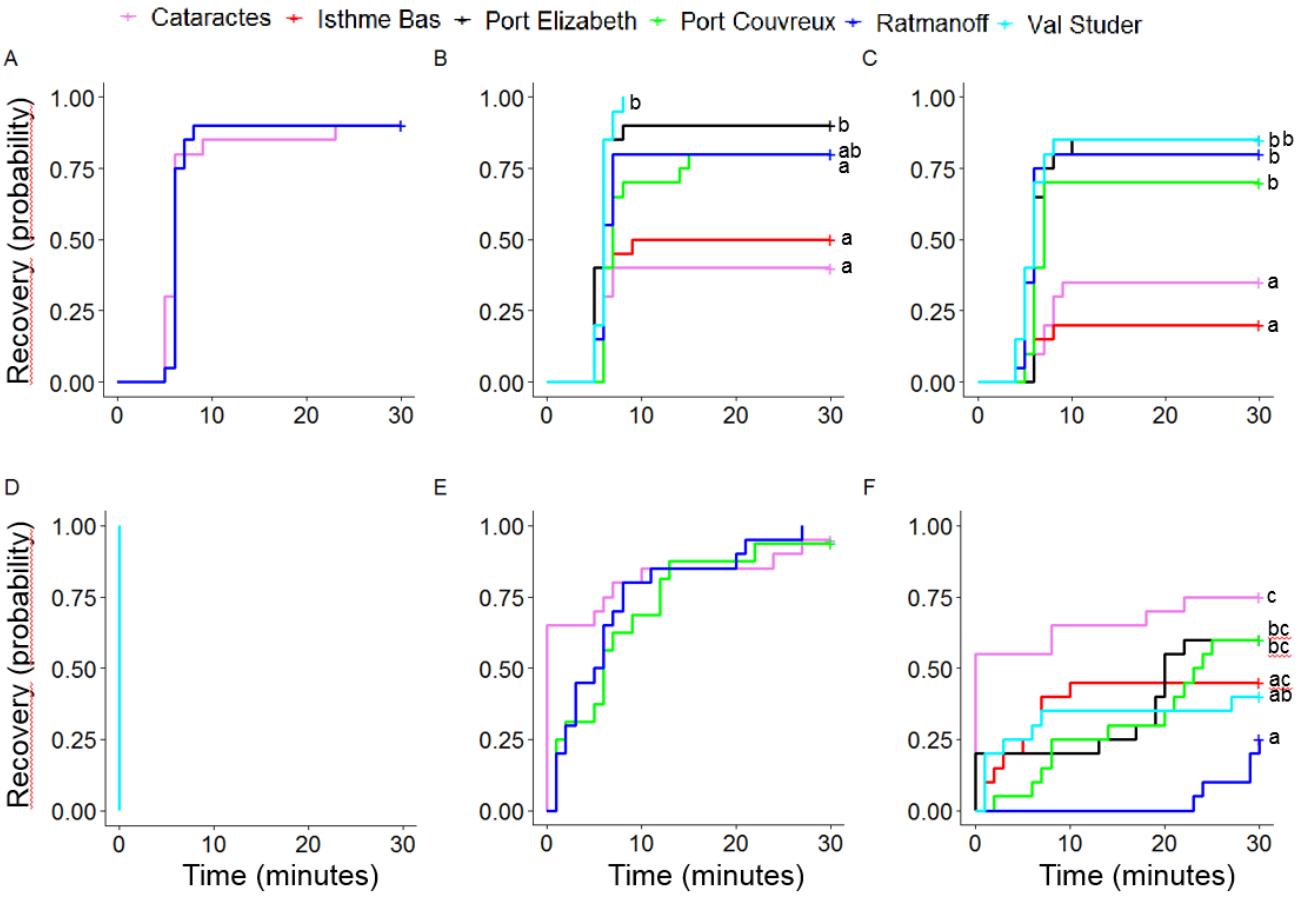
This research was supported by InEE-CNRS (Zone Atelier Antarctique et Subantarctique), and the French Polar Institute (IPEV, program 136 ‘Subanteco’). Peter Convey is supported by NERC core funding to the British Antarctic Survey’s ‘Biodiversity, Evolution and Adaptation’ Team.

Supplementary material

Supp Figure 1

Thermal tolerance patterns of populations of *Merizodus soledadinus* sampled along an invasion gradient at the Kerguelen Islands

Significant differences were observed in the temperature tolerance of the different populations of *M. soledadinus* when the temperature of the assays was more stressful. At -5 °C, there was little difference in the number of recovered insects: insects from Cataractes and Ratmanoff recovered quickly from the cold stress (Supp Figure 1, A). At -6 °C and -7 °C (Supp Figure 1, B and C), there were clear differences in the recovery abilities of the insects, particularly between the cold-sensitive populations of Cataractes and Isthme Bas, and the more cold tolerant insects from the populations of Val Studer and Port Elizabeth. No significant differences were observed in the recovery dynamics of adult *M. soledadinus* after they were exposed at 35 or 36 °C for 15 min (Supp Figure 1 D and E. At 37 °C, insects from Ratmanoff had a significantly lower ability to recover from heat exposure than insects from Cataractes (Supp Figure 1 E), which contrast with their respective cold recovery times. Note that not all populations were exposed to all temperatures, as described in the figure legend.

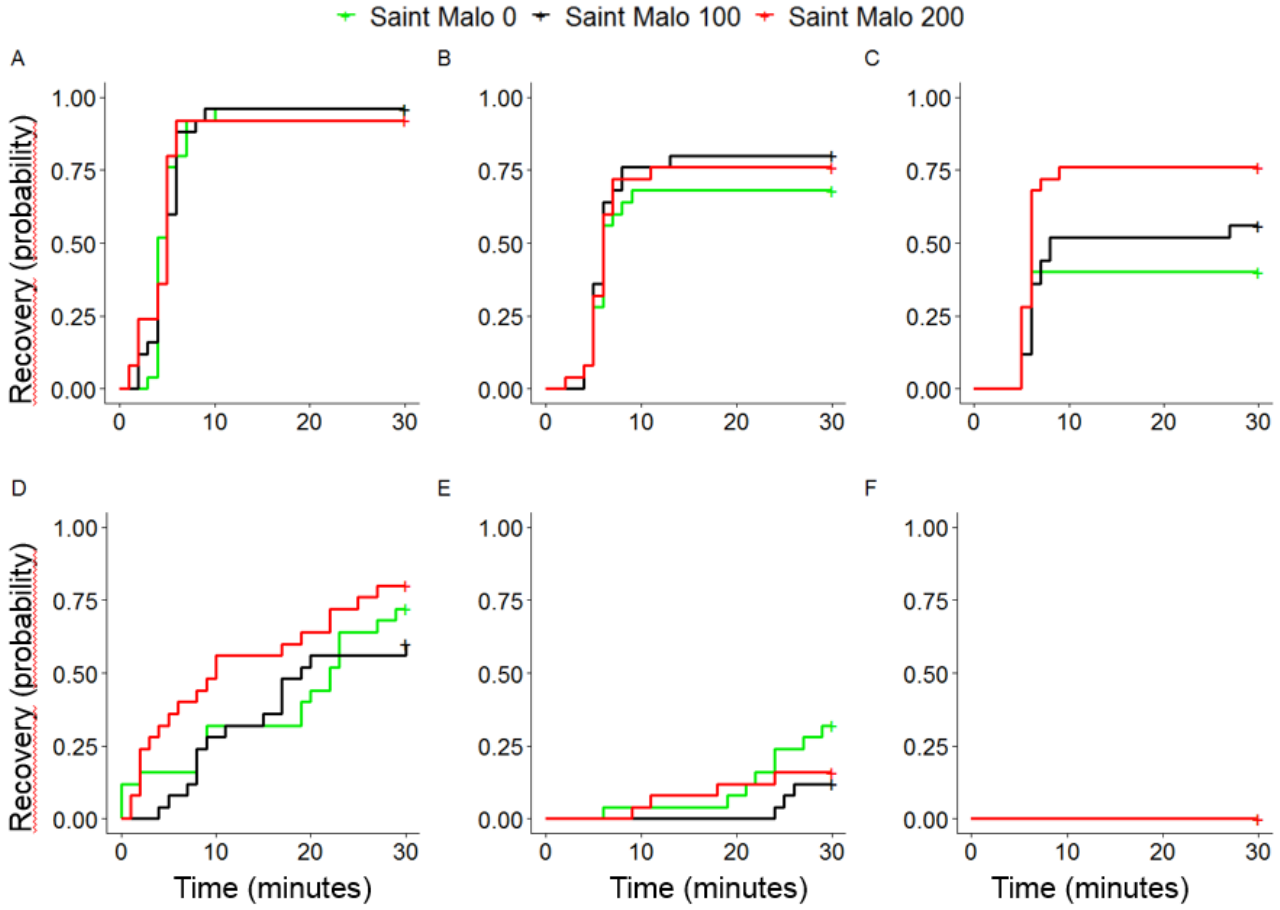


Supp Figure 1 Recovery time of 20-25 individuals from different populations of the carabid beetle Merizodus soledadinus. The recovery curves of the different populations are represented by distinct coloured lines. Recovery curves were estimated using the Kaplan Meier method. Distinct letters indicate different recovery dynamics among the populations; statistical differences were computed using a log rank test with Benjamini & Hochberg adjusted P- values for pairwise comparisons. A) Recovery curves of individuals from the populations of Cataractes and Ratmanoff after they were exposed at -5 °C for 15 minutes (P=0.44). B) Recovery curves of individuals from six populations of M. soledadinus sampled along an invasion gradient at the Kerguelen Islands after they were exposed at -6 °C for 15 minutes. C) Recovery curves of individuals from six populations of M. soledadinus sampled along an invasion gradient at the Kerguelen Islands after they were exposed at -7 °C for 15 minutes. D) Recovery curves of individuals from Cataractes, Isthme Bas and Val Studer after they were exposed at 35 °C for 15 minutes. The recovery curves were identical (P=1). E) Recovery curves of individuals from Cataractes, Port Couvreux and Ratmanoff after they were exposed at 36 °C for 15 minutes (P=0.41). The recovery curve of insects from Port Couvreux was estimated based on 16 individuals in this graph. F) Recovery curves of individuals from six populations of M. soledadinus sampled along an invasion gradient at the Kerguelen Islands after they were exposed at -37 °C for 15 minutes.

Supp Figure 2

Thermal tolerance patterns of populations of *Merizodus soledadinus* sampled along an altitudinal gradient (Saint Malo) at the Kerguelen Islands

No significant difference was detected between the recovery curves of insects sampled from different elevations (approx. 0 m, 100 m or 200 m a.s.l.) at Saint Malo. Insects from these populations struggled to recover after an exposure at 37 °C for 15 min (Supp Figure 2, E), and the two populations (Saint Malo 0 and Saint Malo 200) exposed at 38 °C for 15 minutes did not recover (Supp Figure 2, F).

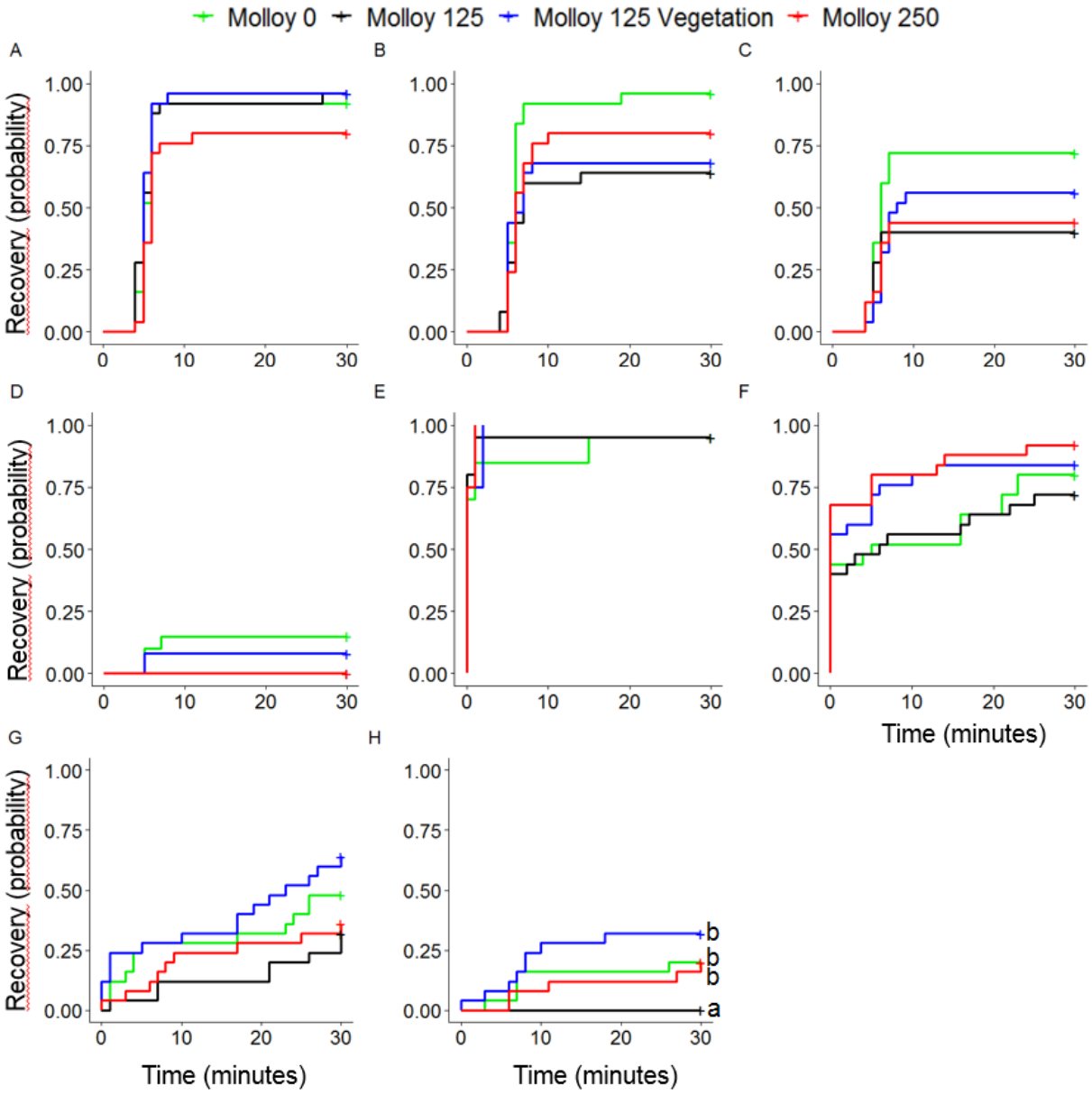


Supp Figure 2 Recovery time of individuals from three populations of Merizodus soledadinus collected from different elevations (0 m, 100 m, 200 m a.s.l.) at Saint Malo (Kerguelen Islands), after they were exposed to A) -5 °C for 15 minutes; recovery curves of the populations were similar (P=0.86). B) -6 °C for 15 minutes; recovery curves of the populations were similar (P=0.66). C) -7 °C for 15 minutes; recovery curves of the populations were similar (P=0.056). D) 36 °C for 15 minutes; recovery curves of the populations were similar (P=0.17). E) 37 °C for 15 minutes; recovery curves of the populations were similar (P=0.17). F) 38 °C for 15 minutes, not including Saint Malo 100; recovery curves of the populations were similar (P=1). The recovery curves of the different populations are represented by different coloured lines. Lines were estimated using the Kaplan Meier Method.

Supp Figure 3

Thermal tolerance patterns of populations of *Merizodus soledadinus* sampled along an altitudinal gradient (Molloy) at the Kerguelen Islands

Thermal tolerance of insects collected at different elevations (approximately 0 m, 125 m and 250 m a.s.l.), and from two distinct habitats of similar elevation, at Molloy is presented in Supp Figure 3. The recovery dynamics were not significantly different among the four populations at most temperatures. The population collected from Molloy 125 did not recover after exposure to 38 °C for 15 min. Conversely, some individuals from the other populations recovered (Supp Figure 3, H).

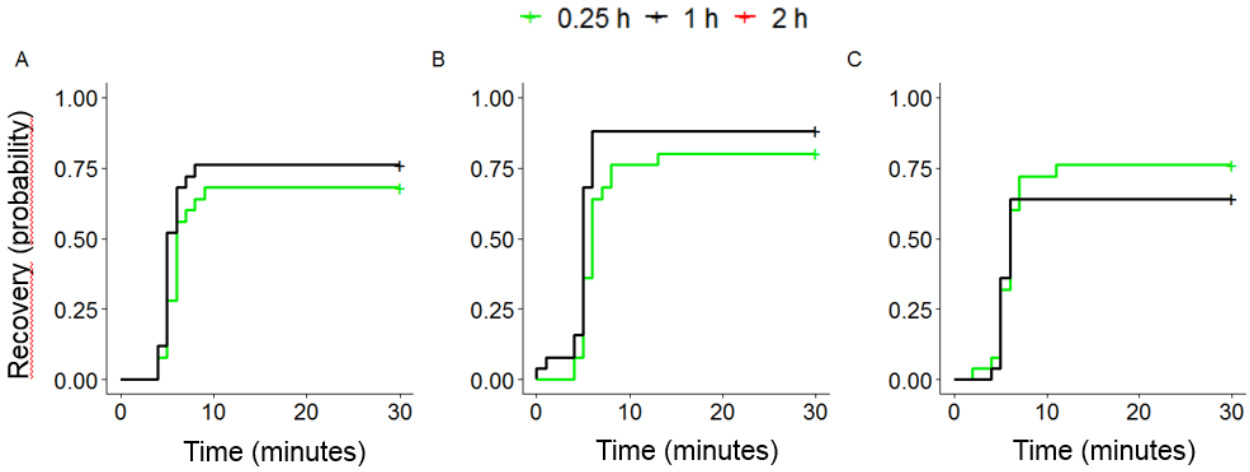


Supp Figure 3 Recovery time of adults from four different populations of *Merizodus soledadinus* sampled from different elevations (0 m, 125 m, 125 m vegetation, 250 m a.s.l.) at Molloy (Kerguelen Islands). The recovery curves of the different populations are represented by the different coloured lines, drawn using the Kaplan Meier method. Dissimilar letters indicate distinct recovery dynamics (log rank test with Benjamini & Hochberg adjusted P-values for pairwise comparison). Recovery curves represent the recovery dynamics of insects after they were exposed to **A**) -5 °C for 15 minutes; recovery curves of the populations were similar (P=0.083). **B**) -6 °C for 15 minutes; recovery curves of the populations were similar (P=0.055). **C**) -7 °C for 15 minutes; recovery curves of the populations were similar (P=0.13). **D**) -8 °C for 15 minutes; recovery curves of the populations were similar (P=0.075). **E**) 35 °C for 15 minutes; recovery curves of the populations were similar (P=0.54). **F**) 36 °C for 15 minutes; recovery curves of the populations were similar (P=0.11). **G**) 37 °C for 15 minutes; recovery curves of the populations were similar (P=0.063). **H**) at 38 °C for 15 minutes.

Supp Figure 4

Recovery dynamics of populations of *Merizodus soledadinus* sampled along an altitudinal gradient at Saint Malo (Kerguelen Islands) and exposed to cold stress for increased durations

Despite varying the exposure period from 0.25 h to 2 h, the recovery dynamics of insects from the three populations at Saint Malo was similar (Supp Figure 4). For all durations of cold exposure, the probability of recovering maximised at around 0.75, and recovered individuals usually recovered after 5 to 7 minutes, indicating resistance to this treatment.

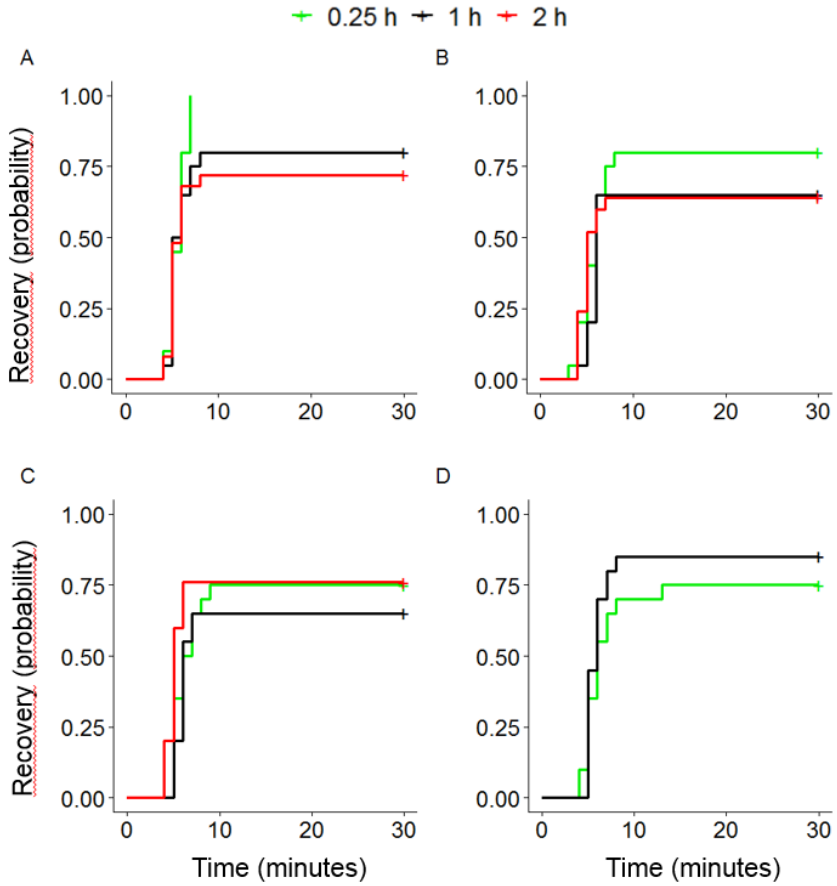


Supp Figure 4 Recovery curves of adult M. soledadinus collected from different elevations (0 m, 100 m, 200 m a.s.l.) at Saint Malo (Kerguelen Islands) after they were exposed at -6 °C for different durations (0.25 h, 1 h or 2h). The recovery curves were estimated using the Kaplan Meier method. No statistical difference was found for the recovery dynamics of the populations after these cold treatments. A) Adult M. soledadinus from 0 m a.s.l. (P=0.29). B) Adult M. soledadinus from 100 m a.s.l. (P=0.08). C) Adult M. soledadinus from 200 m a.s.l. (P=0.56).

Supp Figure 5

Recovery dynamics of populations of *Merizodus soledadinus* sampled along a seashore-inland horizontal transect at Anse des Papous (Kerguelen Islands) and exposed to cold stress for increased durations

Despite varying the exposure period from 0.25 h 2 h, the recovery dynamics of insects from the three populations collected at Papous were similar (Supp Figure 5).

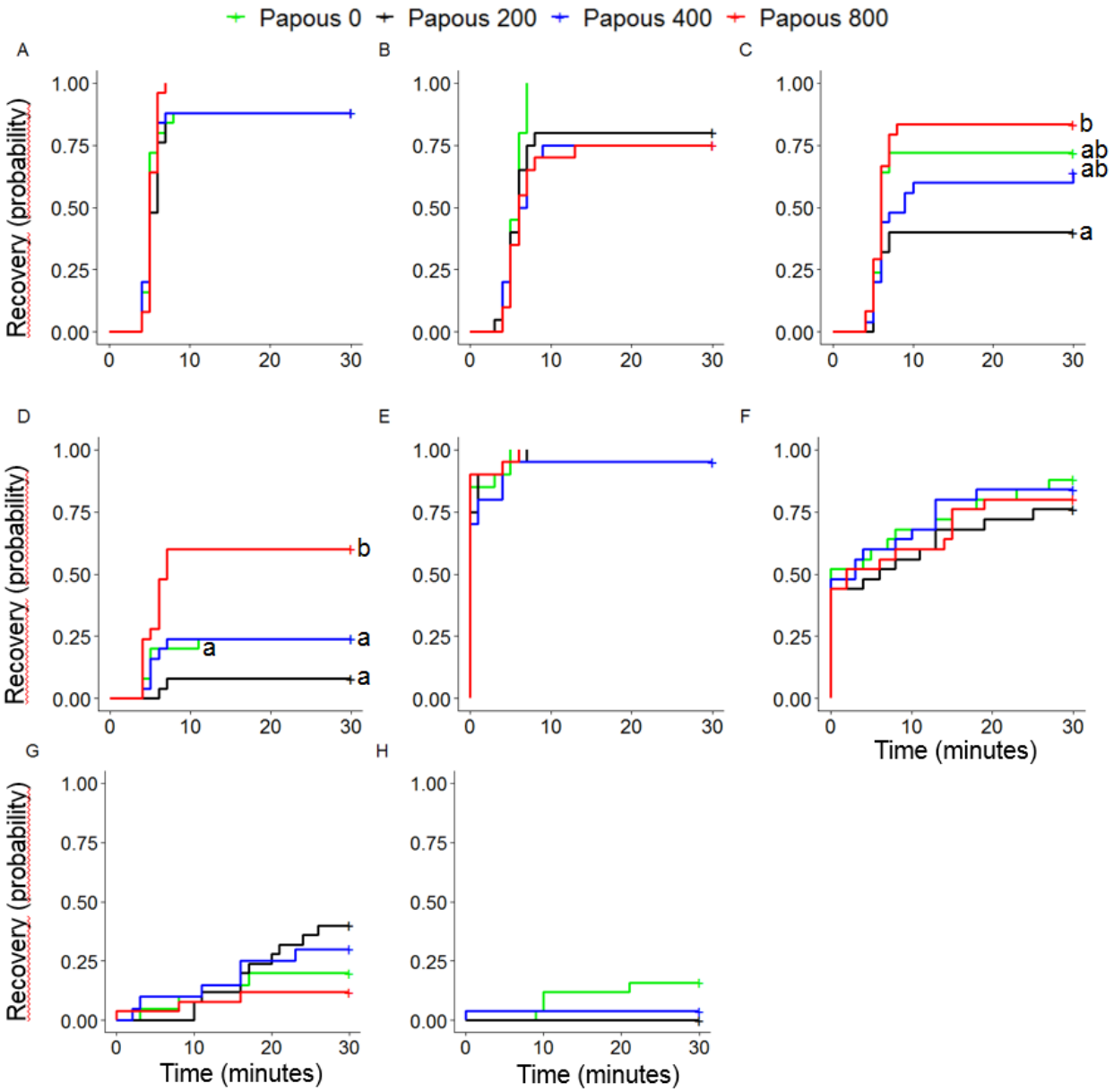


Supp Figure 5 Recovery curves of adult M. soledadinus collected along a seashore-inland transect (0 m, 200 m, 400 m and 800 m from the sea) at Papous (Kerguelen Islands) after they were exposed at -6 °C for different durations (0.25 h, 1 h or 2h). The recovery curves were estimated using the Kaplan Meier method. No statistical difference was found for the recovery dynamics of the populations after these cold treatments. A) Adult M. soledadinus sampled close to the sea (0 m) (P=0.2). B) Adult M. soledadinus sampled at 200 m from the sea (P=0.48). C) Adult M. soledadinus sampled at 400 m from the sea (P=0.24). D) Adult M. soledadinus sampled at 800 m from the sea (P=0.44), not including exposure to -6 °C for 2 hours.

Supp Figure 6

Thermal tolerance patterns of populations of *Merizodus soledadinus* sampled along a seashore-inland horizontal transect at Anse des Papous (Kerguelen Islands)

Differences were found in the recovery dynamics of insects sampled along a seashore-inland horizontal gradient and exposed to extreme cold temperatures: insects from the population located approximately 800 m from the coast recovered significantly faster than the others after they were exposed at -8 °C for 15 min (Supp Figure 6, D) (P = 0.02747, P = 0.00043 and P = 0.02747). These insects also recovered faster than those sampled 200 m from the coast after they were exposed at -7 °C for 15 min (Supp Figure 6, C). In general, insects from these populations were relatively cold tolerant and heat sensitive, as they can recover from -8 °C (Supp Supp Figure , D), and recover well from exposure to -5 and -6 °C (Supp Figure 6, A, B) but struggled to recover from 37 °C (Supp Figure 6, G).



Supp Figure 6 Recovery curves of adult M. soledadinus from four different populations of a seashore-inland transect at Papous (Kerguelen Islands). The recovery curves of the different populations are represented by different colored lines, which were estimated using the Kaplan Meier method. Distinct letters indicate different recovery dynamics (log rank test with Benjamini & Hochberg adjusted P-values for pairwise comparison). Recovery curves represent the recovery dynamics of insects after they were exposed to A) -5 °C for 15 minutes; no statistical difference was found among the recovery curves of the populations (P=0.71). B) -6 °C for 15 minutes; no statistical difference was found among the recovery curves of the populations (P=0.13). C) -7 °C for 15 minutes. D) -8 °C for 15 minutes. E) 35 °C for 15 minutes; no statistical difference was found among the recovery curves of the populations (P=0.68). F) 36 °C for 15 minutes; no statistical difference was found among the recovery curves of the populations (P=0.74). G) 37 °C for 15 minutes; no statistical difference was found among the recovery curves of the populations (P=0.19). H) 38 °C for 15 minutes, not including the population Papous 800; no statistical difference was found among the recovery curves of the populations (P=0.067).

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Chapter 3

The effects of temperature and insecticide, and the combination of these stressors, on the biology of *Alphitobius diaperinus*

Article 1

Thermal plasticity and sensitivity to insecticides in populations of an invasive beetle: Cyfluthrin increases vulnerability to extreme temperature

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Abstract

Climate change increases average temperatures and the occurrence of extreme weather events, in turn accentuating the risk of organism exposure to temperature stress. When thermal conditions become stressful, the sensitivity of insects toward insecticides can be exacerbated. Likewise, exposure of insects to insecticides can subsequently influence their ability to handle stressful temperatures. Here, we investigated the effects of constant temperature and daily heat spikes, in presence/absence of insecticide treatment (cyfluthrin), on the condition (impairment of mobility) and thermal tolerance to cold (- 6 °C) and heat (42.5 °C) of the terrestrial beetle *Alphitobius diaperinus*. The responses of insects from four populations (three farm-collected populations, one laboratory population) to different durations of extreme temperature exposure were compared. The results showed that the laboratory population was generally more sensitive to extreme cold and heat temperatures, with less than 50% of adults recovering after an exposure at -6 or +42.5 °C for 3h. Significant differences in the level of thermal tolerance were also found among insects from poultry farms. Cyfluthrin exposure incurred detrimental effects to insects' condition in all but one population. For two out of the four populations, mobility impairment was increased when adults were exposed to daily heat spikes (6 hours per day at 38 °C) and cyfluthrin simultaneously, compared to cyfluthrin exposure at constant temperatures; yet, no significant interaction between the two stressors was found. Finally, using one farm collected population, effects of pre-exposure to cyfluthrin on extreme temperature tolerance provided another example of the toxicant-induced climate sensitivity in insects.

Keywords:

Pesticide, thermal variation, tolerance, heat spike, pyrethroid, insect

1. Introduction

Over the course of their life, all living organisms, including insects, are confronted with a range of environmental conditions which vary spatially and temporally (Hirose and Nakamura 2005; Mann, Bradley, and Hughes 1998; Soranno et al. 2019). In some instances, environmental constraints may be exacerbated by the currently changing climatic conditions. This increases the occurrence of extreme weather events and the probability of organisms' exposure to higher temperatures (Hansen, Sato, and Ruedy 2012; IPCC 2014; Stillman 2019), heightening the risk of organism exposure to heat stress. In turn, several life history traits of individual insects can be affected (Bozinovic et al. 2011; Kingsolver, Diamond, and Buckley 2013; Renault 2011), with cascading effects on population demography and organism evolution (Chown et al. 2010; Colinet et al. 2015; Katz and Brown 1992; Moe et al. 2013). When temperature varies, heat spikes are more likely to occur, and they are expected to have greater evolutionary significance - and pose greater threat to species performance - than increase in average temperature (Katz and Brown 1992; Vasseur et al. 2014). Yet, whatever the nature of the physiological and biochemical mechanisms triggered by thermal stress, their activation may weaken the ability of organisms to deal with other environmental constraints, or conversely magnify these abilities when the responses elicit cross tolerance phenomena (Chen and Stillman 2012; Noyes and Lema 2015; Sinclair et al. 2013). In this context, investigating insects' ability to cope with stress under and after exposure to varying temperature regimes provides useful information about their sensitivity to environmental stress, and for future insights into their geographic distribution (Bozinovic and Pörtner 2015; Buckley et al. 2010; Ma, Hoffmann, and Ma 2015).

Besides climatic conditions, adverse environmental effects on insects are also caused by the unintentional spread of man-made contaminants, in particular by the use of various pesticides in agriculture and horticulture (Aviles et al. 2019; Calow 1991; Köhler and Triebskorn 2013). The use of pesticides has become widespread in many regions of the world where agricultural intensity has increased (Tilman et al. 2002), representing a threat to biodiversity in general. While valuable insights of the effects of insecticides on behaviour (Dewer et al. 2016), olfaction (Lalouette et al. 2016), reproduction and development (Abbes et al. 2015) have been gathered, these investigations were commonly conducted by exposing the organisms to constant and optimal temperatures, as prescribed in standardized tests (Camp and Buchwalter 2016; Khan and Akram 2014). Yet, the inclusion of daily thermal fluctuations is needed to better simulate the conditions experienced by free-ranging organisms in natural and anthropogenic environments, and thus increase the quality and realism of the outcomes of these investigations (Colinet et al.

2015; Verheyen and Stoks 2019). Moreover, thermal tolerance can vary significantly among insect populations originating from different environments (Engell Dahl et al. 2019; Käfer et al. 2020), as is also the case for pesticide exposure (see Hawkins et al. (2019) for a review of the different mechanisms that may confer different level of insecticide resistance among insect populations). When assessing the effects of environmental stressors on insects' ecology, it has thus become crucial to consider multiple populations (of different exposure or adaptation history) in the experimental designs to get a larger coverage of the range of responses that could be expected at the species level.

The possible combined effects of climate change and environmental pollution on organisms, and wildlife in general, have been reviewed by Noyes and Lema (2015). These authors reported that chemical exposure may affect organisms' sensitivity to climate change by disrupting physiological responses to diurnal and seasonal thermal fluctuations. In particular, the alteration of endocrine system functioning and energy balance in toxicant-exposed organisms may subsequently alter their thermal performance curves, potentially challenging their ability to survive thermal stress. In several instances, it has been suggested that the simultaneous exposure to pollutants, including endocrine disrupting chemicals, and climate change could result in synergistic actions (Cairns, Heath, and Parker 1975; Hooper et al. 2013; Laskowski et al. 2010; Moe et al. 2013). When sequential exposures occur, climatic stress can influence sensitivity towards pollutants, and the reverse is also true, e.g. previous exposure to a pollutant can influence the tolerance to climatic stress (Hooper et al. 2013).

Nowadays, studies assessing the impacts of multiple stressors, including temperature, are increasingly needed (Kaunisto, Ferguson, and Sinclair 2016; Orr et al. 2020), as such investigations greatly improve our estimates of the effects of interactions among environmental stressors on insects. Holmstrup et al. (2010) reviewed the available studies that assessed the combined effects of pesticide and temperature exposures, and reported only few works that considered daily thermal fluctuations, or daily heat spikes, in their experimental designs. Since this review has been published, the number of studies that combined temperature and toxicant exposures has started to accumulate, reporting how thermal conditions could modify the effects of pesticides on insects (Op de Beeck, Verheyen, and Stoks 2018; Camp and Buchwalter 2016; Delnat et al. 2019; Harwood, You, and Lydy 2009; Verheyen, Delnat, and Stoks 2019; Verheyen and Stoks 2019; Willming, Qin, and Maul 2013). For example, Verheyen and Stoks (2019) found that exposure of damselfly larvae to the organophosphate chlorpyrifos did not affect the survival of individuals maintained at constant 20 °C. Conversely, the mortality of the larvae was

increased under daily thermal fluctuations of 5 (17.5-22.5 °C) or 10 °C (15-25 °C). Delnat et al. (2019) reported that daily thermal fluctuations of 7 (16.5-23.5 °C) or 14 °C (13-27 °C) slightly increased the mortality of the larvae of the mosquito *Culex pipiens* exposed to chlorpyrifos, while having no effect on adults. Working with heat waves (constant heat exposure of damselfly larvae over three consecutive days), Arambourou and Stoks (2015) reported that the subsequent exposure to chlorpyrifos did not change mortality rates of the larvae. However, the higher inhibition of acetylcholine esterase suggested that heat waves may increase the sensitivity of damselflies to this chemical. More recent studies have examined the effects of successive heat spikes and pesticide exposure (Meng, Delnat, and Stoks 2020), and the delayed effects of pesticide exposures on life histories of insects (Dinh, Janssens, and Stoks 2016).

Even though Delnat, Janssens, and Stoks (2019) found the effect of warming on pesticide toxicity to be dependent on other factors of their experimental setup, results from the available literature generally underline the importance of incorporating more realism into the experimental designs when investigating the interaction between pesticide and temperature (Böttger, Schaller, and Mohr 2012). Moreover, all but one of these studies used insect larvae, and all investigations were based on aquatic species, meaning that terrestrial insect species and adult life stages are severely underrepresented from this research. Likewise, some studies have investigated the thermal tolerance of insects after they were exposed to pesticides (Op de Beeck, Verheyen, and Stoks 2017, 2018; Delnat et al. 2019; Meng, Delnat, and Stoks 2020). Yet again, all of these investigations have focused on aquatic larvae, and used the organophosphate chlorpyrifos. Other chemicals, including pyrethroids, which are also highly toxic to insects (Tang et al. 2017), should be considered. Finally, no study examined the effects of sequential exposure to daily thermal fluctuations and pesticide on the subsequent thermal tolerance in terrestrial insects.

In order to contribute to the abovementioned research gaps concerning the interaction of climate and pollutant stress, we aimed at investigating the effects of daily heat spikes and insecticide treatment on the thermal tolerance of adults of the lesser mealworm *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae), as well as comparing the performance of populations of *A. diaperinus* of different exposure histories to temperature and insecticide. This insect pest, pullulating in poultry farms from many regions worldwide (Geden and Hogsette 1994; Johnson, Gbon, and Boga 2018; Rueda and Axtell 1997), represents a fitting model for research combining ecophysiological and ecotoxicological investigations. Insects can be collected in large numbers from geographically distant and unconnected habitats (poultry farms), making it

possible to work with different and locally adapted strains. In these anthropogenic environments, the lesser mealworms are exposed to fluctuating temperatures, as broilers optimize poultry production by running a standardized cycle (which has to be closely followed to maintain profitability (Donald 2010)) of weekly decreasing temperatures, i.e. starting from ≈ 31 and reaching ≈ 17 °C over approximately seven weeks (Salin, Delettre, and Vernon 2003). This provides an excellent habitat for lesser mealworms, that have been found to grow well, i.e. with a very high energy conversion efficiency at 23 to 31 °C (Bjørge et al. 2018). Temperature extremes up to 48 °C have also been measured in the litter where *A. diaperinus* thrive (Salin and Vernon 1997), meaning that the insect can be exposed to heat spikes. The poultry houses are disinfected from time to time with chemicals, including the structural type II pyrethroid, cyfluthrin (the use of this insecticide will be banned in France from July 21st, 2021 (ANSES 2020)). This insecticide is an inhibitor of insect nerve cell sodium channels, leaving them open and causing paralysis, tremors, and eventually death (Lushchak et al. 2018). It is sprayed at the end of each broiler flock for controlling outbreaks of *A. diaperinus*, and during the disinfection and cleaning of the poultry farm, the heating system is stopped, doors of the building are left opened, and temperature can vary from -5 °C to 31 °C (Salin, Delettre, and Vernon 2003).

As a first part of the study, we compared the basal thermal tolerance of *A. diaperinus* from four populations, by measuring their ability to recover from exposure to extreme cold or heat temperatures for different durations. We hypothesized that the beetles' recovery from thermal stress would be faster in populations that experienced temperature fluctuations in their original environment, as previously reported on several other insect models (Arias, Poupin, and Lardies 2011; Bozinovic et al. 2011; Cavieres et al. 2019). As a second part of the study, we were interested in comparing the effects of exposure to an environmentally relevant dose of the insecticide cyfluthrin on the condition of insects maintained at constant temperatures or subjected to daily heat spikes. We hypothesized that populations which had experienced frequent pesticide exposure would perform better than laboratory-reared adults (Hamm et al. 2006; Lambkin and Rice 2009). Finally, the population with the highest pesticide tolerance was exposed to different temperature regimes, without pesticide exposure or with different concentrations of cyfluthrin (up to those recommended by commercial formulations). Subsequently, the recovery times of individuals from the different treatments after an exposure to temperature extremes were assessed. Insects pre-exposed to daily heat spikes were expected to recover faster from heat shock, but slower from cold shock treatments, compared to insects kept at constant temperature. Furthermore, insects exposed to insecticides were

hypothesized to be characterized by a lower ability to cope with temperature stress, either due to an increased toxicity of the chemical with daily heat spikes or decreased ability to cope with heat shock after chemical stress (Op de Beeck, Verheyen, and Stoks 2017; Delnat et al. 2019; Meng, Delnat, and Stoks 2020).

2. Materials and methods

2.1. Rearing of the insects

Adults of *A. diaperinus* were collected from three poultry farms in Brittany, France, thereafter referred to as FARM1 (sampled at Miniac-sous-Bécherel, 48°17'10'' N, 1°55'51'' W), FARM2 (sampled at Plaine Haute, 48°26'44'' N, 2°51'16'' W), and FARM3 (sampled at Pommerit-le-Vicomte, 48°37'10'' N, 3°05'15'' W). In addition, larvae of *A. diaperinus* were obtained from Proti-Farm *via* a pet shop (Envies Animales, Saint-Jacques-de-la-Lande, Brittany, France). Insects from this latter population, hereafter referred to as LAB, were reared over many generations under controlled conditions (25 °C, relative humidity 50%, no light) in a supposedly pollutant free environment. Because LAB insects are produced and used as a dietary food supply for pets (e.g. lizards), they are in very good physiological condition (Bjørge et al. 2018).

All adults were kept at 25 °C, in darkness, in plastic containers with sawdust for minimum 4 weeks prior to experimental start. They were supplied with water and food *ad libitum*, in the form of water in microtubes with cotton and dry dog food pellets. This procedure allowed standardisation of the temperature, humidity, and trophic conditions experienced by the adults before the assays. The LAB larvae were additionally provided with a block of polystyrene to burrow in for pupation, and oat bran for the first four weeks to further encourage their development into adults (Rice and Lambkin 2009). The sex and age of the adults was not considered in our experiments.

2.2 Experimental design

2.2.1. Characterization of the thermal tolerance of the insects from different populations

In order to compare the thermal tolerance of the insects from the four populations, adults were exposed to two extreme temperatures, -6 or +42.5 °C, in a cryothermostat (VWR Collection, AP15R-30). This procedure is derived from protocols commonly used for assessing the thermal tolerance in insects (Hazell et al. 2008; Sinclair, Coello Alvarado, and Ferguson 2015). At -6 and + 42.5 °C, the lesser mealworms are expected to fall into coma (see **Supplementary File 1** for the results of the preliminary tests that allowed us designing the methodology). Each insect was exposed to one of these two extreme temperatures for one of six durations: 15 min, 30 min, 1h, 2h, 3h or 4h, in darkness. This range of stress durations allowed us to describe and compare

thermal tolerance among populations. At least four replicates of 10 adults (test tube with rubber cap) were used for each exposure duration and temperature combination. At the end of the thermal treatment (-6 or +42.5 °C), each group of 10 insects was directly transferred into Petri dishes and observed for up to 40 seconds every 5 minutes over 2h at room temperature (19 ± 1 °C) **(Supplementary File 2)**. During each observation period, the recovery of the insects was scored by registering the individuals as either “Active” or “Impaired/immobile”; insects were characterized as “Active” when they exhibited coordinated movements of all limbs when stimulated, all other states were registered as “Impaired/immobile”. The temperatures, both in the cryothermostat and in the room where recovery observations took place, were monitored using iButtons® (iButtonLink, LLC., Whitewater, USA).

2.2.2. Effect of daily heat spikes and cyfluthrin exposure

Here, we combined two abiotic stresses by jointly exposing adult *A. diaperinus* to both moderately stressful heat spikes and insecticide for eight days, before assessing any visible impairment or death of exposed individuals. One temperature cabinet was set to constant 26 °C (control temperature), and another cabinet varied the temperature by holding a 6h period at 38 °C, followed by 18h at 26 °C **(Supplementary File 2)**; the two cabinets were MIR 154 Panasonic programmable incubators. The experiment was run in dark conditions.

Insects were placed in glass Petri dishes (10 cm diameter) with a fitting filter paper covering the bottom. For insecticide application, cyfluthrin (pure molecule standard, CAS-number 68359-37-5, Sigma-Aldrich) was dissolved in acetone, and a volume of 2 mL of appropriately diluted solution was transferred to the filter paper. Pre-testing the insects’ susceptibility to cyfluthrin led to preparation of two distinct insecticide concentrations, so that the applied dose was 20 mg cyfluthrin/m^2 for populations FARM1, 2 and 3, and 0.5 mg cyfluthrin/m^2 for LAB. We had to reduce the cyfluthrin dose significantly for the LAB population, as the 20 mg cyfluthrin / m^2 concentration killed all adults within a day at our experimental temperatures. These cyfluthrin concentrations induced negligible knockdown (<5%) over prolonged exposures in the works of Desvignes-Labarthe (2018) and Renault and Colinet (Submitted). These concentrations also range in the values that may be encountered by the insects in poultry houses (Salin, Delettre, and Vernon 2003). In the other Petri dishes, 2 mL of pure acetone was added to control for any effect of the solvent. All Petri dishes were left to dry under a fume hood for at least 10 min before the tests, which effectively evaporated all acetone. The exiting literature reveals that cyfluthrin remains efficient over seven days (Guillebeau, All, and Javid 1989), with knockdown effects remaining unchanged when the beetle *Tribolium castaneaum* were assessed eight weeks after

the chemical was applied (Arthur 1999) Finally, starting from an initial concentration of 17.3 mg / m², (Nakagawa et al. 2017) reported that cyfluthrin concentration decreased by ca. 17% after 56 days. Thus, even if changes in cyfluthrin concentration were not monitored here, it is assumed that it remained close to the nominal concentration.

For all treatments, the insects were supplied with a water source (0.5 mL microtube containing water and cotton) and a food source (approx. 0.8 g dog food pellet). For each population, a set of Petri dishes treated with the insecticide and a set of dishes without pesticide treatment were placed in the temperature cabinets (8 to 30 dishes of each, as specified in Table 1) along with an iButton® (iButtonLink, LLC., Whitewater, USA) measuring both temperature and humidity. Each Petri dish in experiments with poultry house collected populations contained 30 individuals, and for the LAB population, each Petri dish contained 20 individuals, due to limited number of individuals **(Table 1)**. Previous experiments that examined the sensitivity of *A. diaperinus* to insecticides (including cyfluthrin) revealed that densities from 10 to 30 adults per Petri dish had no effects on the results (Desvignes-Labarthe 2018; Renault and Colinet Submitted).

Table 1 Overview of number of dishes and number of individuals per dish used for assessing the effects of temperature regime (constant temperature or daily heat spikes), with and without the insecticide cyfluthrin, on the locomotory capacities of adult *Alphitobius diaperinus*.

Treatment	**Without insecticide**		**Insecticide-exposed**	
Population	Dishes (N)	Animals (N)	Dishes (N)	Animals (N)
FARM1	20	30	20	30
FARM2	21	30	30	30
FARM3	10	30	10	30
LAB	8	20	12	20

After eight days, and 17 hours after the last peak of heat, the state of each individual was noted: the insects were considered as either “Active” (no apparent effect of the treatment on the locomotory activities of the insects), “Impaired” (locomotor deficits: difficulties in moving limbs, or laying on their backs while moving) or “Immobile” (no observable movement).

2.2.3. Effect of daily heat spikes and cyfluthrin exposure on recovery from exposure to extreme temperatures

In the third part of the study, we measured the capabilities of adult *A. diaperinus* to recover from cold or heat shocks after they had been exposed to insecticide and/or daily heat spikes for eight

days. As a non-negligible part of the adults from LAB, FARM2 and FARM3 were impaired or immobile after they were exposed to cyfluthrin concentration of 20 mg / m² (see the results presented in Figure 3), we had to focus on adults from FARM1 for this experiment. Lesser mealworms from this population survive exposure to cyfluthrin concentrations encountered in the field (as recommended applications contain 20 mg cyfluthrin/m^2, C. Salin et al., 2003), which thus allowed working with this concentration without incurring selection of individuals due to mortality or locomotor deficits during the pre-exposure period.

For this experiment, three temperature conditions were used: (i) constant 26 °C for eight days (control), 18h at 26 °C daily interrupted by exposures of 6h at (ii) 32 °C (low heat spike), or (iii) 38 °C (high heat spike). The exposure ran over eight days in darkness. Glass Petri dishes were prepared with four doses of cyfluthrin (0, 5, 10 or 20 mg cyfluthrin/m^2) as described above, including addition of food and water. Then, 30 adults from FARM1 were added to each dish, and dishes of every pesticide dose were placed into each of the three temperature cabinets (MIR 154 Panasonic Incubators), resulting in a total of four different pesticide exposures at three different temperature regimes.

After eight days, and 17 hours after the last peak of heat, the Petri dishes were removed from the temperature cabinets, and the beetles were directly exposed to either -6 or +42.5 °C for 2h, in darkness **(Supplementary File 2)**. Extreme temperature exposure was performed as described above (section 2.2.1.), including testing the recovery of at least 40 individuals to each combination of treatment (temperature regime, insecticide dose, and temperature regime x insecticide dose), but the temperature of the recovery room was higher (approx. 24 °C instead of 20 °C).

2.3. Data treatment

The recovery time of every single individual was used to compute the temporal recovery curves using Kaplan Meier estimates computed in R version 3.5.2, using the Survminer package (Kassambara and Kosinski 2018; R Core Team 2013). The probability of recovery at any given time point therefore represents the proportion of individuals that have recovered by that time. Pairwise comparisons using Log-Rank test with adjusted P-values (Benjamini and Hochberg 1995) were performed *post-hoc*, with a significance value of 0.05. Generalized linear models (GLMs) with logit link function for proportions outcome (i.e. number of active versus impaired or immobile individuals per dish) were run on data reporting the state of animals after eight days of exposure to the different temperature regimes and pesticide doses. The effect of each variable (temperature, and insecticide dose), and their interactions, were analysed using the analysis of

deviance (Anova function in the car package (Fox and Weisberg 2019)). We assume that this insecticide and heat peaks have different modes of actions, meaning that their individual- and interaction effects on *A. diaperinus* adults are assumed to be multiplicative. Differences among the treatments were finally analysed by Sidak's multiple comparisons using the emmeans and multcompView packages (Graves, Piepho, and Selzer 2019; Lenth 2020), with a significance value of 0.05.

3. Results

3.1. Characterization of the thermal tolerance of the insects from different populations

The recovery probability over time of adult *A. diaperinus* was dependent on the exposure duration to hot or cold temperature extremes **(Figures 1 and 2; Supplementary File 3)**. Adults from LAB recovered more slowly than adults from the poultry house-collected populations for all durations of exposure at 42.5 °C ($P \leq 0.05$), and all but 30 minutes exposure at -6 °C ($P \leq 0.05$) **(Supplementary File 3)**. After a 30 min exposure at -6 °C, the recovery from LAB was comparable to that measured from the three other populations ($P \geq 0.05$); after 1 hour exposure at 42.5 °C, the recovery curve of LAB was also similar to FARM3 ($P = 0.33$). When the duration of exposure to heat or cold temperatures was increased, the proportion of insects that fully recovered was decreased and the duration of recovery was prolonged **(Figures 1 and 2)**.

The recovery probability of the lesser mealworms from FARM1 and FARM2 after they were exposed to heat (42.5 °C) was similar for all experimental conditions. When exposed to temperature extremes for 0.25, 0.5, 1, 2, or 3h, insects from FARM3 were characterised by a lower recovery probability as compared with the two other poultry house-collected populations (Figure 1).

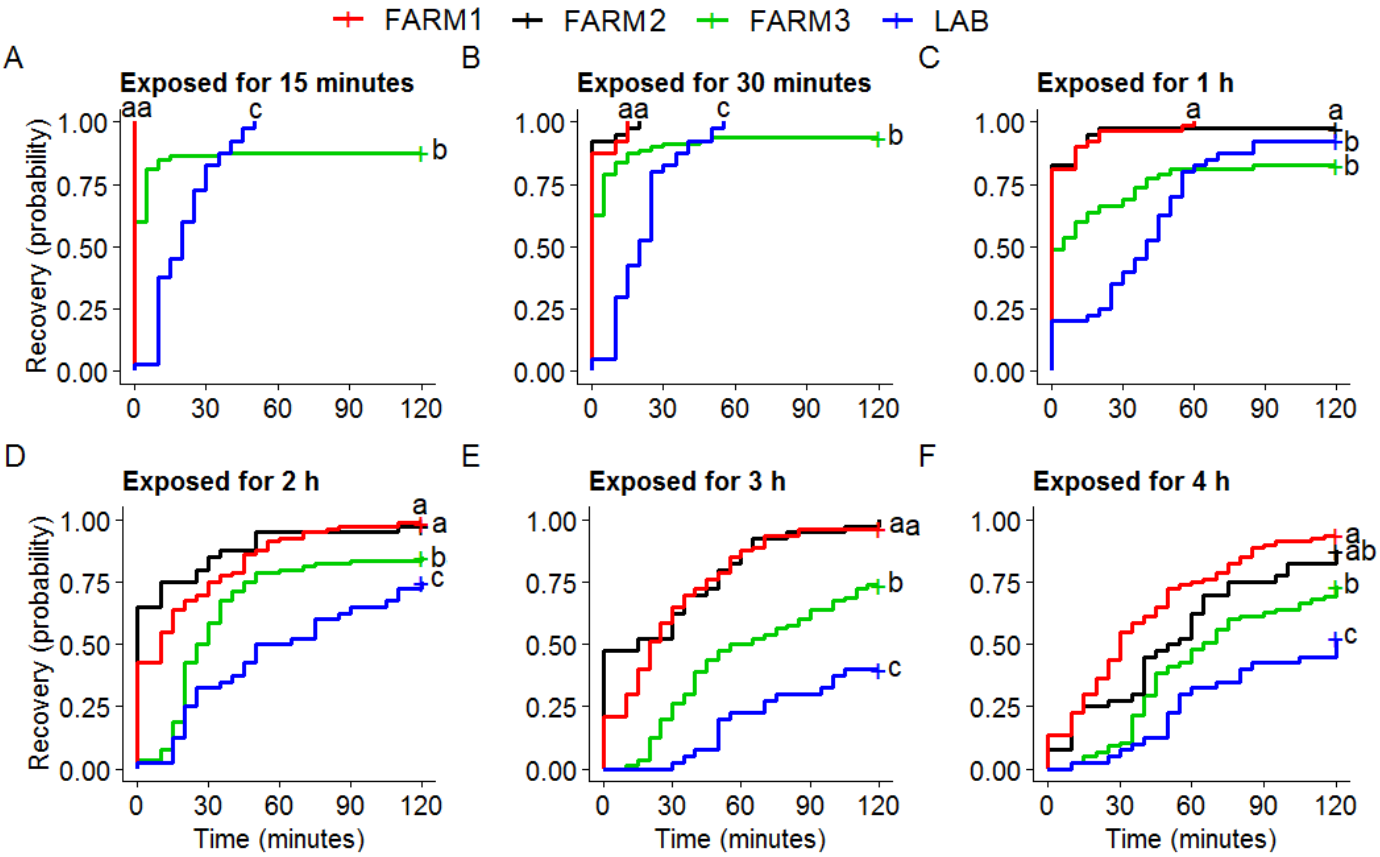


Figure 1. Recovery probability of individuals from four different populations of *A. diaperinus* exposed at 42.5 °C for six different durations: **A**) 0.25 hour (overall P < 0.001); **B**) 0.5 hour (overall P < 0.001); **C**) 1 hour (overall P < 0.001); **D**) 2 hours (overall P < 0.001); **E**) 3 hours (overall P < 0.001); **F**) 4 hours (overall P < 0.001). The red lines represent insects from FARM1, the black lines represent the insects from FARM2, the green lines represent the insects from FARM3, and the blue lines represent the insects from LAB. Different letters indicate differences among the recovery probability curves within each duration.

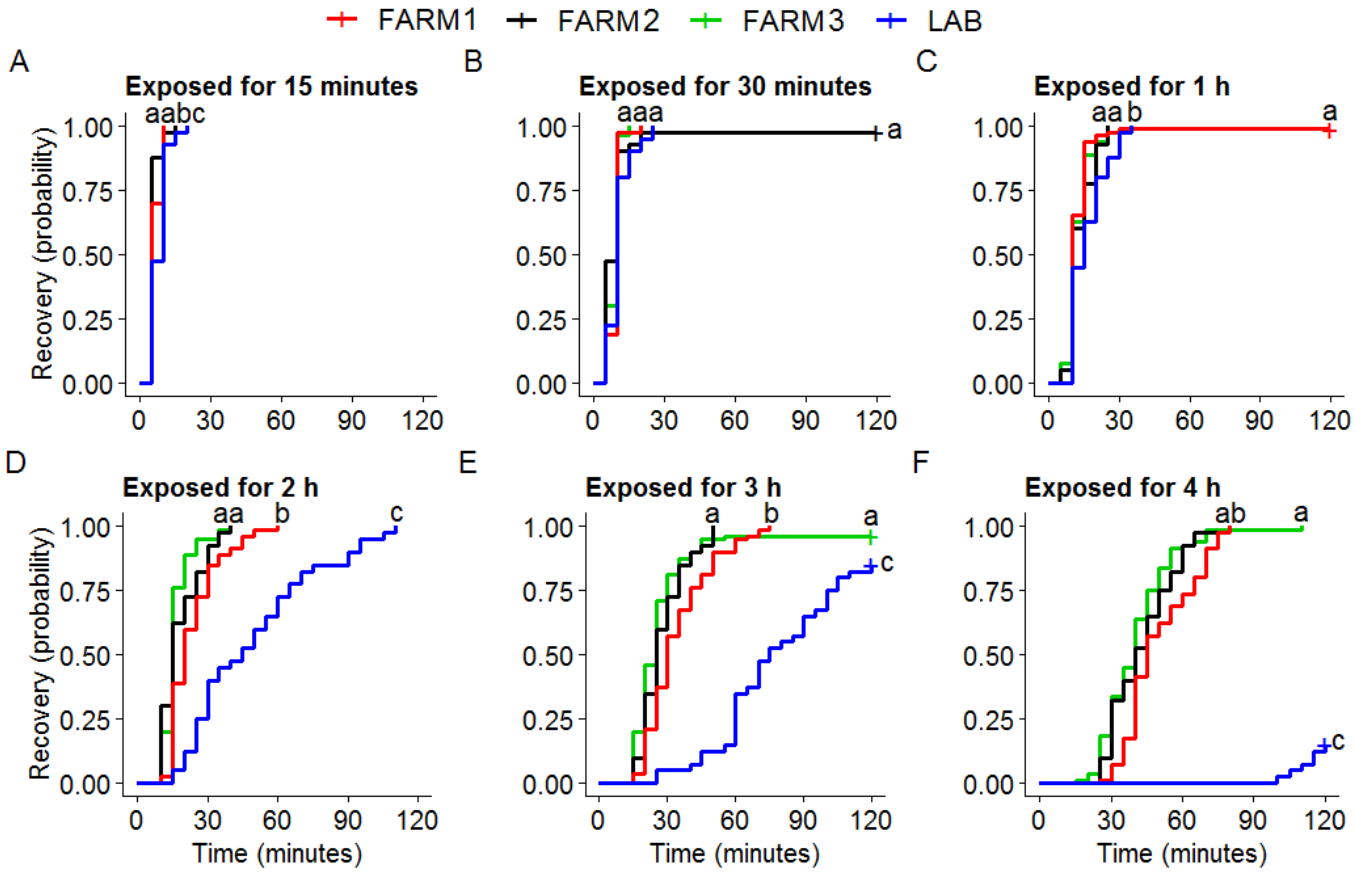


Figure 2. Recovery probability of individuals from four different populations of *A. diaperinus* exposed at -6 °C for six different durations: **A**) 0.25 hour (overall P < 0.001); **B**) 0.5 hour (P = 0.074); **C**) 1 hour (overall P < 0.01); **D**) 2 hours (overall P < 0.001); **E**) 3 hours (overall P < 0.001); **F**) 4 hours (overall P < 0.001). The red lines represent insects from FARM1, the black lines represent the insects from FARM2, the green lines represent the insects from FARM3, and the blue lines represent the insects from LAB. Different letters indicate differences among the recovery probability curves within each exposure duration.

3.2. Effect of daily heat spikes and cyfluthrin exposure

The number of active lesser mealworms was significantly reduced when insects were exposed to cyfluthrin at constant temperature (26 °C) as compared with control insects (P < 0.05 (95% confidence level)) for all populations (Figure 3), except for FARM1 **(Figure 3, A; Supplementary File 3)**. Sidak's multiple comparisons *post-hoc* tests following a two-way ANOVA showed that the insecticide cyfluthrin and daily heat spikes (18h at 26 °C followed by 6h period at 38 °C for 8 days) significantly decreased the proportion of active insects as compared with control and heat treatment conditions (P < 0.05) for all populations **(Figure 3A-D; Supplementary File 3)**. Yet, the proportion of insects that were scored as active remained close to 100% for FARM1. The proportion of insects scored as active significantly differed among the "Cyfluthrin" and "Daily

heat spikes & Cyfluthrin" treatments for FARM2 ($P < 0.05$) and LAB ($P < 0.05$). There was no significant interaction between daily heat spikes and cyfluthrin for any of the populations ($P = 0.28$ or higher) **(Figure 3, A-D; Supplementary File 3)**.

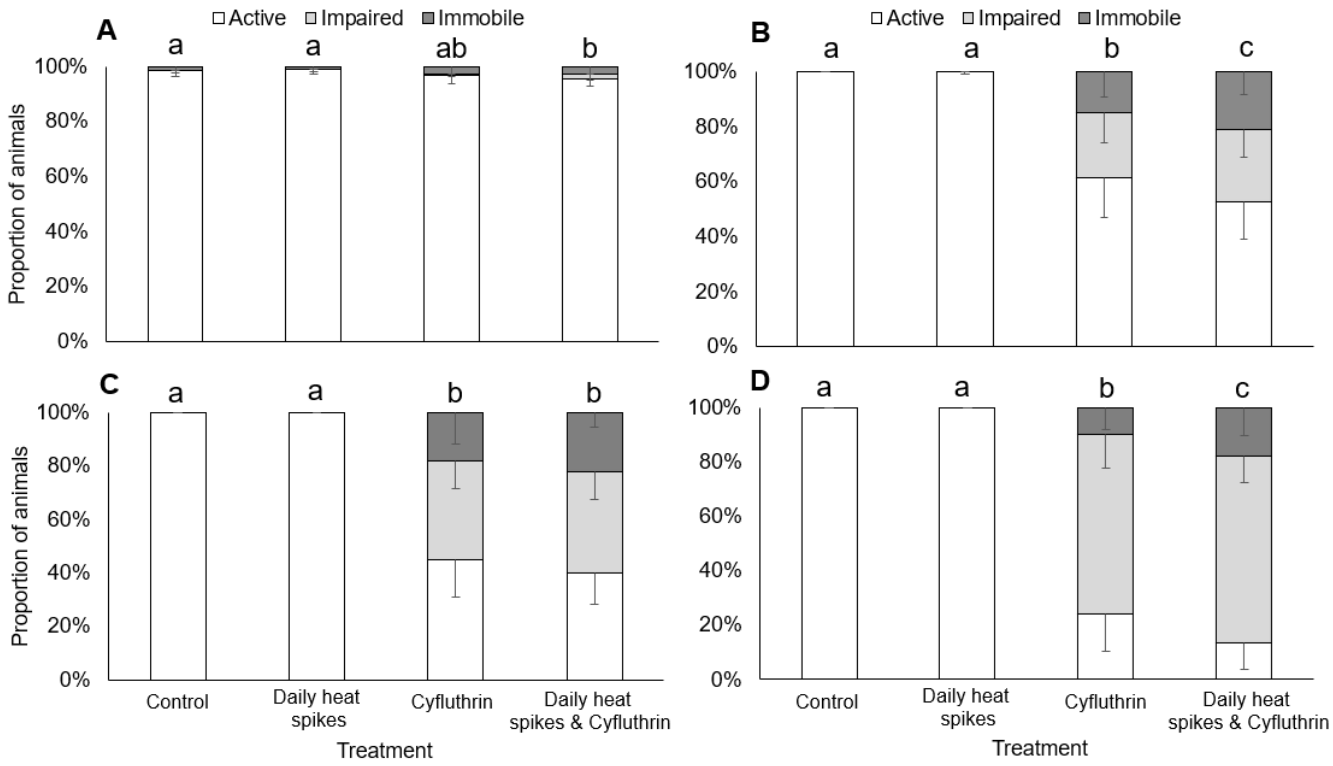


Figure 3. Status of adult *A. diaperinus* from populations **A**) FARM1, **B**) FARM2, **C**) FARM3 after they were exposed for eight days to control conditions (constant 26 °C), daily heat spikes (26 °C for 18h, 38 °C for 6h), cyfluthrin (20 mg/m^2) or daily heat spikes and cyfluthrin and **D**) LAB after eight days of same exposure treatments, but with a cyfluthrin concentration of 0.5 mg/m^2. Different letters indicate differences among the number of active insects within each population.

3.3. Effect of daily heat spikes and cyfluthrin exposure on recovery from exposure to extreme temperatures

The recovery probability of adult *A. diaperinus* from FARM1 decreased with increasing doses of cyfluthrin after the insects were maintained for eight days under different temperature regimes and cyfluthrin concentrations prior to extreme temperature exposure ($P < 0.001$) **(Figure 4; Supplementary File 3)**. This was particularly evident for beetles exposed to heat (42.5 °C) **(Figure 4)**. Pre-exposure to a daily heat spike for eight days **(Figure 4, B-C)** accelerated the recovery of the insects after they were exposed at 42.5 °C for 2h, as compared with animals exposed at constant 26 °C **(Figure 4, A; Supplementary File 3)**. Conversely, the recovery

curves remained comparable for insects that were exposed to extreme cold (-6 °C for 2h; **Figure 4, D-F)**, even though they were previously subjected to different heat spikes for 8 days.

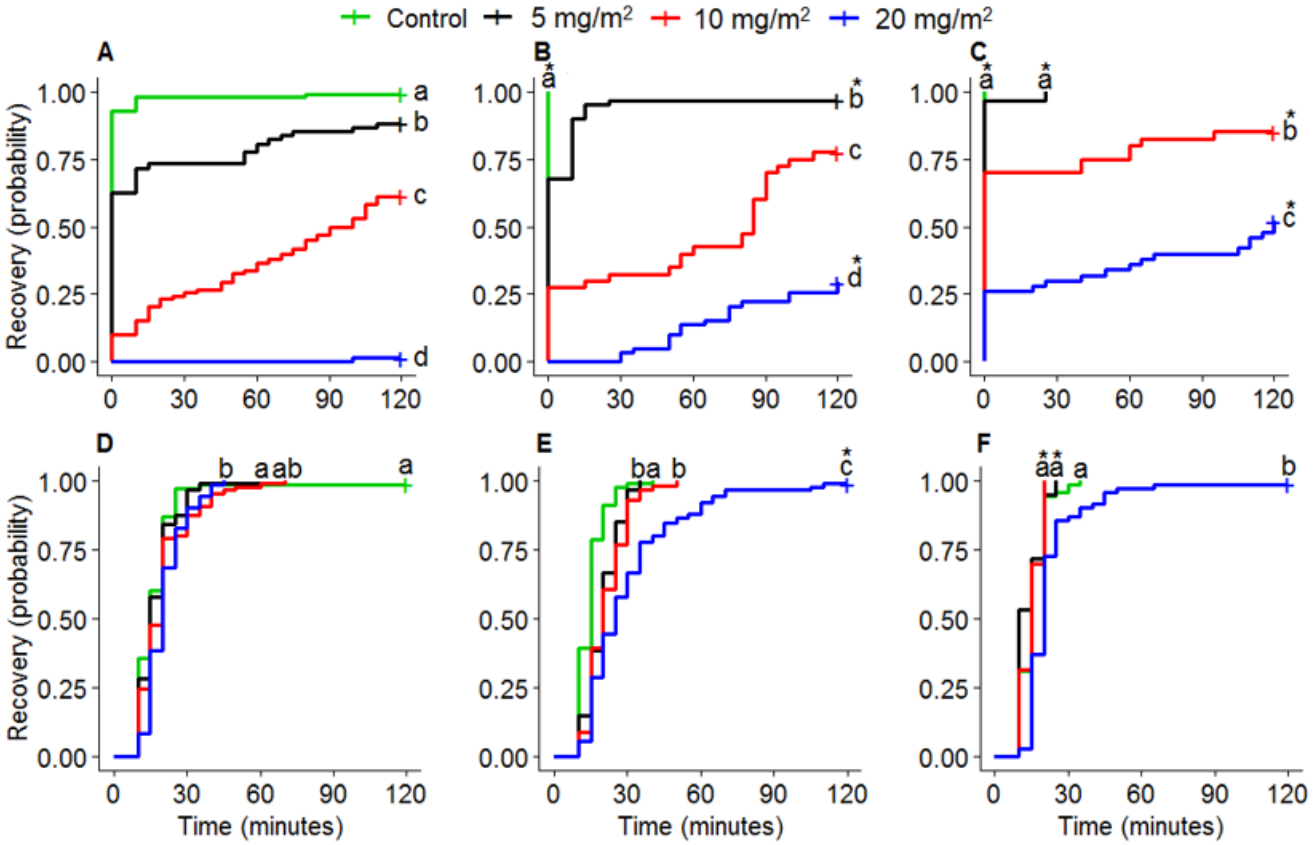


Figure 4. Recovery probability of A. diaperinus from FARM1 observed every 5 minutes after a 2-hour exposure at 42.5 °C (A, B and C) or -6 °C (D, E and F). Before the test, the insects were exposed to different temperature regimes: (A) and (D) constant 26 °C; (B) and (E) 26 °C daily interrupted by 6h at 32 °C; (C) and (F) 26 °C daily interrupted by 6h at 38 °C. For each temperature regime, the insects were exposed to four different concentrations of the insecticide cyfluthrin: 0 (control), 5 (black lines), 10 (red lines) or 20 (blue lines) mg cyfluthrin/m^2. Stars (*) represent significant differences between the marked curve and the curve of the same colour drawn in Fig. 4A (constant temperature regime used as the reference recovery probability). Different letters indicate differences among the recovery probability curves within each temperature pre-treatment group.

4. Discussion

4.1 Characterization of the thermal tolerance of the insects from different populations

In this study, we were interested in comparing the basal thermal tolerance of adult *A. diaperinus* from four populations through assessments of their ability to recover from different durations of exposure to cold or heat. Adults from FARM1 and FARM2 were characterized by the greatest ability to recover from thermal stress, in particular to heat exposure, while laboratory-reared adults exhibited the lowest thermal tolerance, in line with our expectations. The thermal plasticity of lesser mealworm populations is also consistent with the wide range of temperatures that can occur during a poultry house production cycle. Acclimation or adaptation to thermally variable habitats may widen the temperature tolerance range, as reported from other insect models (reviewed in Colinet et al. 2015; Lachenicht et al. 2010). For instance, working with *Drosophila melanogaster* reared at varying temperature regimes, Bozinovic et al. (2011) observed a reduction of the number of flies entering into chill or heat coma when transferred to extreme temperatures, as compared with flies reared at constant temperature. Overgaard, Hoffmann, and Kristensen (2011) evidenced that acclimation of *D. melanogaster* to variable temperatures increased tolerance to both low and high thermal stress. In addition, these authors showed that populations originating from temperate areas exhibited a wider thermal tolerance than those sampled from more thermally stable tropical environments. In sum, the lower thermal tolerance of adult *A. diaperinus* from LAB is consistent with the available literature, and the hypothesis that poultry house-collected insects have higher recovery probabilities than laboratory-bred individuals was confirmed.

In this work, insects from FARM3 exhibited a significantly lower thermal tolerance than their counterparts sampled from the other two poultry farms. However, many other environmental factors experienced by the insects in the poultry farm, such as pH and ammonia concentration in the litter, or the presence and diversity of bacteria and viruses which trigger immune responses that can alter the performance of other physiological traits, may have shaped the thermal tolerance of the insects from poultry farms. Further, we can also expect the populations to be genetically distinct from each other. As a result, variance in thermal tolerance among the insects from the three farm populations is not surprising (Miller, Mohrenweiser, and Bell 2001).

4.2. Effect of daily heat spikes and cyfluthrin exposure

The previous experiment reported differences in the thermal tolerance among populations of *A. diaperinus*. In particular, insects from FARM1 and FARM2 exhibited slightly higher capacity to recover from heat exposure, suggesting that they would be better able to cope with daily heat

spikes, while insects from the LAB population should have had the lowest ability to deal with peaks of heat. Yet, none of the populations were visibly affected by the daily heat spikes on their own.

Insects from FARM1 were capable of coping with the investigated dose of cyfluthrin at constant temperature, while this condition was detrimental for the three other populations. As expected, adults of *A. diaperinus* from LAB were more sensitive to the insecticide than those of the three FARM populations. Differences in cyfluthrin resistance have already been reported in *A. diaperinus* populations from Australia (Lambkin and Rice 2009), where resistance was generally related to the extent and frequency of cyfluthrin applications experienced by the insects. Similarly, Chernaki-Leffer et al. (2011) found strains of *A. diaperinus* of different pesticide (cypermethrin, dichlorvos and triflumuron) susceptibility in Brazil, and Hamm et al. (2006) found strains of *A. diaperinus* with different pesticide (cyfluthrin and tetrachlorvinphos) resistance in the United States. The latter authors suggested that resistance alleles are present in the resistant populations of the lesser mealworm, as may have been the case with our poultry house collected insects.

While daily heat spikes alone did not affect the recovery of the insects of the four tested populations, the combination of insecticide and daily heat spikes significantly reduced the number of active individuals in two (FARM2 and LAB) out of the four assayed populations. Warm temperatures have been found to decrease the toxicity of the pyrethroids cypermethrin and deltamethrin in the fly *Musca domestica* (Khan and Akram 2014), and in the Spotted bollworms *Earias vittella* exposed to pyrethroids at higher temperature regimes (Satpute et al. 2007). Yet, these authors used constant temperatures in their experiments. The increased negative effects of the insecticide for adult *A. diaperinus* experiencing a daily heat spike of 6 hours is consistent with the findings of several other studies (Delnat et al. 2019; Willming, Qin, and Maul 2013; Zhu, Sengonca, and Liu 2006), who found increasing pesticide toxicity in insects subjected to daily bouts of high temperatures. For instance, daily thermal fluctuations as low as 5 °C increased toxicity of chlorpyrifos in damselfly larvae (Verheyen and Stoks 2019). The synergistic effects of thermal variations and insecticides may occur under large thermal variations only, as reported in the vector mosquitoes *Culex pipiens* (Delnat et al. 2019). The magnification of the effects of pyrethroids on *A. diaperinus* we have found emphasizes the importance of considering daily thermal variations and heat spikes in risk assessments of the sensitivity of biodiversity to pollution under different scenarios of climate change, although a higher constant temperature equivalent of the average temperature experienced by animals exposed to daily heat peaks

would allow us to make stronger conclusions on this point. At higher temperatures, pesticide uptake may be increased, as found by Lydy, Belden, and Ternes (1999) and Buchwalter, Jenkins, and Curtis (2003). In parallel, elimination rates of the toxicants, for example through temperature-dependent activation of enzymes, should also be enhanced at warmer conditions. In sum, the available literature and our results could indicate a promotion of insecticide resistant genotypes with increasing occurrence of extreme weather events, which is consistent with documented links between climatic variables and evolution of pesticide resistance in arthropod pests (Maino, Umina, and Hoffmann 2018). Facilitation of insecticide resistance as a result of climate change could be a result of pleiotropy, shared genetic mechanisms or genetic linkage (Pu, Wang, and Chung 2020).

4.3. Effect of daily heat spikes and cyfluthrin exposure on recovery from exposure to extreme temperatures

Using adult *A. diaperinus* from FARM1, we tested how the sensitivity to extreme temperatures was affected by thermal and insecticide pre-treatments. We expected higher proportion of recovery and faster recovery from heat stress in insects exposed to a daily heat spike over eight consecutive days, as extreme temperature tolerance is readily modified by a thermal conditioning processes, even in the short-term (Chidawanyika and Terblanche 2010; Kingsolver et al. 2016; Weldon, Terblanche, and Chown 2011). Our results are consistent with this expectation for heat-shocked insects. Daily peaks at high temperatures may have acclimated the insects to heat, which does not necessarily prepare them for cold exposures. Indeed, cold tolerance requires different mechanisms compared to heat tolerance, by for instance increasing membrane fluidity instead of decreasing it (Neven 2000; Teets and Denlinger 2013). This can explain why heat pre-exposures did not consistently affect cold tolerance in this experiment. Similarly, it has been shown that acclimation to low temperature increased cold tolerance, but did not affect heat tolerance in *Drosophila melanogaster* (Overgaard et al. 2007).

For insects exposed to extreme heat, insecticide pre-treatment significantly reduced recovery probability, both by generally increasing the recovery time and the number of beetles that did not recover within the recovery period. In fact, beetles pre-exposed to the sublethal dose of 20 mg/m^2 at control temperature never recovered from the applied sublethal heat exposure. We also observed that even a relatively low exposure dose (5 mg cyfluthrin/m^2) had a significant effect on the recovery probability of the individuals, thus providing supporting evidence for toxicant-induced climate sensitivity (Hooper et al. 2013; Moe et al. 2013) in adult *A. diaperinus*. Several studies have shown that an increase in temperature generally enhances negative

effects of contaminants (Noyes et al. 2009; Noyes and Lema 2015), but the number of studies that have tested the effects of insecticide pre-treatments is rather limited as compared with investigations assessing the effects of simultaneous exposures. Importantly, when working sequentially, i.e. by applying one stress before the other, results can greatly vary depending on the specific chemical investigated (Matzrafi 2019). Here, we can hypothesize that coma, which is related to nerve dysfunction, through decreased membrane potential and reduced excitability of the neuromuscular system, occurs more quickly and severely during heat treatment after cyfluthrin exposure, as cyfluthrin is an inhibitor of nerve cell sodium channels. Meanwhile, further research is required to reveal the mechanistic processes explaining the recovery patterns we observed here. For the insects exposed to extreme cold, the effect was only observable after exposure to a dose that is close to the one recommended for use of the insecticide in commercial products (20 mg cyfluthrin/ m^2). A previous study did not find an effect of pesticide exposure on freeze tolerance in an earthworm (Bindesbøl et al. 2009), which indicates that cold tolerance is not as sensitive to insecticide exposure as heat exposure.

Our results also fit well with effects of pesticide exposure on temperature tolerance found in other studies. For example, Op de Beeck, Verheyen, and Stoks (2017, 2018) found that damselfly larvae that were sensitive to the pesticide chlorpyrifos also showed a reduction in heat tolerance after exposure to the pesticide. The authors also showed that the reduction in heat tolerance was smaller when the animals had been pre exposed to higher temperatures, a finding that is in line with our study. In addition, Delnat et al. (2019) found that pesticide exposure decreased temperature tolerance in mosquito larvae and males, and Meng, Delnat, and Stoks (2020) showed that pesticide exposure prior to experiencing a heat spike reduced heat tolerance of mosquito larvae. This reduction in temperature tolerance following pesticide exposure is also true for other taxa, as critical thermal maximum was also reduced in fishes after exposure to endosulfan and chlorpyrifos (Heath et al. 1994; Patra et al. 2007).

The effect of fluctuating temperature regime was also observable in animals who had been exposed to insecticide prior to extreme temperature treatment. It is however difficult to determine whether the observed increase in thermal tolerance after varying temperature regimes was caused by increased tolerance to extreme heat, to insecticide exposure or a combination of the two (Oliver and Brooke 2017). Further research into pesticide uptake and elimination in the animals in the course of this experimental setup would be of interest to further elucidate the processes behind the observed effect.

5. Conclusion

The present work reports that populations of the lesser mealworms have distinct abilities to recover from thermal and insecticide stress, and their interactions. This finding emphasises the importance of designing experiments including several populations when making assessments of the sensitivity of a given species to environmental factors, and more particularly when these data nourish models aiming at forecasting the future of biodiversity. No synergistic interaction was found when the lesser mealworms were jointly subjected to daily heat spikes and cyfluthrin; yet, this type of experimental procedure, which simulates the conditions that could be experienced by free-ranging organisms in natural and anthropogenic environments, increase the ecological realism of the study. This is even truer in a global warming context, as daily heat spikes are expected to become more frequent in several habitats. Here, we also found the importance of working with field-collected insects. The thermal tolerance and sensitivity to insecticide of the laboratory population was very different than that of the field specimens, meaning that different conclusions would be drawn if working with the former population only. Finally, and in line with earlier investigations, our study pointed out the importance of considering the effects of multiple stressors, and the lasting effects they can have on other traits of the insects.

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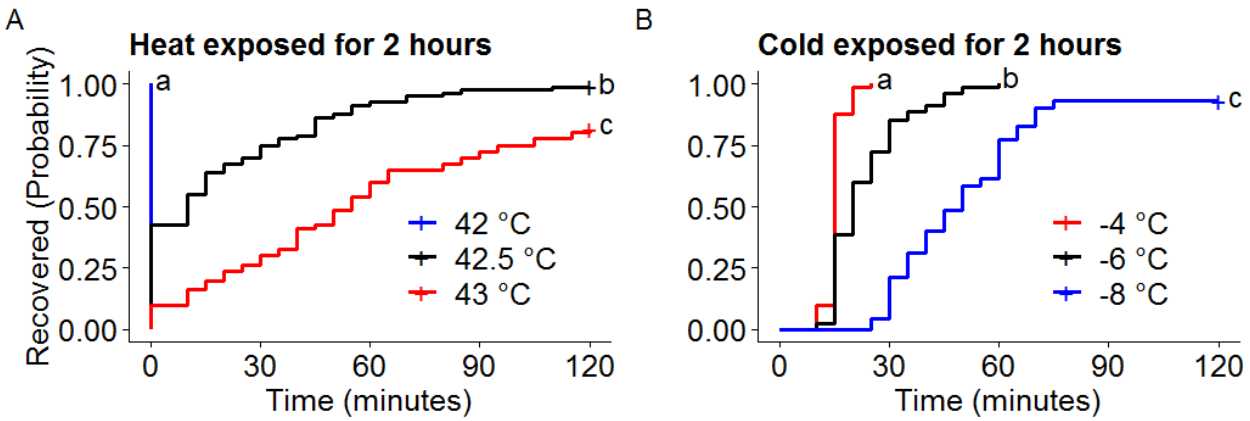
Supplementary File 1

To begin this study, we had to test the effects of different temperatures on the recovery capacity of adult *Alphitobius diaperinus,* that is, the effects of exposure to extreme temperatures (cold, heat) on the recovery ability and duration of the individuals.

Insects from the population FARM1 were used to describe the general temperature tolerance of the species, and to find temperature exposures that would cause the adults to enter chill coma, yet keeping the ability to recover (capacity to regain full movement) afterwards. Four to eight replicates of 10 individuals in each container (test tube with rubber cap) were created for each exposure temperature, starting at -4 °C for cold exposure, and at +42 °C for heat. At the end of the thermal treatment, the insects were directly transferred to Petri dishes and observed every 5 minutes for 2h. Each observation period lasted for up to 40 seconds, and individuals were registered as either “Immobile, Impaired” or “Recovered”. Animals were characterized as “Recovered” when they had regained the ability to move normally. The temperature both in the cryothermostat and the room where recovery observations took place was monitored using iButtons® (iButtonLink, LLC., Whitewater, USA).

Since thermal performance curves are often steep at high temperature extremes, and change less quickly at cold temperature extremes (Huey and Stevenson 1979; Sinclair et al. 2016), we used small increments of temperature in the heat exposure experiments (0.5 °C) and larger increments (2 °C) for cold exposure. When having run three temperatures for a duration of 2 hours, we were able to see the temperatures that would best describe the effects of different durations of exposure on the thermal tolerance of adult *A. diaperinus* from different populations. These experimental conditions were also used for comparing the effects of different daily heat spikes and insecticide exposures prior to extreme temperature exposure.

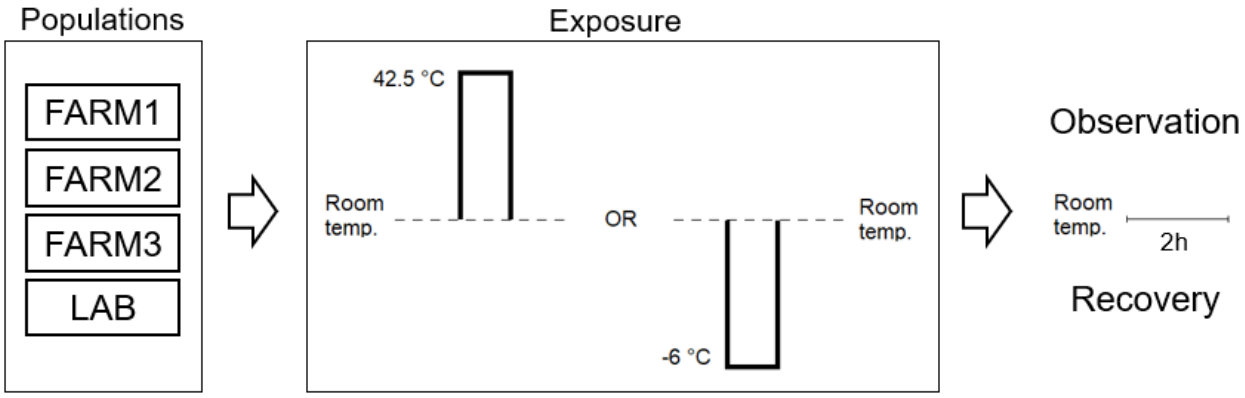
In Appendix Figure 1, we see that the insects recovered very quickly from the least extreme investigated temperatures; exposure to +42 °C did not even elicit heat coma from which the animals could recover. Furthermore, the animals showed a low ability to recover from the most extreme temperatures used: after an exposure to +43 °C, many insects were not able to recover within the recovery period (Appendix Figure 1, A), and after 2 hours at -8 °C it took around 45 minutes before half of the individuals had recovered (Appendix Figure 1, B).



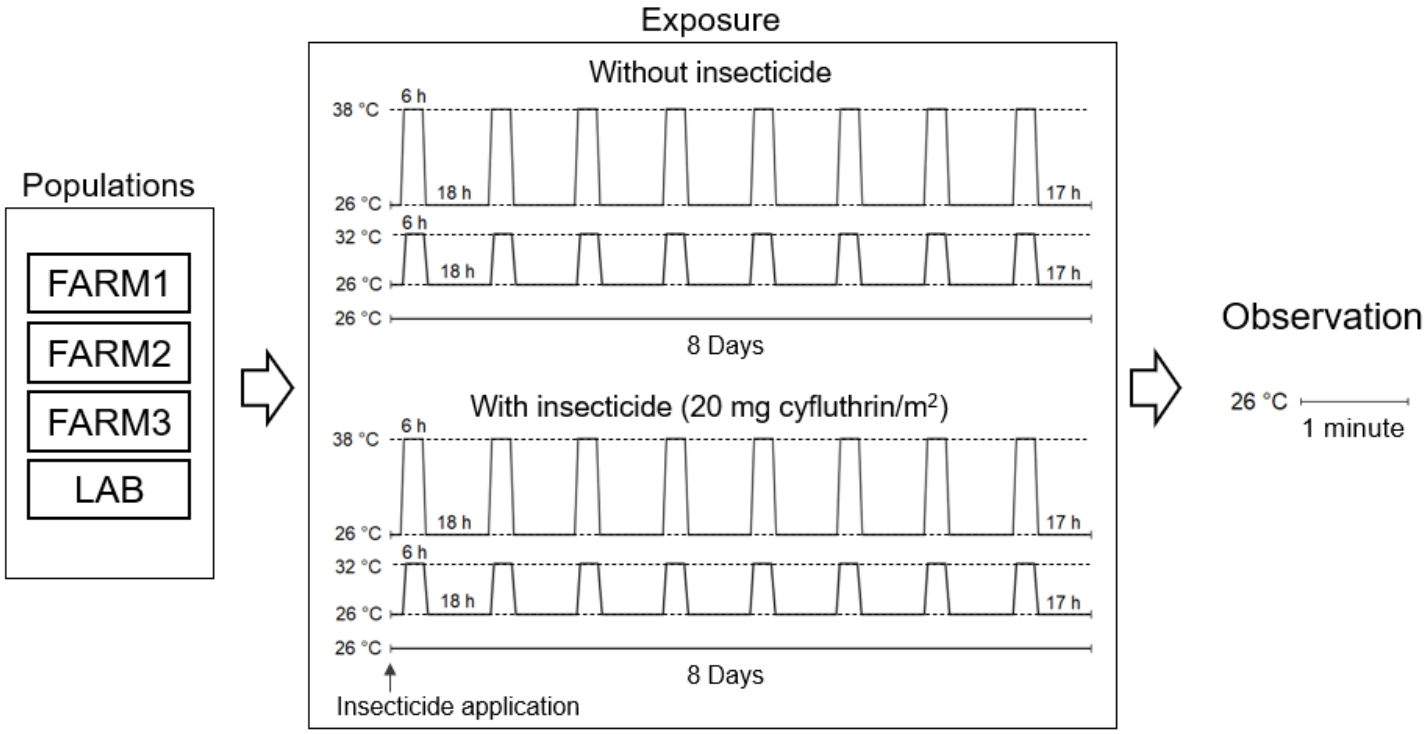
Appendix Figure 1 illustrates the recovery time of adult Alphitobius diaperinus from FARM1 after exposure to different extreme temperatures (represented by different coloured lines) for 2 hours. A) Recovery curve of individuals exposed to extremely warm temperatures (42 to 43 °C) (overall $P < 0.001$). B) Recovery trajectory of individuals exposed to extremely cold (-4 to -8 °C) temperatures (overall $P < 0.001$).

From these results, it can be concluded that adults of *A. diaperinus* have an extreme temperature tolerance that is within the range that commonly characterizes insects (Addo-Bediako, Chown, and Gaston 2000). We also see that the changes in recovery time with changing temperature follows a pattern typical from thermal performance curves (Colinet et al. 2015; Huey and Stevenson 1979). Specifically, great changes in thermal tolerance happens across a narrower temperature range at extreme heat (here between 42 and 43 °C) compared to extreme cold (here -4 to -8 °C). We also note that adults of *A. diaperinus* were stressed (went into chill coma), but all individuals recovered from sudden exposure at 42.5 °C and -6 °C. Therefore, these conditions represent suitable temperatures for comparing differences in recovery time among populations or treatment groups.

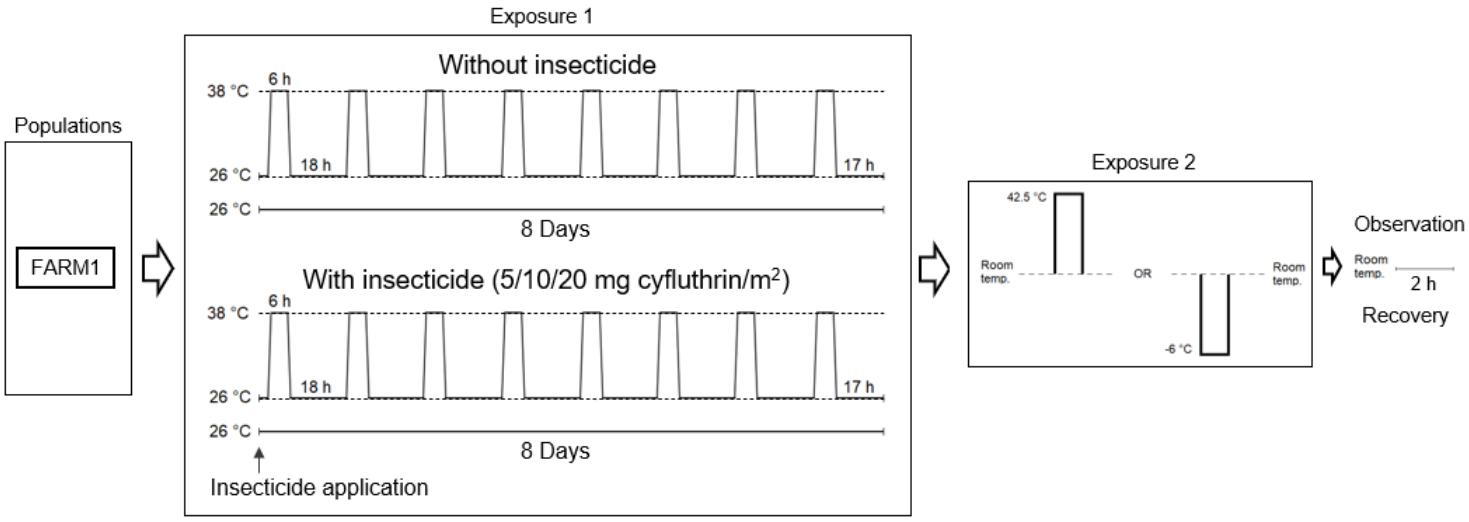
Supplementary File 2



Appendix figure 1 Schematic presentation of the design of experiment 1



Appendix figure 2 Schematic presentation of the design of experiment 2



Appendix figure 3 Schematic presentation of the design of experiment 3. The second part of the experiment (Exposure 2 and recovery observations) was identical to experiment 1, and can be seen in larger version in Appendix figure 1.

Supplementary File 3

Appendix table 1: Detailed statistical results of experiment 1 (corresponding to results presented in Figures 1 and 2 in the main text of the article). Pairwise comparisons (Log-Rank tests; graphs A-F) were performed using the Survdiff function in R. Significant p values are presented in bold.

Recovery from extreme heat (42.5 °C)

Duration	15 minutes exposure		
Population	FARM 3	FARM 2	FARM 1
FARM 2	**4.90E-06**	-	-
FARM 1	**4.90E-06**	1.0000	-
LAB	**0.004**	**< 2.00E-16**	**< 2.00E-16**

Duration	30 minutes exposure		
Population	FARM 3	FARM 2	FARM 1
FARM 2	**0.0020**	-	-
FARM 1	**0.0020**	0.8152	-
LAB	**0.0004**	**3.20E-14**	**< 2.00E-16**

Duration	1 hour exposure		
Population	FARM 3	FARM 2	FARM 1
FARM 2	**0.0001**	-	-
FARM 1	**4.90E-07**	0.9446	-
LAB	0.3298	**2.00E-08**	**2.70E-12**

Duration	2 hours exposure		
Population	FARM 3	FARM 2	FARM 1
FARM 2	**2.80E-06**	-	-
FARM 1	**2.30E-05**	0.2720	-
LAB	**0.0190**	**1.00E-07**	**1.70E-08**

Duration	3 hours exposure		
Population	FARM 3	FARM 2	FARM 1
FARM 2	**1.70E-08**	-	-
FARM 1	**3.40E-10**	0.6024	-
LAB	**0.0002**	**5.30E-14**	**1.50E-14**

Recovery from extreme cold (- 6 °C)

Duration	15 minutes exposure		
Population	FARM 3	FARM 2	FARM 1
FARM 2	0.7078	-	-
FARM 1	**0.0105**	0.1059	-
LAB	**7.60E-06**	**0.0013**	**0.0082**

Duration	30 minutes exposure		
Population	FARM 3	FARM 2	FARM 1
FARM 2	0.6470	-	-
FARM 1	0.1430	0.1430	-
LAB	0.0720	0.1430	0.2050

Duration	1 hour exposure		
Population	FARM 3	FARM 2	FARM 1
FARM 2	0.5049	-	-
FARM 1	0.8946	0.5049	-
LAB	**0.0039**	**0.0449**	**0.0080**

Duration	2 hours exposure		
Population	FARM 3	FARM 2	FARM 1
FARM 2	0.3069	-	-
FARM 1	**1.80E-07**	**0.0042**	-
LAB	**< 2.00E-16**	**2.20E-11**	**1.60E-10**

Duration	3 hours exposure		
Population	FARM 3	FARM 2	FARM 1
FARM 2	0.4633	-	-
FARM 1	**0.0019**	**0.0080**	-
LAB	**2.00E-13**	**< 2.00E-16**	**< 2.00E-16**

Duration	4 hours exposure		
Population	FARM 3	FARM 2	FARM 1
FARM 2	0.0591	-	-
FARM 1	**5.00E-06**	0.0591	-
LAB	0.0121	0.0002	**1.10E-08**

Duration	4 hours exposure		
Population	FARM 3	FARM 2	FARM 1
FARM 2	0.4142	-	-
FARM 1	**0.0002**	**0.0137**	-
LAB	**< 2.00E-16**	**< 2.00E-16**	**< 2.00E-16**

Appendix table 2: Detailed statistical results of experiment 2 (corresponding to results presented in Figure 3 in the main text of the article). Overall ANOVA tests (type II) to the left, Sidak adjusted *post hoc* test to the right. All data made using 95% confidence level, significant P-values are presented in bold.

FARM1	Chisq	Df	P-value
Daily heat spikes	0.6414	1	0.4232
Cyfluthrin treatment	16.6845	1	**4.41E-05**
Daily heat spikes & Cyfluthrin	1.1667	1	0.2801

FARM1	LCL	UCL	Group
Daily heat spikes & Cyfluthrin	0.931	0.973	a
Cyfluthrin treatment	0.947	0.983	ab
Control	0.968	0.994	b
Daily heat spikes	0.973	0.996	b

FARM2	Chisq	Df	P-value
Daily heat spikes	13.7	1	**0.000214**
Cyfluthrin treatment	973.44	1	**2.20E-16**
Daily heat spikes & Cyfluthrin	0.08	1	0.775383

FARM2	LCL	UCL	Group
Daily heat spikes & Cyfluthrin	0.484	0.567	a
Cyfluthrin treatment	0.569	0.651	b
Control	0.981	0.999	c
Daily heat spikes	0	1	abc

FARM3	Chisq	Df	P-value
Daily heat spikes	1.52	1	0.218
Cyfluthrin treatment	603.76	1	**2.00E-16**
Daily heat spikes & Cyfluthrin	0.02	1	0.8857

FARM3	LCL	UCL	Group
Daily heat spikes & Cyfluthrin	0.329	0.469	a
Cyfluthrin treatment	0.377	0.519	a
Control	0.961	1	b
Daily heat spikes	0.961	1	b

LAB	Chisq	Df	P-value
Daily heat spikes	9.53	1	**0.002018**
Cyfluthrin treatment	631.06	1	**2.20E-16**
Daily heat spikes & Cyfluthrin	0.27	1	0.604759

LAB	LCL	UCL	Group
Daily heat spikes & Cyfluthrin	0.084	0.193	a
Cyfluthrin treatment	0.177	0.315	b
Control	0.929	0.999	c
Daily heat spikes	0.929	0.999	c

Appendix table 3: Detailed statistical result of experiment 3 (corresponding to results presented in Figure 4 in the main text of the article). Pairwise comparisons using Log-Rank test were performed using the Survdiff function in R. Significant p values are presented in bold.

Extreme heat (42.5 °C) tolerance data

Insecticide conc.	Pre-treatment peaks:		non
mg cyfluthrin/ m2	0	5	10
5	**1.60E-06**	-	-
10	**< 2.00E-16**	**3.40E-09**	-
20	**< 2.00E-16**	**< 2.00E-16**	**9.20E-14**

Insecticide conc.	Pre-treatment peaks:		32 °C
mg cyfluthrin/ m2	0	5	10
5	**2.2E-06**	-	-
10	**2.0E-14**	**9.4E-09**	-
20	**< 2.0E-16**	**< 2.0E-16**	**5.9E-07**

Insecticide conc.	Pre-treatment peaks:		38 °C
mg cyfluthrin/ m2	0	5	10
5	0.17	-	-
10	**2.7E-05**	**1.3E-05**	-
20	**1.4E-14**	**1.9E-15**	**7.7E-05**

Extreme cold (- 6 °C) tolerance data

Insecticide conc.	Pre-treatment peaks:		non
mg cyfluthrin/ m2	0	5	10
5	0.4598	-	-
10	0.0627	0.1072	-
20	**0.0064**	**0.0341**	0.7462

Insecticide conc.	Pre-treatment peaks:		32 °C
mg cyfluthrin/ m2	0	5	10
5	**1.0E-05**	-	-
10	**3.6E-07**	0.25036	-
20	**5.2E-16**	**1.2E-05**	**0.00038**

Insecticide conc.	Pre-treatment peaks:		38 °C
mg cyfluthrin/ m2	0	5	10
5	0.25	-	-
10	0.7	0.65	-
20	**5.0E-07**	**8.3E-08**	**1.3E-07**

Appendix table 4: Bold numbers represent p values showing significant differences between graphs, bold and italicized p values showing significantly slower recovery in insects having experienced daily heat spikes in pre-treatment.

Pre-treatment	constant VS 32 °C peaks		constant VS 38 °C peaks	
Extreme temp.	42.5 °C	-6 °C	42.5 °C	-6 °C
Cyfluthrin conc. 0	**0.036**	0.092	**0.045**	0.29
Cyfluthrin conc. 5	**0.02**	0.07	**< 0.0001**	**0.0016**
Cyfluthrin conc. 10	0.068	0.62	**< 0.0001**	**0.00041**
Cyfluthrin conc. 20	**< 0.0001**	***< 0.0001***	**< 0.0001**	0.4

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Article 2

Effect of heat and pesticide exposure on *Alphitobius diaperinus*

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In preparation for Comparative biochemistry and physiology C

1 Introduction

In several ectothermic animals, tolerance to temperature changes are essential as their life cycle is driven by external temperatures, which vary both spatially and temporally (Soranno et al. 2019). Moreover, the global climate has already changed in the past decades, and the accelerating impact of human-mediated changes on climatic conditions is expected to increase further in the near future (IPCC 2014). In this context, ecophysiological studies aiming at improving our understanding of the effects of both chronic and acute thermal stress, as well as the physiological mechanism at play for coping with a changing climate have become increasingly important. With predictions of climate change, the interest in studying animals' tolerance to extreme heat exposure has greatly increased, mainly because the occurrence of hot periods is expected to rise (Stillman 2019). Similarly, the use of temperature variations in experimental setups in the context of thermal tolerance have increased in recent years, so that the experimental plans have increased resemblance with natural situations, thereby making extrapolations to field scenarios more realistic. In turn, this knowledge feeds models predicting the future fate of insect populations (Buckley et al. 2010; Thompson et al. 2013).

The fate of insect populations in our environment is also affected by the massive use of pesticides, and more particularly insecticides which have been massively sprayed in the modern agriculture. Yet, studies looking at the effects of insecticide exposure combined with temperature variation remain scarce (see Holmstrup *et al.*, 2010 for a review), even though the use of heat waves, heat peaks and fluctuating temperature regimes in combination with pesticides are now receiving increasing attention. Especially research on aquatic life stages of insects (mosquito and damselfly larvae) exposed to chlorpyrifos provided evidence of both pesticide-induced

temperature sensitivity (Op de Beeck, Verheyen, and Stoks 2017, 2018; Delnat et al. 2019; Meng, Delnat, and Stoks 2020) and temperature-induced pesticide sensitivity (Arambourou and Stoks 2015; Dinh, Janssens, and Stoks 2016; Verheyen, Delnat, and Stoks 2019; Verheyen and Stoks 2019; Willming, Qin, and Maul 2013). However, the use of adult life stages and terrestrial species in multiple stress research, as well as using insecticides with a different mode of action to the organophosphate mentioned above remain absent from the available literature.

In order to address the abovementioned research gaps, this study investigates the effects of combining insects' exposure to the pyrethroid insecticide Cyfluthrin with daily heat spikes using adults of the widely distributed poultry house pest beetle, *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae) as a model. The goal of this study was to investigate the effects of repeated moderate stress exposure (daily heat peaks) alone, and combined with constant pesticide exposure, on the survival of this terrestrial insect, thus allowing to reveal the joint effects of these two stressors. Furthermore, we have measured the level of antioxidant capacity in the insects, because ROS (Reactive Oxygen Species) scavengers (which are a group of antioxidant substances) have often been reported in higher amounts in several organisms handling stressors, including pesticides (Sulmon et al. 2015; Verheyen and Stoks 2019). Lastly, metabolite composition in animals from the different experimental treatments was determined, in order to see if, and how, the different stressors individually, or in combination, affected the insects' homeostasis.

We expected that metabolites fueling the energetic needs of the insects would be consumed at a higher rate in stress exposed beetles, as they would be using these as substrates for maintaining homeostasis and/or repair damages inflicted by the stressor. Such increase in energy metabolism could for example be reflected in increased number of metabolites in the citric acid cycle. Also, we expected that these effects would increase when increasing the number of exposure events (in the form of daily spike of heat). We also expected that indicators of ROS scavenging would be elevated when beetles were exposed to heat and cyfluthrin, and more so if these two stressors occurred in combination.

Further, *A. diaperinus* adults were also used to investigate if these stress exposures affected the fitness of the beetles over the long-term. To reach this objective, we estimated the impacts in terms of reproduction, by measuring the number of offspring produced per female. We expected that insecticide and heat exposures would result in reduced number of offspring, because of adverse effects on reproductive function in the beetles (David et al. 2005; Galvan,

Koch, and Hutchison 2005). However, as stressful environments can result in an increased reproduction response, the outcome will be driven by the nature of the counteracting effects at play (Lee 2000). The outcome of heat exposure was particularly difficult to predict, as the ectothermic nature of these animals means processes linked to reproduction may be elevated at higher temperatures (Adamo and Lovett 2011). However, we expected that the dominant effect of heat on reproductive output would be negative, through damage or resource allocation towards thermal tolerance (Aira et al. 2007), at the expense of reproductive output.

2 Methods

2.1. Rearing of the insects

Adults of *A. diaperinus* were collected from two turkey farms, thereafter referred as to Farm A (sampled at Plaine Haute, 48°26′44″ N, 2°51′16″ W) and Farm B (sampled at Miniac-sous-Bécherel, 48°17′10″ N, 1°55′51″ W) in Brittany, France. After collection, insects were maintained in sawdust at 25 +/- 1°C in temperature cabinets (Lovidond incubators, TC 225 S, Tintometer GmbH, Sarasota, USA), with *ad libitum* water and food in the form of dry dog food pellets, oat bran and water in microtubes with cotton. The insects were reared in plastic boxes (30 x 30 x 13 cm) in the dark.

2.2 Experimental chamber preparation

Before experimental start, a fitting piece of filter paper was placed at the bottom of glass Petri dishes (10 cm diameter). To evaluate the survival capacity of adults of *A. diaperinus* to pesticides, insects were treated with the insecticide cyfluthrin (pure molecule standard, CAS-number 68359-37-5, Sigma-Aldrich). The insecticide was diluted in acetone so that the recommended application dose of this chemical in the field could be achieved (20 mg cyfluthrin/m^2) by depositing 2 ml of a cyfluthrin/acetone solution on the filter paper covering the bottom of each Petri dish. Control treatments were prepared by spreading 2 mL of acetone to the filter paper, so that the solvent effect could be disregarded. The Petri dishes were left open in fume hoods for at least 10 minutes, corresponding to the time required for the full evaporation of acetone.

For the beetles collected at Farm A, a total of 30 dishes with only acetone (control), and 42 dishes with cyfluthrin was prepared for insects further used for metabolomics and antioxidant assays. For Farm B, 40 acetone treated control dishes and 40 dishes with cyfluthrin on the filter paper were prepared and further used for testing the effects of the treatment on the reproduction ability of the insects. The animals from farm B tolerated the relevant level of pesticide and temperature stress so well that selection pressure from these stressors between generations

was considered negligible (Dahl et al. 2021). For all treatments, 30 adults were placed in each petri dish and supplied with a microtube with water (0.5 mL tube with cotton, refilled every second day) and a dog food pellet (0.8 g), for *ad libitum* access to food and water during the experiment.

2.3 Exposure treatment

After the individuals were added to the glass Petri dishes, dishes were placed in temperature cabinets. One temperature cabinet (cabinet 1) was set at a cycle of 6 hours (h) at 32 °C followed by 18h at 26 °C (referred to as control condition) and a cabinet (cabinet 2) programmed with a 6 h duration at 38 °C, followed by 18 h at 26 °C (referred to as daily spike of heat) (Figure 1). Half of the insecticide free dishes and half of the dishes with insecticide in them were placed in each cabinet. An iButton® (iButtonLink, LLC., Whitewater, USA) was also placed in each cabinet to monitor the temperature.

The effect of treatment on the animals was registered as number of active (could move normally) or impaired (any problems moving limbs or completely paralyzed) animals in each Petri dish after 8 days. Beetles from Farm A were also observed after 1 and 4 days.

After 1,4 or 8 days, Farm A animals were noted as active or not, and removed from pesticide and/or heat exposure (Figure 1) (7 dishes from each pesticide exposure treatment and 5 dishes from each treatment without pesticides), and 40 healthy/active individuals from each condition were used for immediate sampling for antioxidant capacity and metabolomics testing. Samples for antioxidant capacity and metabolomics were shock-frozen in liquid nitrogen and then stored in a freezer (-25 °C). After 8 days exposure, animals from a chamber of each exposure treatment were to recover for 4 days in clean Petri dishes? in cabinet 1. After 4 days of recovery, beetles from each treatment were sampled again, and snap-frozen for metabolomics analysis.

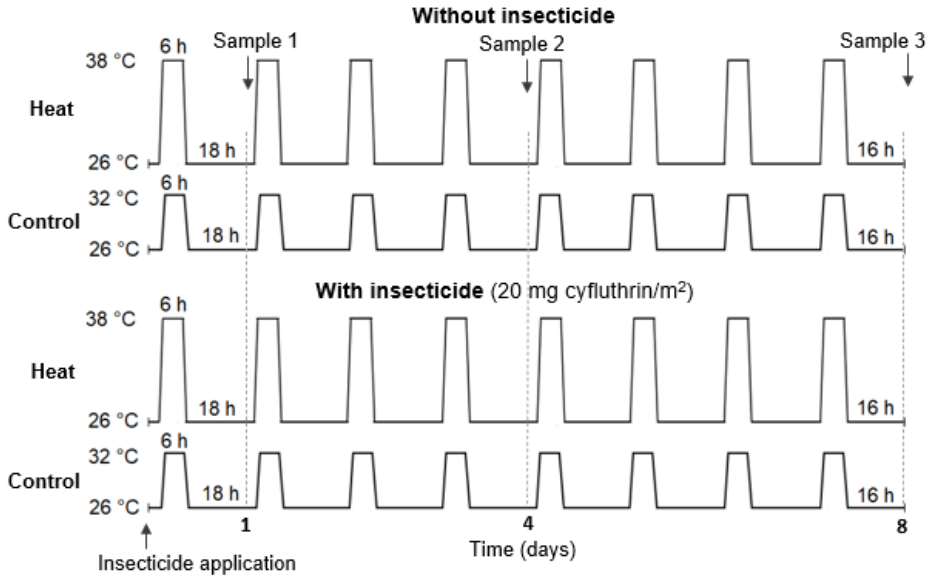


Figure 4 Illustration of exposure temperatures of the different treatments and sampling times. For farm A insects, the sampling times 1 to 3 represent the time points where insects were observed and noted as active, mobile or immobile, and then immediately sampled for physiological analyses. After 8 days exposure, they were removed from any exposures and set to recover for 4 days in control cabinet before sampling for physiological analyses.

2.4 Physiological Analyses

Samples for metabolomics and antioxidant capacity measurement were taken from the freezer (-25 °C) and three animals at a time from each treatment were weighed on a Sartorius Analytic scale, and placed in marked 2 mL Eppendorf tubes. Six replicates for each treatment were made and to measure antioxidant capacity, samples were treated as described in the Antioxidant Assay Kit (Cayman Chemicals). Following antioxidant assays, protein analysis was conducted using Bradford's technique (colorimetric assay, wavelength 595 nm) and antioxidant values were normalized to protein content.

Gas Chromatography/Mass Spectrometry (GC-MS) was used to compare the metabolic profiles of the beetles at the end of their respective exposure treatment, and after 4 days recovery. Samples were crushed in 500 µl buffer, and 500 µl methanol and 250 µl chloroform was then added before all tubes were mixed using a vortex and then centrifuged. Subsequently, 90 µl aliquots of the upper aqueous phase (containing the polar metabolites) were transferred to glass vials and vacuum-dried. The dried sample was then suspended 30 µl methoxyaminehydrochloride (Sigma-Aldrich, St. louis, MO, USA) in pyridine at 20 mg mL^{-1} and after incubation at 40 °C for 90 min, 30 µl of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA;Sigma, #394866) was added. The derivatization was conducted at 40 °C for 45 min

under agitation. Metabolite analysis was conducted as described in detail by Laparie & Renault (2016).

2.5 Reproduction

For all treatment groups, after the 8 days, samples of 50 Farm B individuals from the same treatment were placed together in each of 10 plastic boxes (15 x 25 x 10 cm), resulting in a total of 40 plastic boxes. In each plastic box there was 80 ml of fine wheat flour, topped with 80 ml of oat bran. A piece of carrot cut along the center core, approx. 5 cm long, was placed in each container as a source of water for the animals, and to provide a moist area under which the adults could lay their eggs. The boxes had their lids on to retain moisture, with an approx. 1.5 cm wide opening to allow fresh air to enter. After 7 days, the food was run through a sieve that eliminated the adults only, after which 80 ml of new oat bran was added and the piece of carrot was changed every second day to avoid mold growth, while any offspring hatched and grew. Adults were then placed in microtubes with ethanol, for subsequent gender determination. Gender was determined based on the apical spurs on the second pair of legs, as well as the shape of their genitalia, according to Esquivel, Crippen, & Ward (2012). After about 7 weeks, the number of offspring in each box were counted.

3 Results

Control and heat exposed animals all survived the experimental period, whereas around 5 animals in each experimental chamber died when exposed to the insecticide cyfluthrin, with or without daily heat peaks (Figure 2). After 8 days exposure, it appears that animals experiencing heat peaks in addition to cyfluthrin exposure had significantly fewer active individuals than any other treatment group (Figure 2).

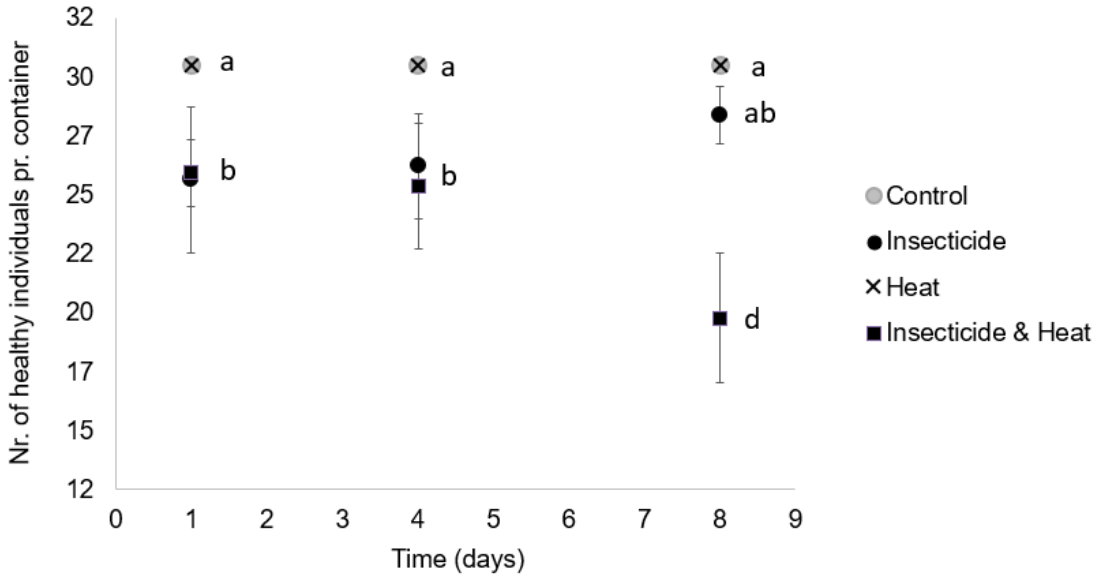


Figure 2. The mean number of active individuals (out of 30) (±SE) of *A. diaperinus* (from Farm A) after 1, 4 and 8 days of exposure. Different symbols represent the number of animals treated with either insecticide (Black circle – 20 mg/m^2 cyfluthrin), Heat (Cross - 6h period at 38°C daily), a combination of the two stresses (Black squares) or Controls (Gray circles). See text for further explanation of treatments. Different letters indicate significant differences between means of treatments at each exposure time.

Based on the results presented in Fig. 3, there were no significant differences between antioxidant capacity of animals from different treatments across time.

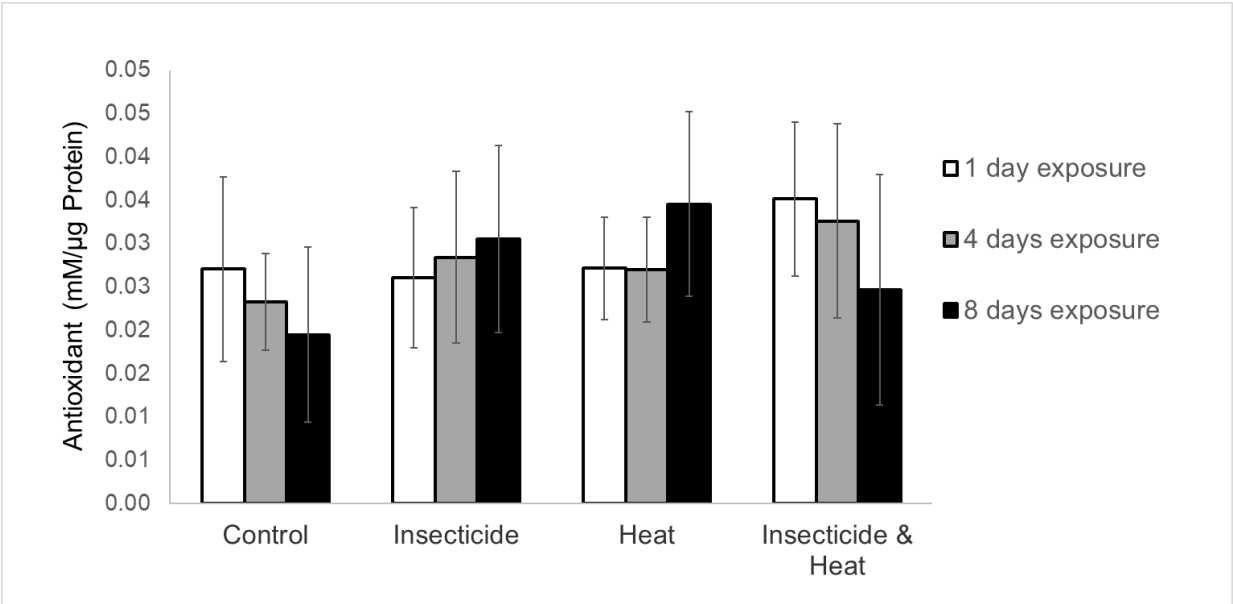


Figure 3. Average Antioxidant capacity +/- SE in *A. diaperinus* after 1 (white), 4 (gray) or 8 (black) days exposure to 4 different treatment groups (Control, Pesticide exposed (20 mg/m^2, cyfluthrin) Heat exposed (6h at 38°C) or combined pesticide & heat exposure. No significant differences in antioxidant capacity was observed between the different treatment groups.

We see in Fig. 4 that we cannot distinguish between overall fingerprints of the different groups.

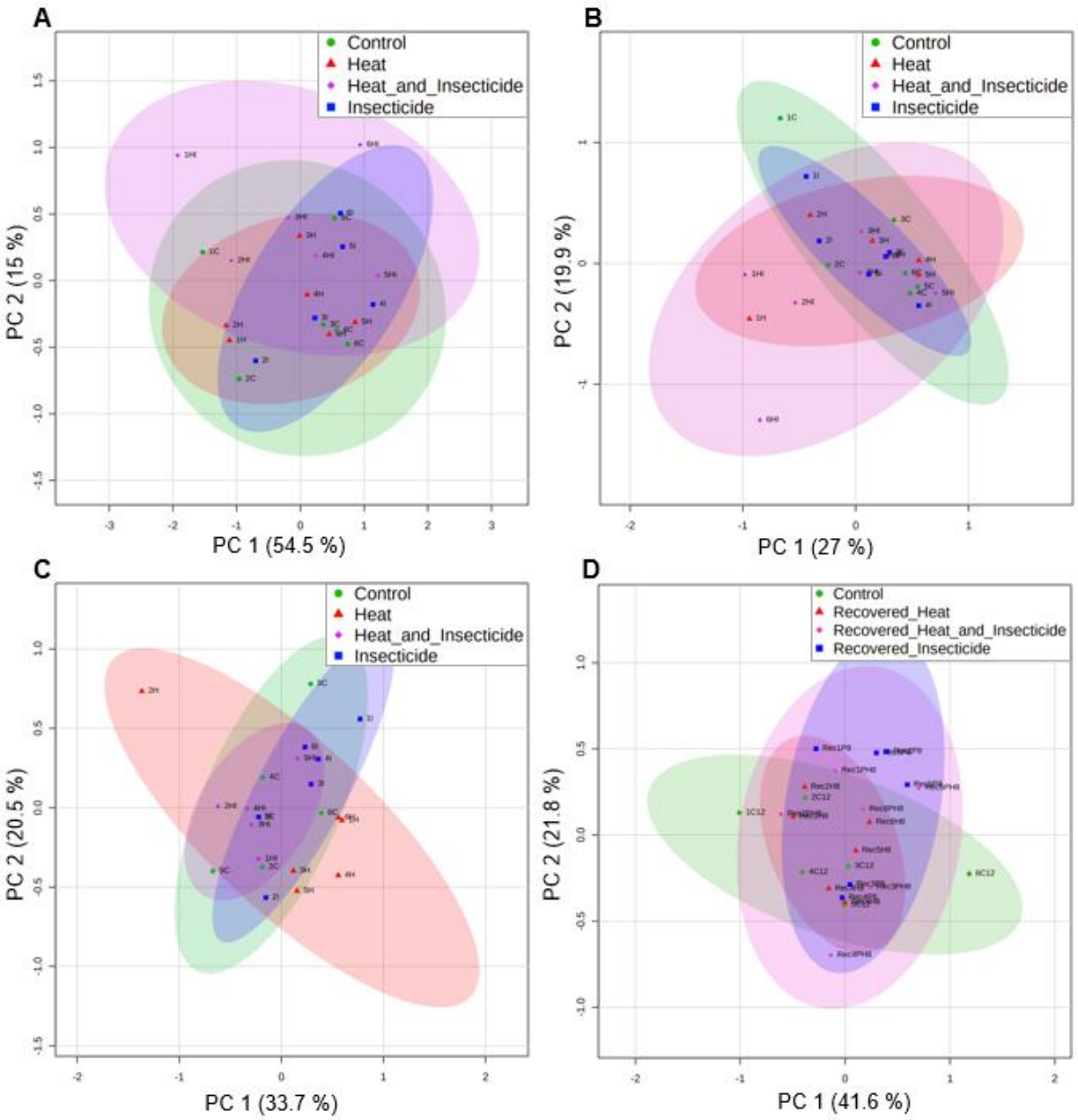


Figure 4 Illustrates the metabolite profile of *A. diaperinus* after 1(A), 4(B) or 8(C) days exposure to 4 different treatment groups (Controls, Pesticide exposed (20 mg/m^2, cyfluthrin) Heat exposed (6h at 38°C) or Pesticide & Heat exposed). Panel D illustrates the metabolite profile in animals recovered for 4 days after 8 days exposure to the different treatments. Note that the different figures have different scales on the X and Y axis.

Number of offspring were significantly increased for animals experiencing a daily heat peak, compared to controls, but animals experiencing daily heat peaks while being exposed to insecticide had significantly fewer offspring compared to the animals who experienced daily heat

peaks with no other stress. However, pesticide exposure alone did not significantly reduce number of offspring per female (Figure 5).

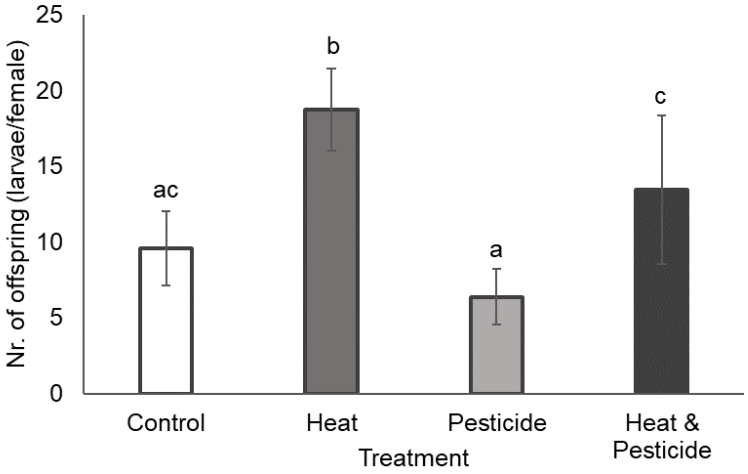


Figure 5. Average total number of offspring per female (+/- SE) after 8 days exposure to one of four different treatment groups (Controls, Pesticide exposed (0.02 g/m^2, Cyfluthrin) Heat exposed (6h at 38°C) or Pesticide & Heat exposed, followed by 7 days of reproduction and 30 days for larvae development without adults present.

4 Discussion

Similar to earlier findings, the present study found that the Farm A population has a high resistance to the insecticide Cyfluthrin (Dahl et al. 2021; Hamm et al. 2006; Lambkin and Rice 2009). In the present study, tnsecticide treatment resulted in slightly lower number of active animals than in controls after 1 and 4 days, but after 8 days this effect was no longer significant. However, it seemed that the effect of the insecticide was relatively stable throughout the experimental period, indicating that the pesticide concentration did not change substantially. Despite observing no effect at all of the heat exposure treatment on number of active individuals, and only a small effect of cyfluthrin, the two stressors combined resulted in significantly fewer active individuals after eight days of exposure. This suggests that the two stressors interacted synergistically considering number of active animals. However, this effect is not reflected in metabolic changes, that is, throughout the experimental period in all treatments, the metabolic profile of the animals remained similar. This indicates a high level of tolerance to the investigated stressors, that has not required metabolic changes or incurred system failure.

The antioxidant capacity assay did not reveal any significant changes in any of the treatment at any time point sampled in our experiment. This indicates that the animals were not experiencing

a change in the oxidative stress from our exposure treatments. This result is unexpected, as Verheyen & Stoks (2019) found increased antioxidant activity of specific enzymes when *Ischnura elegans* damselfly larvae were exposed to chlorpyrifos. In addition, Zhang et al. (2015) found increased antioxidant capacity in the ladybeetle, *Propylaea japonica*, exposed to high temperature stress. Meanwhile, neither insecticide exposure, daily heat peaks, or a combination of the two, resulted in increased antioxidant capacity in *A. diaperinus*. It could be that these wild collected populations have experienced a level of pollution in their environment which has geared their antioxidant capacity to a constant high level, however, the antioxidant capacity measured here are all in the range of the initial antioxidant capacity measured in ladybugs by Zhang et al. (2015), so we would not expect this to be the case.

The reproduction data could indicate that the maintenance of homeostasis under insecticide exposure comes at the cost of reproductive output. Whether the animals are exposed to insecticide only, or insecticide combined with daily heat peaks, there is a trend in the data which indicates that pesticide exposure has a negative effect on number of offspring. This fits well with other studies such as Abd-Elghafar and Appel (1992), Hajjar and Ford (1989) and Bariola and Lingren (1984) who found a decrease in fecundity resulting from exposure to a number of insecticides, including cyfluthrin. The negative effect of insecticide exposure alone (albeit statistically insignificant), and the positive effect of intermittent heat exposures seemed to be nearly perfectly additive when combined, and still significantly higher than controls. However, this interpretation should be backed by an appropriate formal statistical test (Schäfer and Piggott 2018). The positive effect of heat exposure on the number of offspring per female per sample was substantial (almost twice as high as controls). Since we measured reproduction only after exposure, this effect could be the result of delayed reproduction. Huang, Chen, & Kang (2007) found that heat stress can lead to delayed ovipositioning during the stress period, with the implication of increased ovipositioning in the period immediately following, which would result in an increase number of offspring compared to controls in our study. However, further studies on fecundity of the animals after stress would be needed to further discuss the underlying mechanisms of the effect we have described.

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Chapter 4

The effects of desiccation in the lesser mealworm *Alphitobius diaperinus*

Desiccation in the lesser mealworm *Alphitobius diaperinus*

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Abstract

In order to improve predictions of the impacts of climate change on insects, this study aimed to uncover how exposure to dry conditions can affect the biology of the invasive pest beetle *Alphitobius diaperinus* in terms of longevity, activity, water content, metabolic profiles and fecundity. We measured desiccation tolerance in *A. diaperinus* by recording the time the beetles could survive desiccation stress. We found that the species was highly desiccation resistant, with 50% of the experimental animals surviving 30 days, and some individuals surviving up to 50 days in 14% ± 2 air humidity. There was no evidence of active upregulation of sugars or other metabolites which the animals could have used to better tolerate desiccation. However, food deprivation appears to have affected both control and treatment group animals, as their metabolite composition changed similarly after 1 week of treatment, and the activity of animals from both desiccation treatment and controls was increased, two weeks after experimental start. Despite not revealing any physiological changes in the animals experiencing desiccating conditions, these animals had a significantly reduced reproductive output compared to controls, after 8 days of treatment and a week of subsequent reproduction. This indicated a physiological cost of drought resistance or that damage from drought exposure occurred, which has not yet been described for tenebrionid beetles, and could have negative effects on *A. diaperinus* populations.

Keywords: Dehydration, Beetle, Osmolarity, Metabolites, Sugar, Activity.

1 Introduction

In the context of climate change, warming and the increasing frequency of dry spells threaten terrestrial biodiversity by increasing the likelihood of individuals' desiccation (IPCC 2014; Trenberth 2011). In particular, small animals such as insects, which have a large surface-to-volume ratio, are particularly susceptible to evapotranspiration and exposures to dehydrating conditions (Hadley 1994). Therefore, they have developed a wealth of behavioural, morphological and physiological adaptations aiming at limiting loss of body water and/or the detrimental consequences thereof (Hadley 1994; Thorat and Nath 2018). Knowledge from investigating the nature of these adaptations, and the variation in such traits within and across species, can contribute to improve predictions of the impacts of climate change (Chown and Gaston 2008; Helmuth, Kingsolver, and Carrington 2005).

Insect behaviour is an important adaptation to alterations in humidity conditions (Rowley and Hanson 2007). If possible, insects will avoid dry microhabitats, and relocate towards more humid environments, as recently shown in the yellow-spined bamboo locust *Ceracris kiangsu* (Yu et al. 2010), as well as several other species (Heger, Guerin, and Eugster 2006; Kessler and Guerin 2008; Rowley and Hanson 2007). If a more optimal microhabitat is not available, or if the distance to reach it is too high for the species, insects must then rely on morphological and physiological adaptations to survive stressful dry spells. The main physiological responses to desiccation include limiting the rate of water loss (desiccation resistance) (Ahearn 1970; Zachariassen 1991) or increasing tolerance to water loss (desiccation tolerance) (Arlian and Staiger 1978; Hadley 1994) and the main routes of water loss for insects are excretion, transpiration and respiratory loss (Chown, Sorensen, and Terblanche 2011).

For many desert beetles, cuticle permeability represents a prominent source of body water loss, and adjustment in cuticle composition can thus greatly contribute to enhance desiccation resistance (Ahearn 1970; Cloudsley-Thompson 2001). Other studies have shown that the tenebrionid sub-elytral cavity can also effectively reduce transpiration (Ahearn and Hadley 1969; Draney 1993), further increasing the level of desiccation resistance of the insects. Reducing transpiration by lowering activity and reducing metabolic rate also enhance the capabilities of insects to handle dryer environmental conditions (Williams, Rose, and Bradley 1998). For instance, a study comparing *Drosophila* flies from desert and mesic regions, showed that species from dry regions mainly increased resistance to desiccating conditions by reducing activity and metabolic rate as compared to mesic species, thereby decreasing respiratory water loss (Gibbs, Fukuzato, and Matzkin 2003).

Desiccation resistance or tolerance can be enhanced by modulation of haemolymph osmolarity, by for instance accumulation of sugars and polyols in the insects' body fluids (Gray and Bradley 2005). In terms of desiccation tolerance, trehalose (but also other sugars and polyols) has been often reported as an important metabolite for insect subjected to harsh desiccating conditions, and is even indispensable for those invertebrates capable of anhydrobiosis (Crowe 2002; Mensink et al. 2017; Thorat and Nath 2018; Watanabe et al. 2002). Trehalose helps to maintain the integrity of cell membranes and proteins (Kikawada et al. 2007). Interestingly, similar metabolic changes have been reported from insects exposed to drought and other abiotic stressors, such as salt and temperature stress. For example, levels of amino acids, sugars, and sugar alcohols typically increase in response to different stress conditions (Colinet et al. 2012; Guy et al. 2008; Krasensky, Jonak, and Mendel 2012). The accumulation of these low molecular weight compounds (often referred to as compatible osmolytes) can help cells to reduce water loss during desiccation (Yancey 2005). In addition to helping cells maintain osmotic pressure, these compounds may have other protective functions, such as protein (Mensink et al. 2017) and membrane stabilization (Crowe 2002) and may have antioxidative properties (Liang et al. 2013). Therefore, describing changes in metabolite composition in animals during stressful conditions can help elucidate the animals' coping strategy.

When insects have to actively maintain homeostasis, for example by maintaining water balance, they can reallocate resources from other biological processes to improve the overall individual's fitness (Chown and Nicolson 2004). In *Drosophila*, increased stress tolerance has often been negatively correlated with life-history traits other than longevity (Hoffmann and Parsons 1989). For example, coping with stress can affect reproductive fitness either in terms of gametes or progeny quality (Schreck, Contreras-Sanchez, and Fitzpatrick 2001). In addition, probably in consequence of resource allocation in parents, studies on costs of reproduction indicated that reproduction increases stress susceptibility and oxidative stress (Salmon, Marx, and Harshman 2016; Wang, Salmon, and Harshman 2001). In addition, some studies have found negative associations between dry conditions and reproduction in insects in the field (Vessby 2001; Yaro et al. 2012). The scarcity of knowledge on how desiccation affects insects is surprising, as this clearly could have consequences of great ecological relevance.

In the present study, we investigated the desiccation resistance of the tenebrionid beetle, *Alphitobius diaperinus* Panzer, which is a tropical species that has invaded Europe, and nowadays represents one of the most abundant pest insects in poultry houses (Rueda and Axtell 1997). Owing to its origin, the beetle was expected to have a good desiccation resistance

(Zachariassen and Einarson 1993). Our aim was to uncover how exposure to dry conditions can affect the biology of this beetle in terms of longevity, activity, water content, metabolic profiles and fecundity. We measured desiccation tolerance in *A. diaperinus* by recording the time the beetles could survive desiccation stress. We hypothesized that stress exposed animals would not live as long as control animals. We also hypothesized that animals exposed to desiccating treatment would lose body water content over time compared to controls, but increase the internal concentration of soluble sugars to increase desiccation resistance. Furthermore, we expected the activity of stressed animals to be lower, to reduce metabolism and thereby reduce respiratory water loss. In order to measure any effect of desiccation on fecundity, we determined the number of offspring animals desiccated for 8 days had, after one week of reproduction and approximately one month of development. We expected animals having experienced desiccation to produce fewer offspring than control animals.

2 Methods

2.1 Collection and rearing of insects

Adults of *A. diaperinus* were hand-collected from a poultry house at Miniac-sous-Bécherel (Brittany, France; 48°17'10'' N, 1°55'51'' W) in October 2019. Directly after collection, the insects were transported to the laboratory (UMR EcoBio, University of Rennes, Rennes, France) and kept in plastic containers (30 x 30 x 13 cm) in the dark at constant 25 ± 1 °C (Lovidond incubators, TC 225 S, Tintometer GmbH, Sarasota, USA). The insects were fed *ad libitum* with pellets of dry dog food and oat, and supplied with tap water in 2 mL microtube closed by wet cotton. The age and sex of the insects was not controlled for the experiments, as we were aiming at getting estimates of the desiccation tolerance that would be representative of the responses at the populational scale.

2.2 Effects of desiccating conditions on the survival and physiology of the insects

2.2.1 Survival of the insects exposed to desiccating conditions

To measure the survival capacities of adult *A. diaperinus* continuously exposed to desiccating conditions in comparison to controls, groups of 10 beetles were transferred to 24 round plastic containers (5 cm diameter, 4 cm tall), without food. To assess the survival of the beetles maintained at low relative humidity conditions, four containers were placed into each of three distinct larger and transparent plastic boxes (35 x 25 x 10 cm), whose bottom was covered by 3 cm of desiccant (silica gel). The plastic boxes were hermetically closed. Three additional large plastic boxes, each again containing four round containers of 10 beetles, were realised and served as controls (wetted cotton balls were placed at the bottom of the plastic boxes to maintain

relative humidity optimal). All insects were deprived from food during the whole duration of the experiment to avoid survival differences that would have inevitably occurred among stressed insects progressively falling into coma and control ones. The groups of 10 beetles were scored for survival twice a week for six weeks at room temperature (22 ± 2 °C). At each observation, the beetles were categorized as alive (walking and fit beetles) or dead, corresponding to insects without visible movement of legs or other body appendage, even when the plastic boxes were gently knocked to induce vibrations that stimulated responses of alive insects. In the boxes, relative humidity and temperature was monitored with hygrochron ibuttons (Mouser electronics, Brive La Gaillarde, France). Over the course of the experiment, relative humidity was 14 ± 2 and 60 ± 5% in relative and optimal humidity conditions, respectively.

2.2.2 Measurements of body water and sugar content

Changes in body water and sugar contents were monitored by setting two additional plastic boxes for each experimental condition (control, desiccating environment). Here, five containers, each containing groups of 20 insects were set up in each box. Once every week, eight replicates of three individuals were taken from each treatment. The wet mass of each pool of three insects was measured directly after collection, and the samples were then transferred in a dryer (60 °C) for three to four days, before dry mass was measured. Water content was then calculated as the difference between wet and dry mass for each replicate of three individuals, in relation to their respective wet mass. After five weeks of experiment, the number of survivors was drastically reduced, and only three replicates of three insects were made for water and sugar content determination from insects exposed to desiccating conditions.

Total soluble sugars were measured from the samples whose fresh and dry mass was determined (six replicates of three insects for each experimental condition, except for the insects exposed for five weeks at the desiccating condition); we used the method described by Foray et al. (2012). Briefly, two tungsten beads (Ø = 3 mm) was added to each sample, before they were homogenized in 1200 µL of a 2:1 (v:v) mixture of methanol:chloroform with a bead beater (Retsch) at 25 Hz for1 min 30. Then, a volume of 800 µL of water was added to each sample, and they were vortexed before being centrifuged at 4,000g for 10 min at 4 °C. 400 µL of the upper alcoholic phase of the supernatant was pipetted, and let dried for 30 min under a fume. Total soluble sugars were measured by adding 1000 µL of a solution of Anthrone mixed in 70% H_2SO_4. The samples were plunged in a water bath at 90 °C for 15 min. The absorbance of the samples was then determined using a spectrophotometer at 625 nm wavelength, for which a

reference curve was made using known concentrations of glucose, and this was used to estimate soluble sugar content in the samples based on their respective absorbance.

2.2.3 Metabolomics

The beetles (3 insects / replicate) were homogenized in 750 µL of a solution of ice-cold methanol/chloroform (2:1, v:v). Two tungsten beads were added to the sample which were crushed with a bead beater (25 Hz for 1 min 30). Then, 500 µL of ultrapure water was added, and the samples were centrifuged at 4 °C, 4000 g for 10 min. 850 µL of the upper phase of the supernatant was pipetted and stored the samples at -25 °C until being processed for GC-MS analyses.

300 µL of the extract was transferred to new glass vials. Samples were vacuum dried (Speed Vac Concentrator, MiVac, Genevac Ltd., Ipswich, England). We used the derivatization process described by Khodayari et al. (2013), which relies on the use of a CTC CombiPAL autosampler (CTC Analytics AG, Zwingen, Switzerland) ensuring an on-time preparation and analysis of the samples. The analyses were performed with a GC-MS platform comprising an Agilent 7890B gas chromatograph coupled to a 5977B mass spectrometer. We used the same settings as those described in Thiebaut et al. (2020). The detected peaks (electron energy: 70 eV; in full scan mode) were annotated with MassHunter. Concentration of each metabolite was calculated using individual quadratic calibration curves.

2.3 Effects of desiccating conditions on activity and reproductive capacities of the insects

2.3.1 Measurement of locomotor activity

For each experimental treatment (control, desiccation), activity of individual beetle was measured once at the start of the experiment, and after three weeks of experiment. Petri dishes with squared paper ($1cm^2$) on the bottom were used to measure beetle's activity, recorded as the number of times they crossed a line during one minute. Thirty individuals were used from each treatment for these observations, taken from the setup used for water and sugar content determination.

2.3.2 Measurement of the reproduction capacities of the beetles

Finally, we aimed at assessing the long-lasting effects desiccating conditions could have on the fitness of the beetles by measuring their subsequent reproductive capacities. To that aim, two sets of seven plastic boxes (30 x 25 x 10 cm) were setup for controls and desiccation treatment, as described in the survival experiment section. Six small plastic containers (5 cm diameter, 4

cm tall), each containing 15 insects were placed in each plastic box for eight days at 25 °C. Humidity was measured in the air inside the desiccation boxes to 14 % relative humidity (RH) ± 2 %, by Hygrochron iBbuttons® (Mouser electronics, Brive La Gaillarde, France) measuring every second hour. As the duration of the exposure would not induce a comatose state in any animals, and as we were interested in testing the impact solely of desiccation on reproductive capacities of the beetles, the plastic containers inside the desiccation boxes were set up with a dog food pellet; control containers were all additionally provided with a micro tube with water and cotton for *ad libitum* water access. After 8 days of experimental treatment, plastic boxes were briefly opened to assess the health status of the individuals, where no mortality (mortality was defined as no movement and lack of response when disturbed) was observed. Then, groups of 50 insects from the same treatment were transferred to new plastic boxes (15x25x10 cm)) with lids that had an approximately 10cm^2 aeration hole. The bottom of the plastic boxes was covered with approximately 0.5 cm of fine wheat flour, topped with approx. 0.7 cm of organic oat bran. A piece of organic carrot weighing approximately 8 grams was placed in each container as a source of water for adults, and to provide a moist area under which the adults could lay their eggs. After one week in these boxes for the adults to reproduce, the food was run through a sieve that eliminated the adults only. The food and carrot they had was then returned to their respective boxes for the eggs, and later larvae to develop and grow. After 30 days (during which food and water (oats and tap water in 1.5 mL microtube closed by wet cotton) was refilled to ensure *ad libitum* access) the food was finally sieved with a finer sieve to get the larvae separated from the food and counted.

2.4 Statistics

All statistical tests were run in R version 3.5.2. Difference in survival between controls and desiccation treated animals was compared using Kaplan Meier plot with a log rank test, using the survminer package in R. Changes in water and sugar content over time was measured using a two-way ANOVA with *post hoc* multiple comparisons using multiple t-tests and Bonferroni adjusted P-values. Identifying the key features of any changes in the metabolite concentrations was done by first running a Principal Components analysis, after cube root-transformation and auto scaling the data using www.metaboanalyst.ca (loadings of the components are described in appendix 1). Osmolality in the animals based on the total quantity of metabolites in the animals was calculated and compared using a two-way ANOVA with type III sum of squares, as the number of samples in each group was not always identical. Differences in activity and number of offspring in animals from different treatment groups were tested using one-way ANOVAs with pairwise comparison using t-tests for the activity measures.

3 Results

3.1 Survival of the insects exposed to desiccating conditions

Desiccation significantly affected the duration of survival of the beetles ($P < 0.001$), with about 50% of the individuals being dead after 30 days of experiment, while almost no mortality was recorded in the controls at the same time (Figure 1). All insects from the desiccating conditions were dead at day 50, while approximatively 60% of control beetles were still alive.

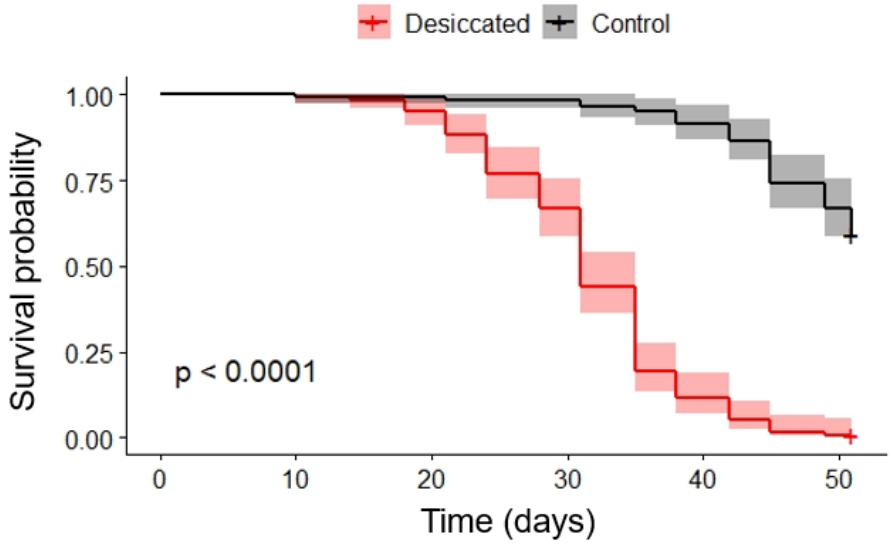


Figure 1. Survival time of food deprived animals kept at 14% ± 2 R.H. (desiccation, gray line) compared to animals kept at approximately 60% R.H (black line). P value resulting from log rank test comparing the two Kaplan Meier plots, plotted with 95% confidence intervals. Censored data marked by +.

3.2 Effects of desiccating conditions on sugar content and metabolic profiles

The water content was significantly reduced in desiccation treated animals after 2 weeks of desiccation (P Values compared to week 0), hereafter it stabilized (Figure 2, A). Water content in controls never changed significantly from the water content in animals before experimental start (Figure 2. A). The sugar content in the animals was greatly reduced during the first experimental week, after which it stabilized for both treatments.

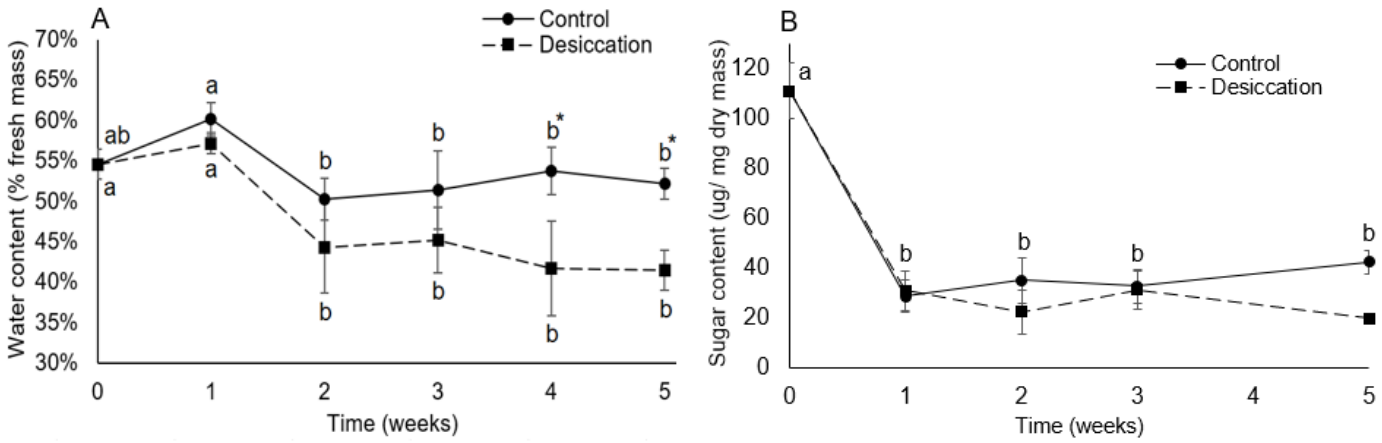


Figure 2. Shows water content (A) and sugar content (B) of food deprived animals kept at 14 % ± 2 R.H. (desiccation, dotted line) compared to animals kept at approximately 60% R.H (solid line). Letters denote significant differences within treatment groups. Stars (*) indicate significant difference between treatment groups at that timepoint using a two-way ANOVA

Metabolite composition was significantly different (95% confidence intervals) after 1 week, compared to the remaining weeks, but there were no observed differences between treatment groups.

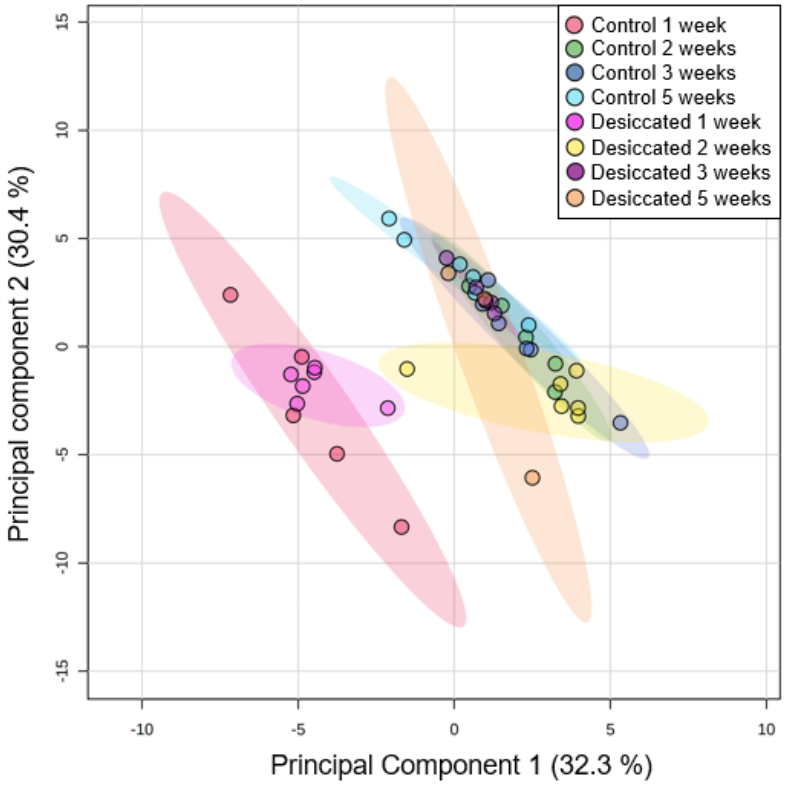


Figure 3. Diagram representing principal component analysis performed using Metaboanalyst v 5.0. A Principal component analysis representing desiccation treatment and control group separation based on the two most significantly contributing components. Colored circles represent 95 % confidence intervals. Colored dots represent individual samples. b Loading plots of the first two components for the metabolites. Loading plots for component 1 and 2 are in appendix 1.

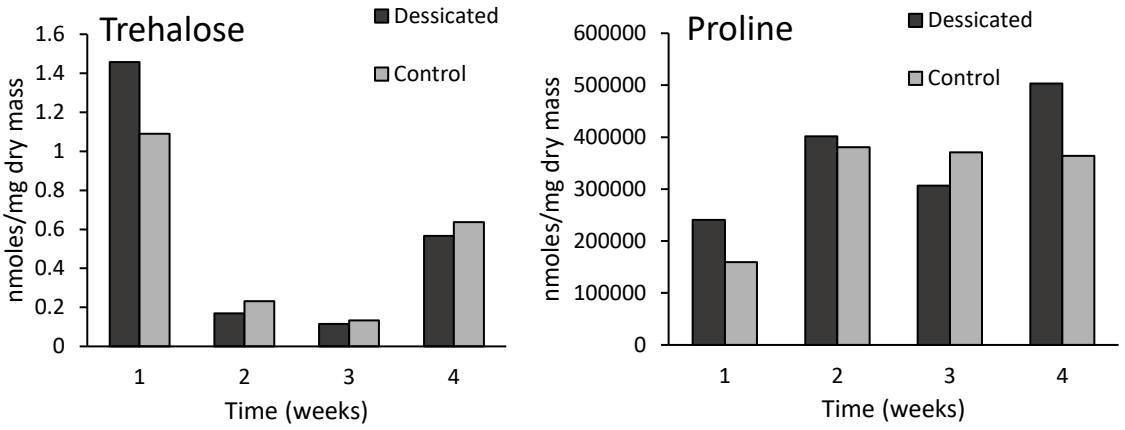


Figure 4. Example of compatible osmolytes measured with the GC-MS, showing no consistent difference between treatments, but trehalose (fig a) showing an effect of food deprivation over time. All metabolites quantified are illustrated in appendix 2.

Osmolality of measured metabolites in the haemolymph of the animals remained constant over the experimental period for both treatments, illustrating that no active upregulation of osmolytes took place (Figure 5).

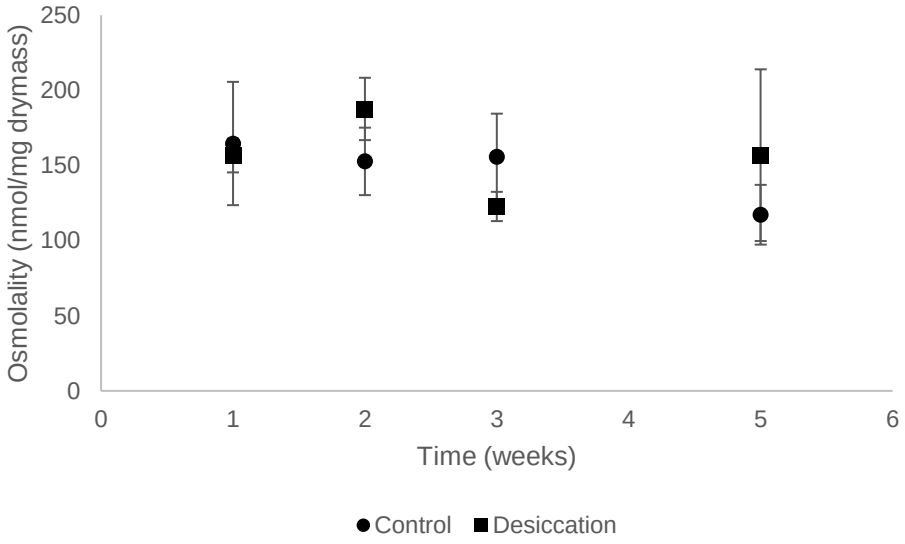


Figure 5. Total osmolality in the animals based on concentrations of the metabolites quantified using GC-MS. A Two-way ANOVA (using type III sum of squares) showed no effect of time ($p = 0.063$) or treatment ($p = 0.669$).

3.3 Locomotor activity of the beetles

The activity of the animals increased significantly after 2 weeks of experimental treatment ($P < 0.001$) but the activity level for the two treatment groups remained similar (Figure 6).

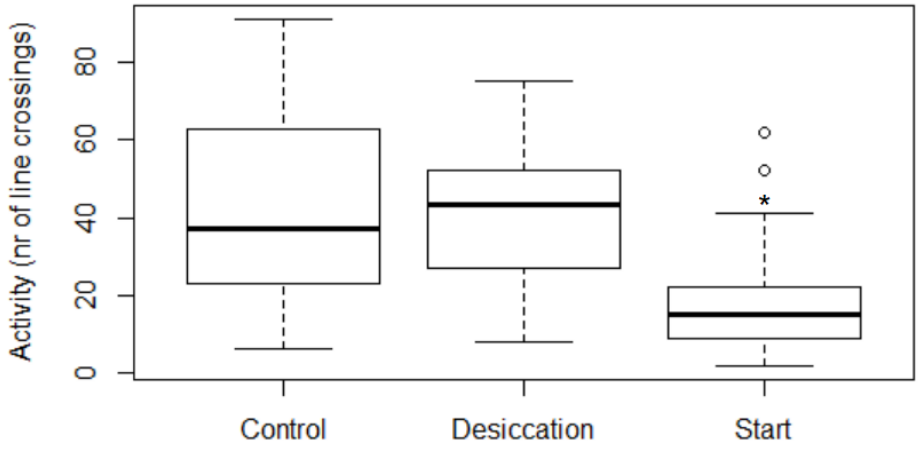


Figure 6. Activity of food deprived animals kept at 14% ± 2 R.H. (desiccation) compared to animals kept at approximately 60% R.H. Activity was measured as number of times an individual crossed a line in a petri dish with a line grid in the bottom. Star (*) indicates significant difference from the other groups found by pairwise t tests ($P < 0.001$) following a one-way ANOVA.

3.4 Reproductive capacities

Animals having experienced one week of desiccation had significantly fewer offspring after one week of reproduction and 30 days of offspring development, compared to control animals ($P < 0.001$).

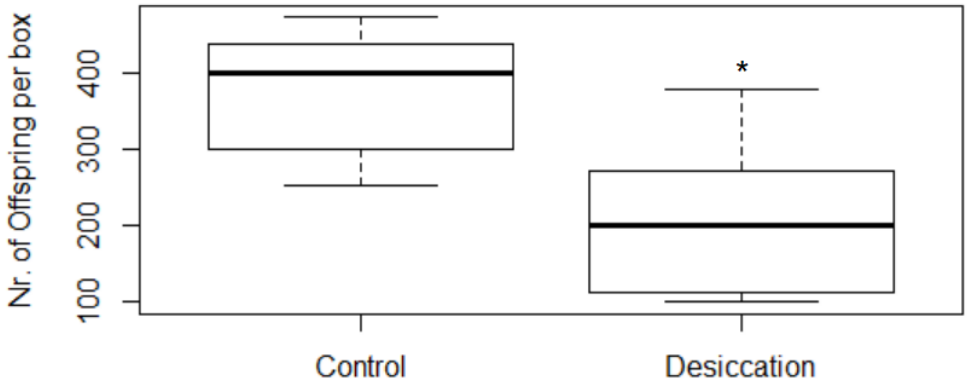


Figure 7. Number of offspring in each replicate of animals after having been kept at approximately 14% R.H. (desiccation) compared to animals kept at approximately 60% R.H for one week, followed by one week of reproduction and 30 days of development of the offspring. * indicating significant difference shown by a one-way ANOVA ($P < 0.001$).

4 Discussion

We can see from our results that adult *A. diaperinus* are tolerant to desiccating conditions. Some individuals survive over 45 days without water in dry air, as is shown for the similar species *Tenebrio molitor* kept at 25 ºC at 12 % R.H. (Punzo and Mutchmor 1980). Comparable results were also found by Renault and Coray (2004), where *A. diaperinus* survived up to 30 days at slightly higher temperature (29 ± 1°C) and lower humidity (5 ± 1% r.h.) compared to the present study. As expected, mortality in our study was much higher in the desiccated treatment group compared to controls during the experimental period. For example, 25% mortality was reached after approximately six weeks in controls compared to three weeks for desiccated animals.

The initial body water content was determined to approximately 55% of the animals' fresh mass. This is comparable to the 58.5% body water previously determined for the same species by Salin, Vernon, & Vannier (1999) and to Bjørge, Overgaard, Malte, Gianotten, & Heckmann (2018) who determined the body water content of the same species to vary from 58% to 67% depending on temperature. Despite a drop in body water in animals from both treatments between the first and second week of treatment, the water content in control animals did not change significantly from the initial water content of the animals during the experimental period, and it took more than three weeks for the water content of the desiccated animals to become significantly smaller than in controls. Since water content in these animals was largely conserved until after three weeks of desiccation treatment, it makes sense that mortality in the desiccated animals did not increase quickly until after around three weeks of desiccation. As mentioned, tenebrionid beetles have been found to have a low cuticular permeability and it has been indicated that they have highly controlled respiratory mechanism for water regulation (Ahearn 1970; Zachariassen 1991), which can explain the long retention of body water even in dry conditions. Several insects have been shown to cope with desiccation by resisting dehydration, where ability to tolerate dehydrating by for example accumulation of sugars can be seen as a contrasting strategy (Nakahara et al. 2008), which is supported here, as these animals do not take use of both strategies.

Total soluble sugar content in the animals was significantly reduced in both treatments within the first week of the experiment, likely as a result of food deprivation. Graves et al. (1992) showed that even though desiccation selected *drosophila* had a higher content of sugars, those sugar stores were depleted during stress exposure, which is in line with our study. Looking at the metabolome in the animals over time, we see that the two treatment groups follow a similar pattern over time. In the first week, control and desiccation treatment are both different from the

remaining experimental time points, indicating that food deprivation and not desiccation, caused the observed effect. For example, we see that the sugar trehalose (which is the main energetic sugar in insects (Thompson 2003)) content falls after one week, and remains low also in the desiccation treated animals. This illustrates that this beetle does not actively increase sugars or other compatible osmolytes (for example proline) when exposed to desiccation stress in combination with food deprivation. In fact, osmolality in the animals calculated based on the metabolites determined here was constant throughout the experimental period for both treatments groups. Ofcourse, keeping in mind the reduction in body water during the exposure period, we know that there is an increased concentration of metabolites in the animals' body water, however, there is no active mechanism of upregulation beyond this effect, as such upregulation would be apparent per dry mass as well. As such, the increased concentration of metabolites in the insects body water as a result of water loss does not represent the upregulation response we were looking for. To our knowledge, the osmolality of metabolites has not been investigated for tenebrionid beetles previously, but total osmolality and ions was measured in the tenebrionid *Stips stali*, in a study that found that the species increased osmolality through sodium, potassium and chloride concentration augmentation during dehydration (Naidu and Hattingh 1986), so it would be interesting to also measure the ion contribution to osmolality in *A. diaperinus* under desiccation to see if this is also the case for this species.

We hereafter wanted to investigate if the animals changed their activity level when exposed to a desiccating environment. We observed an increase in activity in both control and desiccated animals. This could be due to foraging behaviour (Lorenzo and Lazzari 1998), or looking for a more suitable habitat (Yu et al. 2010). Since the same change in activity is seen for both treatment groups, it is likely that foraging behaviour is at least in part causing the observed change, and it would be interesting to see what the results would be if both treatments had food access. In a former study, desiccated *A. diaperinus* were observed to be inactive most of the time (Renault and Coray 2004) while Renault et al. found that starved *A. diaperinus* would have an initial hyperactivity, followed by a sit and wait strategy (Renault, Hervant, and Vernon 2003). It could therefore be hypothesized that the animals observed here are in the hyperactive state following food deprivation, possibly because they are introduced to the new environment of the activity measurement petri dish.

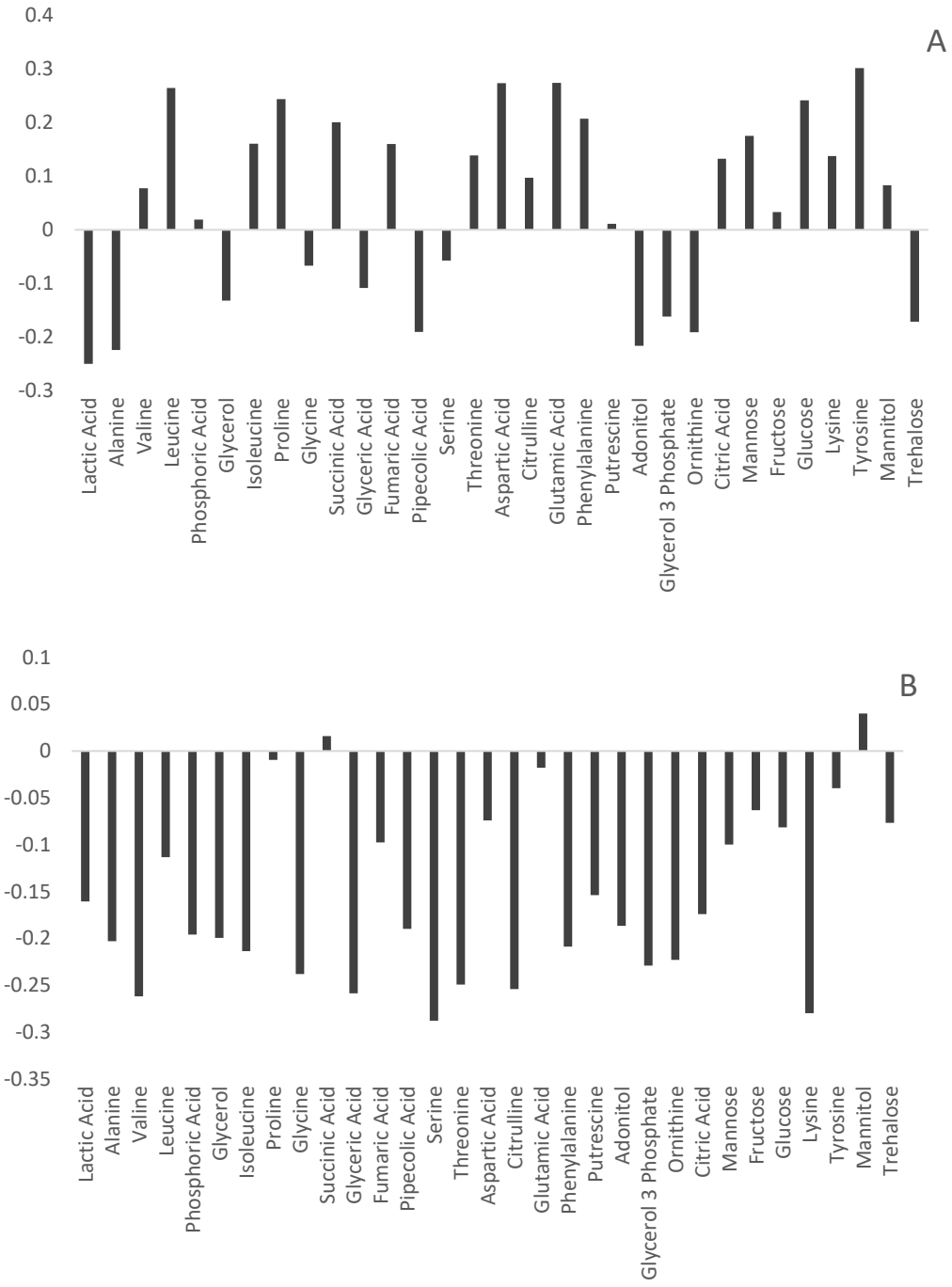
Despite exhibiting high desiccation tolerance, costs of dealing with the stress, as well as potential non mortal damage from desiccation, can reduce fitness of the animals. To investigate

if such costs are associated with desiccation for this species, we exposed them to desiccation for 8 days, which is a period that did not induce any mortality or significant change in metabolite composition as shown above, after which we observed number of larvae produced in the following week, and saw a great effect of desiccation on the number of offspring of *A. diaperinus*. As the desiccation occurred prior to egg laying and development, this treatment had an effect on the adults themselves or their gametes prior to egg laying. Since these experimental animals were fed, we don't expect the explanation to be reduction in energy stores. Reduction in egg laying due to dryer habitat has been shown in the dung beetle *Aphodius fossor*, which could explain the observed difference in offspring (Vessby 2001). Furthermore, cessation of reproduction after exposure to dry conditions has been shown in Collembola (Waagner, Bayley, and Holmstrup 2011). Further investigations into the time it takes *A. diaperinus* to reach maximum reproduction recovery would be interesting. Considering effects on eggs and larvae are often more severe than in adults, this study on adults indicates that desiccating conditions can have detrimental effects on population size of *A. diaperinus* (Holmstrup 2019; Urs and Hopkins 1973).

Conclusion

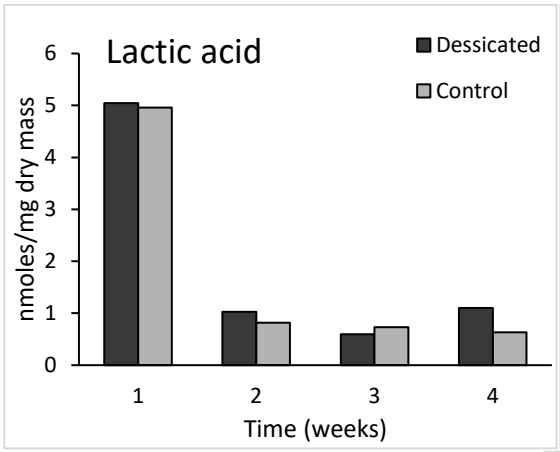
We have found that *A. diaperinus* is highly resistant to a desiccating environment, which can explain its success as a pest in very dry habitats. We see no evidence that the method of desiccation tolerance for this species involves upregulation of compatible osmolytes or reduction in activity. These results lead us to hypothesise that the animals rely on their highly impermeable cuticle and use of the elytral cavity for respiration, whereby they can achieve very low respiratory water loss and cuticular transpiration. Despite high desiccation tolerance, number of offspring from adults reproducing for one week after eight days of desiccating conditions was significantly lower than controls, indicating that dry conditions still can have a detrimental effect on *A. diaperinus* populations.

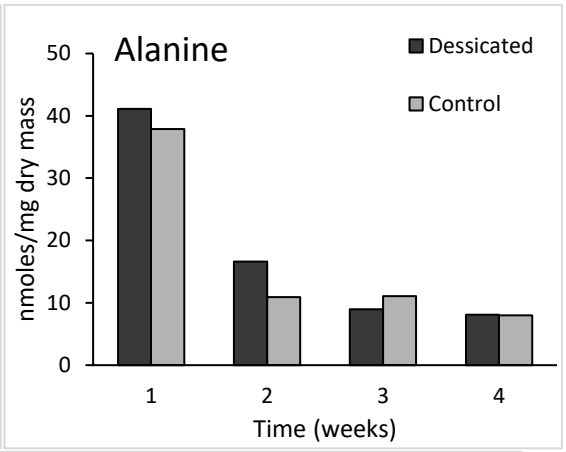
Supplementary Material

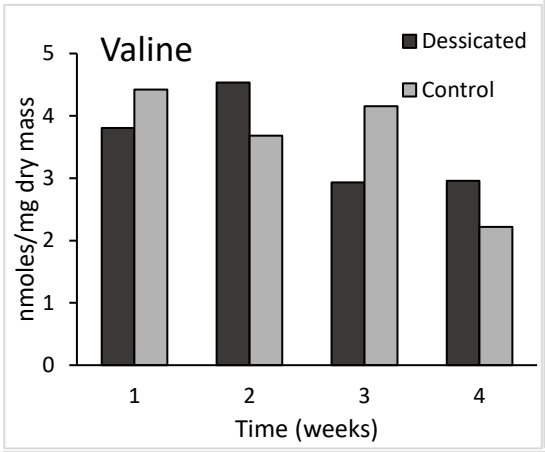


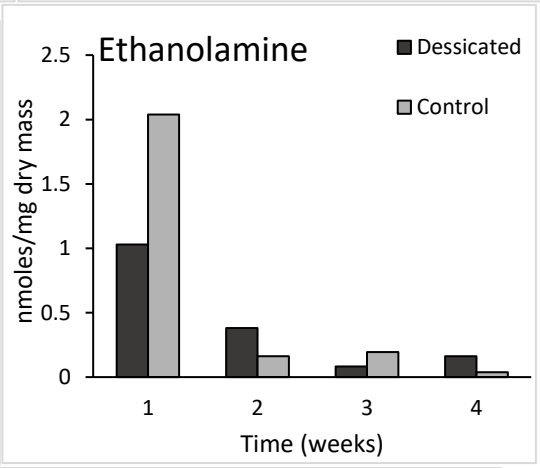
Appendix Figure 1 Loadings plot of component 1 (A) and component 2 (B) from the PCA in the results.

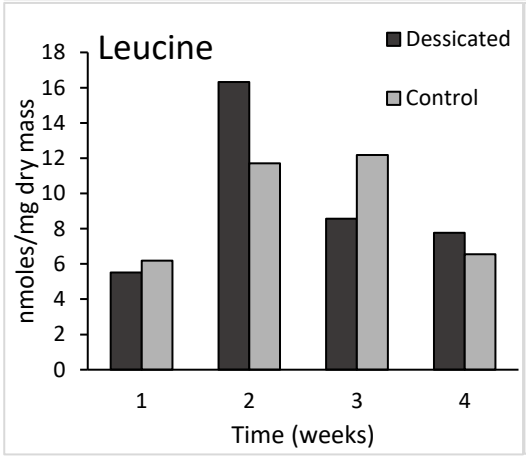
The quantified metabolites after desiccation:

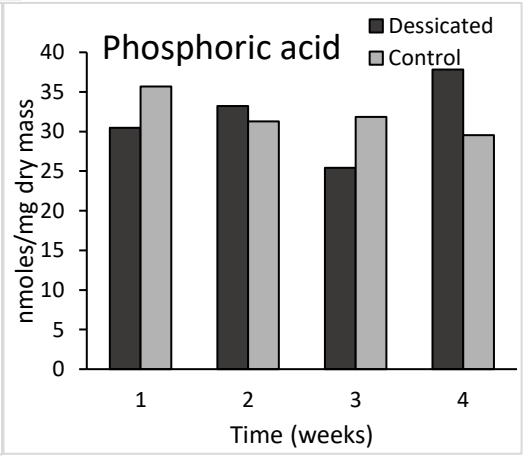


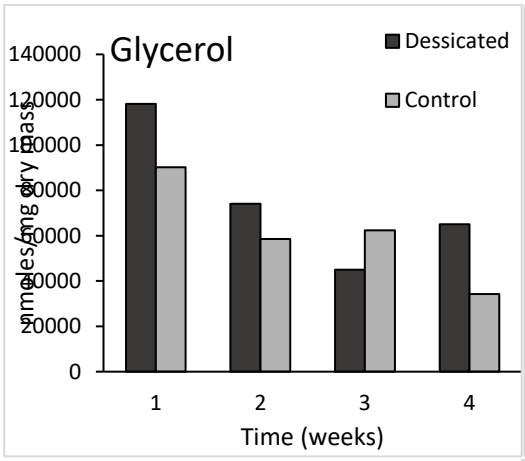


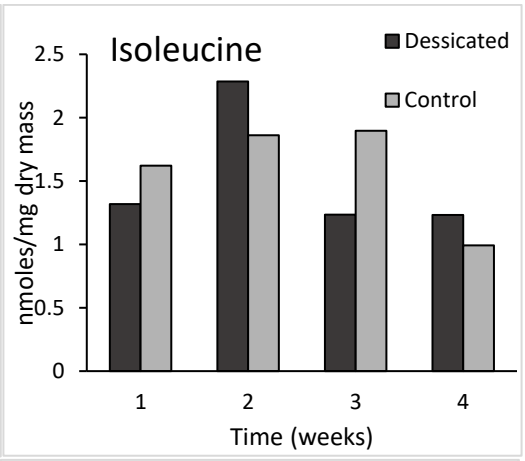


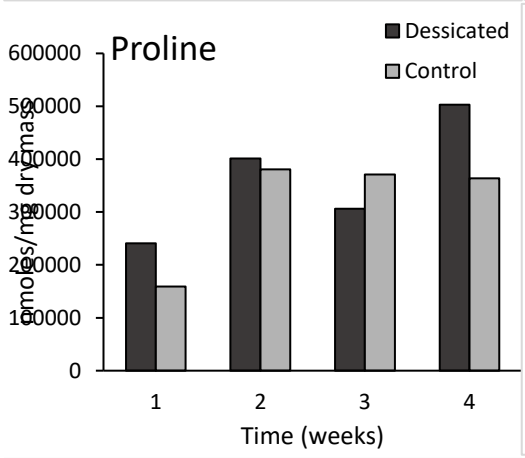


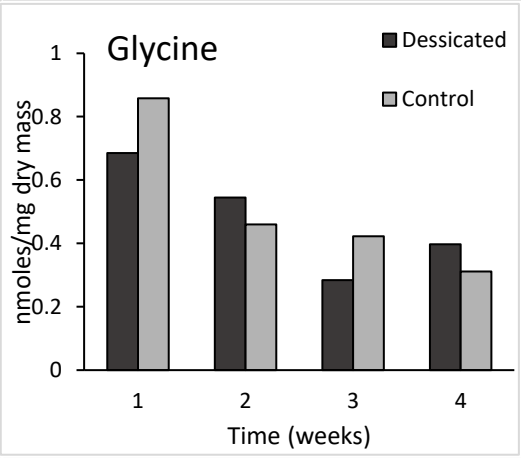


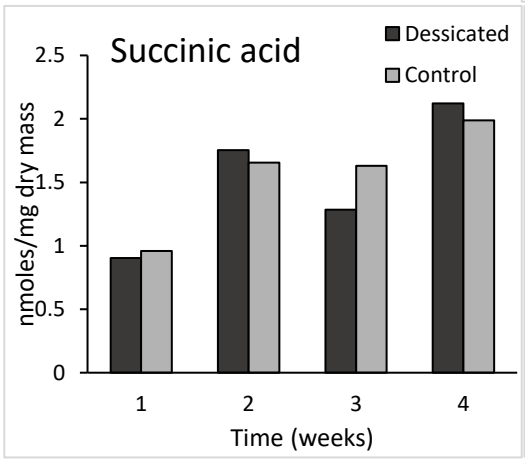


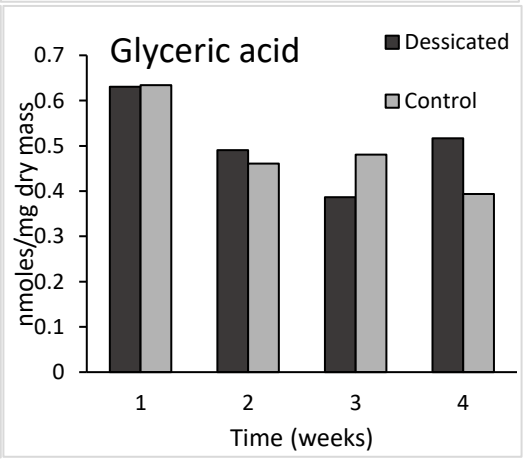

Glycerol
Dessicated
Control
nmoles/mg dry mass
140000
120000
100000
80000
60000
40000
20000
0
1
2
3
4
Time (weeks)

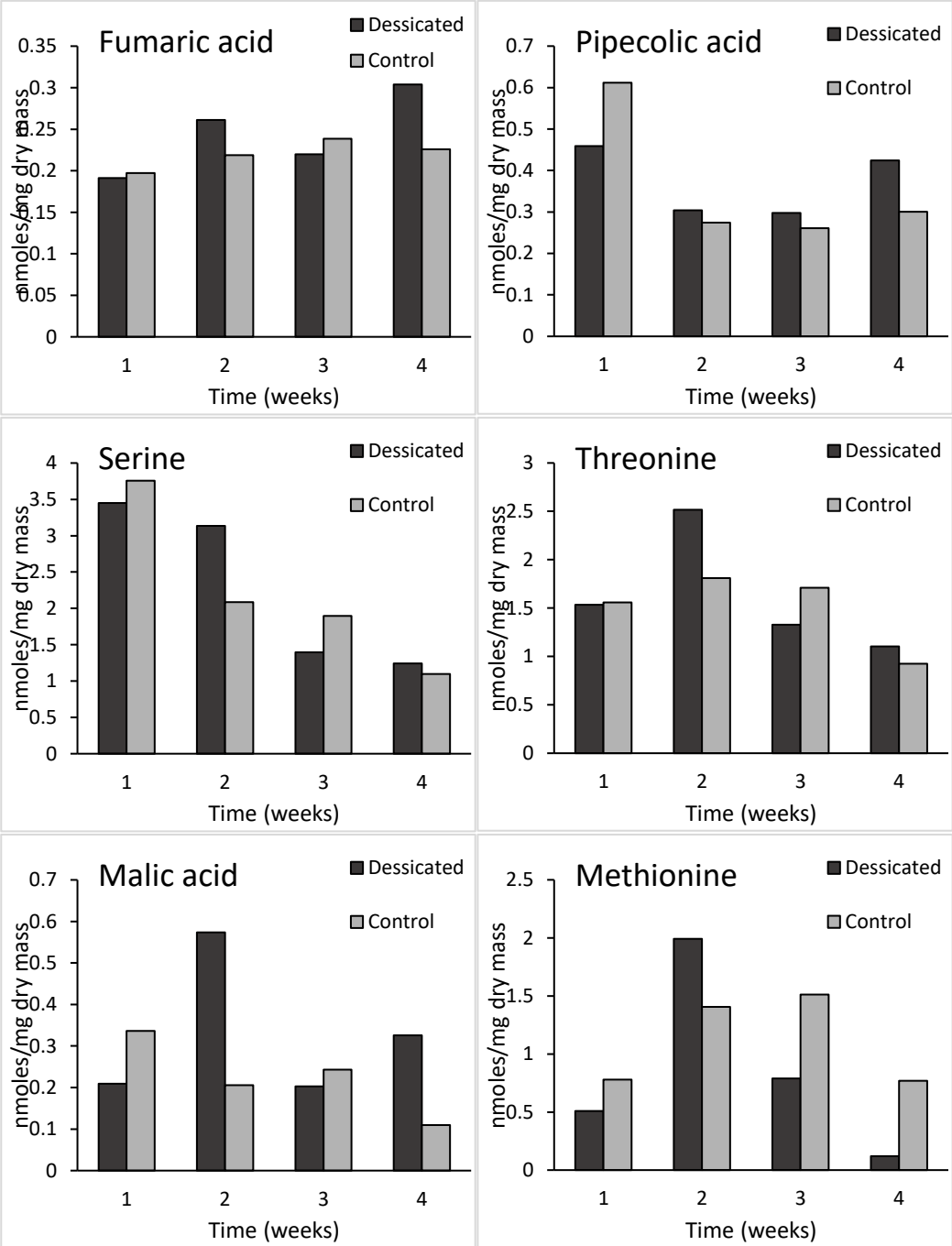

Isoleucine
Dessicated
Control
nmoles/mg dry mass
2.5
2
1.5
1
0.5
0
1
2
3
4
Time (weeks)

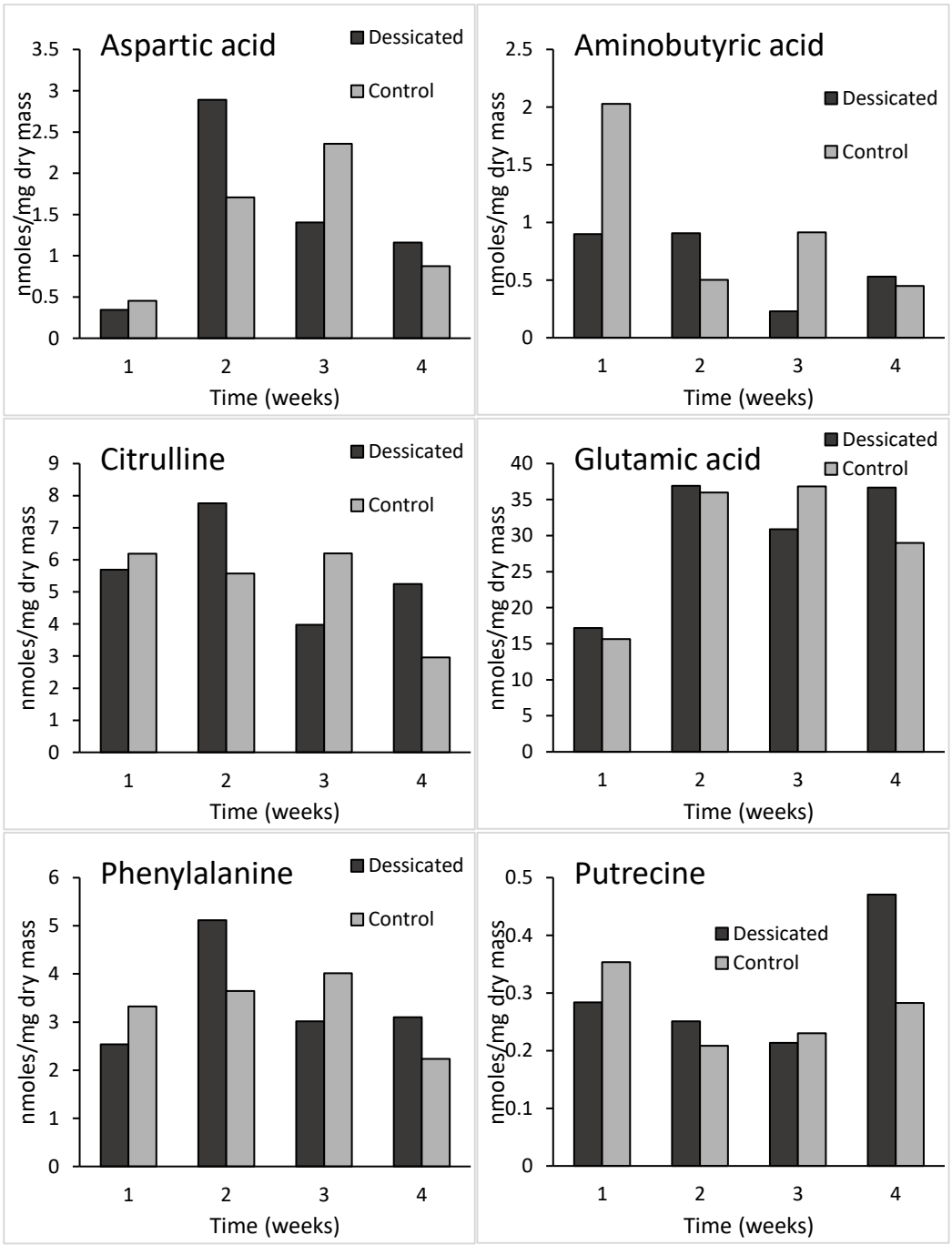

Proline
Dessicated
Control
nmoles/mg dry mass
600000
500000
400000
300000
200000
100000
0
1
2
3
4
Time (weeks)

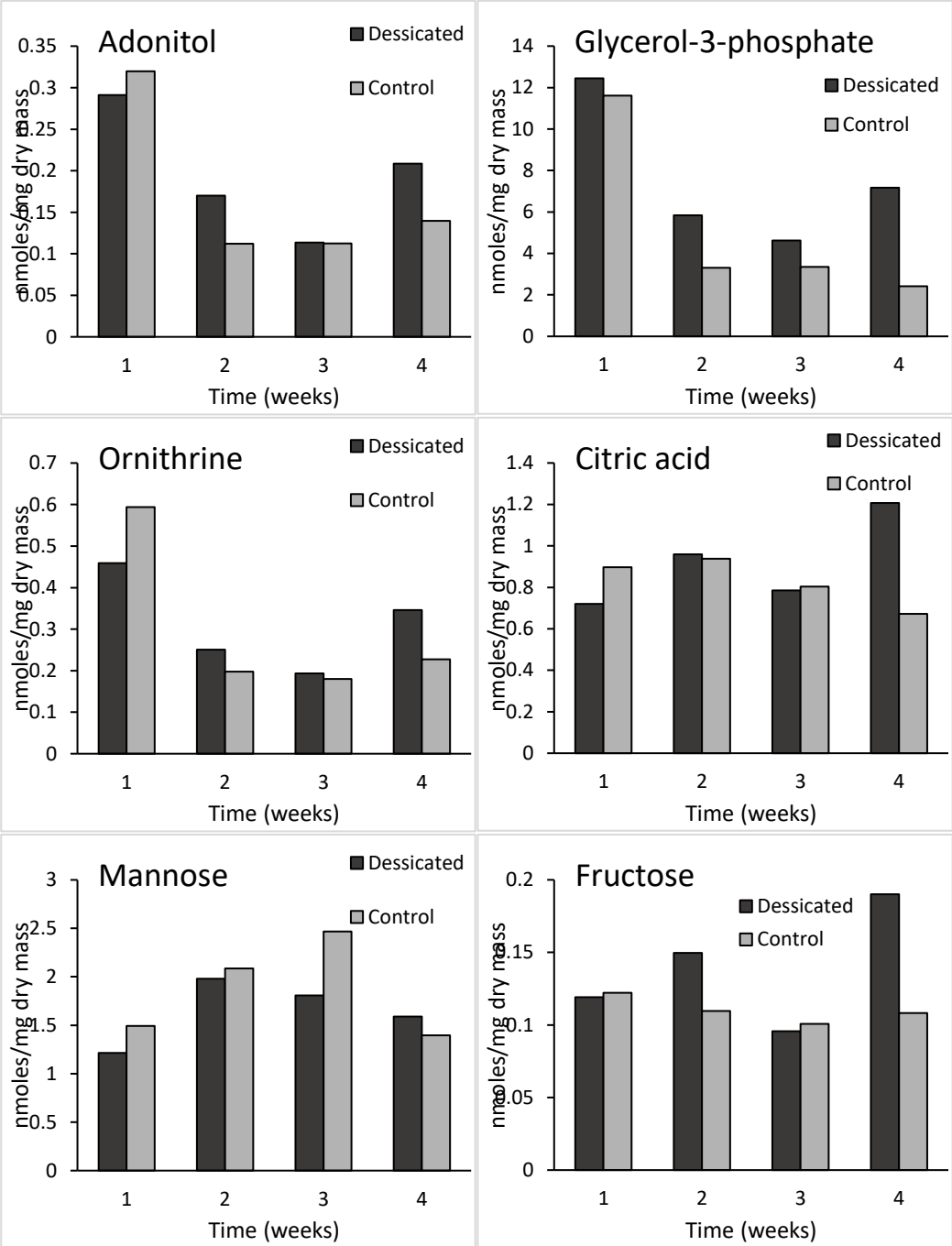

Glycine
Dessicated
Control
nmoles/mg dry mass
1
0.8
0.6
0.4
0.2
0
1
2
3
4
Time (weeks)

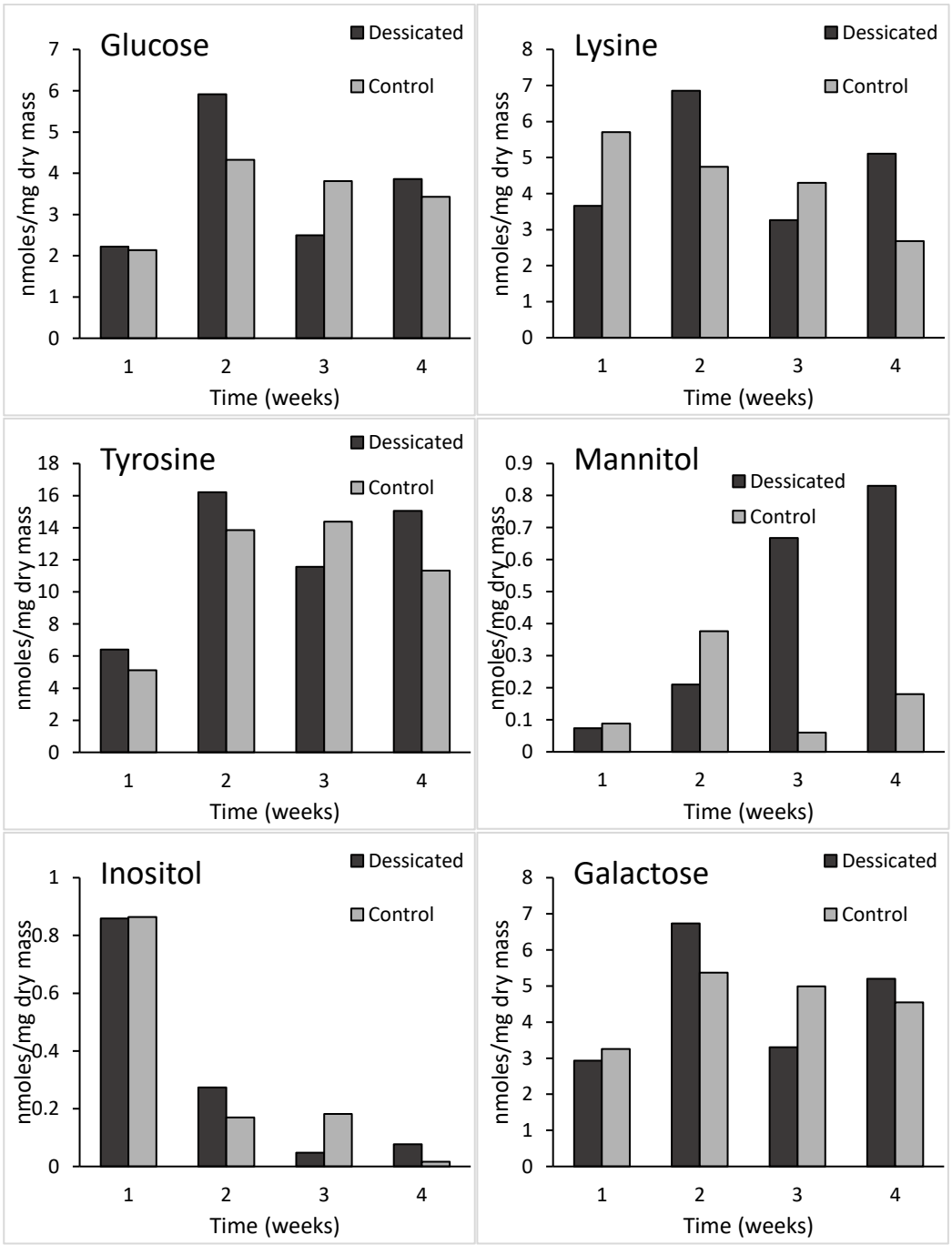

Succinic acid
Dessicated
Control
nmoles/mg dry mass
2.5
2
1.5
1
0.5
0
1
2
3
4
Time (weeks)


Glyceric acid
Dessicated
Control
nmoles/mg dry mass
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
Time (weeks)


Fumaric acid
Dessicated
Control
nmoles/mg dry mass
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
1
2
3
4
Time (weeks)
Pipecolic acid
Dessicated
Control
nmoles/mg dry mass
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
Time (weeks)
Serine
Dessicated
Control
nmoles/mg dry mass
4
3.5
3
2.5
2
1.5
1
0.5
0
1
2
3
4
Time (weeks)
Threonine
Dessicated
Control
nmoles/mg dry mass
3
2.5
2
1.5
1
0.5
0
1
2
3
4
Time (weeks)
Malic acid
Dessicated
Control
nmoles/mg dry mass
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
Time (weeks)
Methionine
Dessicated
Control
nmoles/mg dry mass
2.5
2
1.5
1
0.5
0
1
2
3
4
Time (weeks)


Aspartic acid
Dessicated
Control
nmoles/mg dry mass
Time (weeks)
Aminobutyric acid
Citrulline
Glutamic acid
Phenylalanine
Putrecine


Adonitol
Dessicated
Control
nmoles/mg dry mass
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
1
2
3
4
Time (weeks)
Glycerol-3-phosphate
Dessicated
Control
nmoles/mg dry mass
14
12
10
8
6
4
2
0
1
2
3
4
Time (weeks)
Ornithrine
Dessicated
Control
nmoles/mg dry mass
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
Time (weeks)
Citric acid
Dessicated
Control
nmoles/mg dry mass
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
2
3
4
Time (weeks)
Mannose
Dessicated
Control
nmoles/mg dry mass
3
2.5
2
1.5
1
0.5
0
1
2
3
4
Time (weeks)
Fructose
Dessicated
Control
nmoles/mg dry mass
0.2
0.15
0.1
0.05
0
1
2
3
4
Time (weeks)


Glucose
Dessicated
Control
nmoles/mg dry mass
Time (weeks)
Lysine
Dessicated
Control
nmoles/mg dry mass
Time (weeks)
Tyrosine
Dessicated
Control
nmoles/mg dry mass
Time (weeks)
Mannitol
Dessicated
Control
nmoles/mg dry mass
Time (weeks)
Inositol
Dessicated
Control
nmoles/mg dry mass
Time (weeks)
Galactose
Dessicated
Control
nmoles/mg dry mass
Time (weeks)

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Chapter 5

General discussion

General discussion on the scientific contributions of the thesis' results and future perspectives

Human activities have resulted in a number of climatic changes, and we must rapidly improve our understanding of the impact this may have on the ecosystems whose function we depend on (Clark et al. 2001; IPCC 2014). This knowledge is also critical for setting policy and adequate attenuation measures aiming at limiting the effects of climate change on biodiversity and ecosystem services (IPBES 2019). To this aim, uncovering the effects of environmental disturbances induced by human activities on insects, and on biodiversity in general, is crucial. Insect species indeed provide many ecosystem services, and cover several ecological roles such as pests, vectors, predators and prey for other species, and so changes in insect populations can have ripple effects through communities, food webs and entire ecosystems (Köhler and Triebskorn 2013). In this thesis, I have conducted a wide range of experiments, using beetles as model species, in order to improve our understanding of the responses of insects thriving in a quickly changing world.

Warming and geographic expansion of alien species

The predatory carabid beetle *Merizodus soledadinus* has long been restricted to altitudes below 110m after it has been introduced to the Kerguelen Islands in 1913. However, in the last few decades, and more particularly since the middle of the 90s, the insect has spread more widely and can now be found up to 400m a. s. l. The colonization of habitats at higher elevations has likely been made possible by the milder climate, increasing the species' range on the island (Berthier et al., 2009; Laparie & Renault, 2016; Ouisse et al., 2020). This is a very clear example of how either milder winters or warmer summer periods have facilitated the spread of an invasive species (Lebouvier et al. 2020). The spread of this beetle is continuously monitored, and, looking at the predicted warming at the horizon 2050, it is likely that its expansion will be further increased toward higher elevations.

This insect represents a valuable model for testing a range of hypotheses crossing concepts related to biological invasions and species range dynamics, including the evaluation of the impact of the founder effect on the subsequent responses of the insect to environmental stress. Also, as the geographic expansion of this insect at the Kerguelen Islands has mainly occurred without human assistance, the knowledge of establishment dates of the populations and range edges (invasion fronts) allow investigations of the impact of the "Olympic village effect" on the thermal plasticity of the individuals in this warming archipelago. The Olympic village effect is the

effect on generations of insects born at the invasion front, that are offspring of the “fastest and fittest” (individuals with high dispersal rate and success), making them more adapted to the new environment and to spread on, than animals born further back from the leading edge of the species distribution. This effect has been illustrated on a wide range of traits, from behavioural, such as dispersal propensity (Ochocki and Miller 2017), to physiological, such as thermal tolerance (Hillaert et al. 2015). Despite evidence that animals at an invasion front have a wider thermal breadth, no such pattern was found in the invasive species *M. soledadinus* on Kerguelen (**chapter 1**). This illustrates how a specific field scenario may not follow a general pattern, and investigating special cases such as this alien species spreading in the Sub Antarctic, can provide new perspectives. A wide range of factors could influence *M. soledadinus'* thermal tolerance (and other physiological limits) locally, as the environment is highly complex. For example, environmental factors such as desiccation resistance and salinity tolerance may have driven the physiological performance of the range-expanding populations of *M. soledadinus* (Hidalgo et al. 2013; Ouisse et al. 2016). Other, un-investigated factors may also be at play, such as quality of shelter, available food sources over the year or local temperature variations, not to mention interactions between species as well as for example the possible influence of residence time on the quality of available food. I have shown that altitude and residence times of the populations are not directly associated with temperature tolerance breadth, and so looking into if and how the un-investigated factors vary between locations, and to what extent they can influence temperature tolerance, would be interesting to explain the observed temperature tolerance patterns. Knowledge of these factors and their implications are also useful to determine which areas are at high risk of future invasion by the beetle, and if anything can be done to prevent this.

Making predictions of a species' future distribution and performance is further complicated by the ability of animals to acclimatize or adapt to environments that the species has not yet encountered in its natural range. To determine if this is the case, laboratory investigations are very helpful, to simulate conditions the animals do not encounter in the field, in addition to studying mechanisms. However, the results may not be directly reflecting how field situations develop, as the number of factors influencing an animal in its natural habitat is, as mentioned, so large. This is why combining field studies and laboratory work to improve our understanding of species invasions is necessary. In Kerguelen, modelling the distribution of *M. soledadinus* from field observations, along with information on the environmental factors mentioned above, could reveal if there is a pattern that can determine which factors limit the spread of the species.

Thermal tolerance breadth

When we look at the general temperature tolerance patterns of populations of *M. soledadinus* and *A. diaperinus*, we see that the overall thermal range is larger for *A. diaperinus* populations, who are living in temperate areas (**Chapter 2 and Chapter 3**). Even though heat tolerance is not generally as variable as cold tolerance across species (Sunday, Bates, and Dulvy 2011), *M. soledadinus* populations exposed to 38 °C for 15 minutes had only few survivors, whereas most *A. diaperinus* individuals collected from farms (and about half of the laboratory reared ones), recovered after 4 hours at 42.5 °C. As for cold tolerance, most *M. soledadinus* did not recover from 15 minutes at -8 °C, whereas all individuals collected from the farm and exposed at -8 °C recovered. As such, *M. soledadinus* appears to be more specialized to temperatures closer to those commonly encountered in its environment, and not to sudden extreme temperature changes, that is, a trade-off between acute and chronic thermal tolerance may be at play (Magozzi and Calosi 2015). Conversely, *A. diaperinus* had a high temperature tolerance breadth, even demonstrating a higher cold tolerance than *M. soledadinus*, despite *A. diaperinus* having tropical origins.

In addition, I showed that *M. soledadinus* recovery time and probability did not seem to depend on duration of exposure to an extreme temperature (**chapter 2**), whereas both were affected with exposure duration of *A. diaperinus* (**chapter 3**), which could also be connected to adaptation to better chronic rather than acute temperature tolerance in *M. soledadinus*. Another difference between the two species, was that recovery time for *M. soledadinus* individuals was very consistent, either they recovered in around 10 minutes, or they never did, whereas recovery time of *A. diaperinus* varied a lot between individuals, to up to two hours. It would be interesting to uncover the differences in mechanisms behind extreme temperature tolerance and recovery thereof in the two species, and see how it relates to these observed differences in tolerance to duration *versus* intensity of stress, and the difference in recovery time.

In a fairly stable environment such as that found on Kerguelen, it is likely that costs associated with increased extreme temperature tolerance makes it unbeneficial, as the risk of encountering such temperatures is low. This fits well with our discovery that certain populations of *M. soledadinus* were more heat or cold tolerant, but non where both, indicating a trade-off between extreme acute hot and cold tolerance, and other trait(s) of importance to these beetles (**chapter 2**). We can see that the laboratory reared *A. diaperinus* population also had a narrower thermal range than the farm collected ones, again probably due to trade-offs that made reduction in thermal breadth a better strategy for these beetles. In addition, as farm collected individuals

experience regular thermal fluctuations, it is likely to have been beneficial for them to have increased tolerance to such stress. This capacity to tolerate extreme temperatures may never have developed in insects living in less variable environments, such as *M. soledadinus*, living in the fairly stable Kerguelen Islands, and originating from the similarly stable Falkland Islands (Lebouvier et al. 2020). For this reason, it could be relevant to look at trait performance in *M. soledadinus* towards the invasion front and along the elevation gradients, at different temperatures closer to those measured on average in their natural habitat. This could unravel if different populations differ in their thermal optima and performance breadth along these (elevation and altitude) gradients. Interesting traits could for example be activity (fouraging/ mating) or egg laying, as their performance level can greatly influence the population success, and as such, this would fit well under the “olympic village” framework.

Effects of climate change and pesticides on *A. diaperinus*

Using both the pyrethroid insecticide cyfluthrin, daily heat exposures and a desiccation period to represent likely stressors to occur in the environment due to climate change, I attempted to discover physiological changes resulting from (multiple) stress exposure in *A. diaperinus* (**chapter 3 and chapter 4**). However, I found that this species excels at maintaining homeostasis at the stress levels I exposed them to. It is interesting to see that this generalist pest beetle can handle harsh conditions so well, as it tells us the likelihood that it will have a high fitness in a changing world.

Even though I did not uncover a change in metabolite composition or antioxidant activity in the insects, they were clearly affected in a detrimental way by the insecticide, temperature peaks and desiccation. Longevity was affected, although more slowly by extreme drought than extreme temperature or relatively high exposure concentrations of cyfluthrin. However, the results were highly dependent on the population I investigated (in experiments where more than one population was used). We showed striking reduction in tolerance to extreme heat after exposure to a tolerable concentration of insecticide, which has not been illustrated for a terrestrial insect in this degree before. The number of studies on the effects of insecticides on temperature tolerance, both with consecutive and simultaneous exposures, are far too few, especially considering the large effect we found. In fact, all the studies presented in this thesis have underlined the importance of increasing ecological realism in laboratory experiments. By combining stressors and capturing interactions, we can improve the predictability of field scenarios based on laboratory results. In this respect, and in the context of climate change, it is

crucial that more studies on interacting effects of heat stress and other stressors are described for terrestrial insects.

In addition, studies on tolerance of different populations of a wider array of terrestrial species, and their different life stages, should be studied to uncover the extent to which pesticide exposure can influence their survival at extreme temperatures, and *vice versa*. Knowing more about for example what life stages are more sensitive, can help us aim conservation efforts to the periods where it makes the most difference, and knowing more about interactions also tells us which stressors should be given more focus. In such studies, it would also be interesting to work with populations of (verified) different genetic background, or similar genetic background but different (stress exposure) history, to test the relative importance of each of these two aspects on tolerance.

Extending on the studies in this thesis and similar studies, as suggested in the previous paragraph, uncovering the mechanisms behind the observed effects could provide valuable information for ecologists attempting to predict future biodiversity and species distributions. In addition, it may help pesticide developers in their work towards developing more specific pesticides, to reduce consequences to non-target species. In the case of *A. diaperinus*, I would expect the reason heat tolerance is worse after pesticide exposure, is that the animals increase their oxygen consumption when dealing with the pesticides, which causes a mismatch between oxygen consumption and supply to occur sooner when the animals are then exposed to extreme heat, which can be the limiting factor to the beetles tolerance to high temperatures (Verberk et al. 2016). This could be tested by exposing the animals to pesticides as done in the work in chapter 3, and then place the animals in a respirometer instead of exposing them to extreme heat. Simultaneously measuring their activity would be ideal, in order to know how this affects the animals´ respiration as well.

Further elucidating the mechanisms these insects use to tolerate pesticides and extreme temperatures would be interesting, especially comparing the different populations to see if they depend on the same defences or not, and if they have mutated to render the pesticide mode of action less effective or if they depend on an increase in other functions such as degradation of the pesticide.

Since mosquitos are vectors of several very serious diseases, attempts to control their distribution by use of pesticides have been widespread and long lasting. This has resulted in development of resistance for example to pyrethroids, which has been well documented due to

the large economic and health implications of these insects. Mosquitoes tolerate pyrethroids through reduced target site sensitivity due to a single point mutation (called knock down resistance (kdr)) or metabolic resistance mechanisms, mainly associated with the enzyme families; cytochrome p450 mono-oxygenases (p450s), carboxylesterases (CEOEs) and glutathione-S-transferases (GSTs). (Awolola et al. 2018). Thus, using biochemical assays to evaluate the activity of these insecticide metabolizing enzymes would be a logical step towards elucidating the mechanisms behind these insects pesticide tolerance (Punchihewa et al. 2019). Furthermore, if possible for this species, sequencing the (relevant regions of the) gene for voltage gated sodium channel regulatory protein for possible kdr mutations would also be of interest. Finally, comparing results from laboratory reared (sensitive) populations with farm collected (tolerant) ones, to see why the laboratory reared populations are so much more sensitive to pesticide as well as comparing the different farm collected populations could give interesting insights. First, seeing which mechanisms are not present in the sensitive strain indicates that these are associated with a cost, which renders them unfavorable in an environment where this stress in not encountered. It also could indicate which mechanisms appear more often in field populations, and which mechanisms are rare. If a very resilient populations also has a very rare mutation, efforts to completely eradicate such a strain of a pest species could save a lot of resources in the long run.

Climate change, i.e. the changes in precipitation regimes, mean temperature, and occurrence of extreme weather events, is having massive consequences on the physical environment. For instance, it can make soils dryer, or increase drought events. As a result, many species are increasingly exposed to desiccating conditions, in turn affecting the ecology and physiology of the organisms. Despite reproduction being a very costly process, and effects on reproduction under stressful conditions being known for many species (Aira et al. 2007; Marshall and Sinclair 2010; Schreck, Contreras-Sanchez, and Fitzpatrick 2001; Waagner, Bayley, and Holmstrup 2011), the lack of knowledge of effects of desiccation on reproduction in insects literature is striking. We showed that an exposure of one week of very dry conditions had no visible effect on the behaviour and survival of the tenebrionid beetles. Yet, this experimental condition reduced the number of *A. diaperinus* offspring significantly, even though mating and egg laying all took place in the same conditions as controls (**Chapter 4**). Further research into the mechanism behind this reduction in reproductive output would be interesting. In our experimental setup, we cannot deduce if the number of offspring was reduced due to a reduction in fertilized eggs, egg laying, hatching or development issues in the offspring. Thus, experiments

where development of the eggs in the female is monitored, as well as number of eggs laid, how many of them hatch and then develop into pupae and adults would be a natural first step further. Since female functions generally involves higher costs than male, it is likely to be affected to a greater extent than male, thus I would initially focus on effects on gamete production and function on the females (Aira et al. 2007). Furthermore, looking at effects of stress exposure prior to, as well as simultaneously to reproduction and offspring development are both highly relevant, to separate effects of stress directly on the parents, and effects on egg laying, eggs, and larvae. Vessby (2001) found that reproduction in the dung beetle *Aphodus fossor* was more successful in wetter, shaded cow pats than in exposed drier ones, both in terms of number of eggs and larvae found 26 days after adults were added to the pats, and number and size of adult beetles having developed at the end of her experiment. In addition, Marshall & Sinclair (2010) observed fewer offspring in *drosophila melanogaster* flies exposed to cold for a longer period (-0.5 °C for 10h), and a change in sex ratio resulting in more males. Such change in sex ratio could be because there is an increased cost associated with producing females (which has not yet been shown), or that female progeny are more stress sensitive in the developmental stages, rendering them unable to complete development. Such effects would therefore also be interesting to investigate for desiccated *A. diaperinus*, as these factors are all unknown. When the functions affected by water stress in A. diaperinus have been described, research to understand why this effect occurs would be interesting, as it may provide new insights into desiccation sensitivity of desiccation resistant insects, as well as give possible novel methods of controlling the pest beetle. However, which mechanisms that may be at play, depends on which functions turn out to be affected by the stressor, and in what way, and should be hypothesized when we have more knowledge of these effects.

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Résumé

1 Introduction

Dans le contexte des changements environnementaux liés aux activités anthropiques, les conséquences sur la biodiversité sont aujourd'hui l'une des principales préoccupations des écologistes. En effet, les changements climatiques ont des implications pour tous les organismes, affectant la qualité de l'habitat, la physiologie, les traits d'histoire de vie, la phénologie, les interactions entre les espèces et les migrations (Bellard et al. 2012; Bozinovic and Pörtner 2015; Doney et al. 2012; Foden, Mace, and Butchart 2013; Pacifici et al. 2015; Vasseur et al. 2014; Walther et al. 2002). En particulier, le développement, la survie, la forme physique et la distribution spatiale des ectothermes dépendent des conditions environnementales, en particulier de la température et de l'humidité (Angilletta et al. 2002; Bowler et al. 2017; Chown, Sorensen, and Terblanche 2011). Afin d'accroître la fiabilité des prévisions des effets du changement climatique, améliorer notre compréhension des mécanismes et de l'impact des facteurs de stress environnementaux affectant la dynamique des populations, et donc la composition des communautés et les fonctions écosystémiques,est nécessaire (Clark et al. 2001).

La nature ectothermique des insectes implique que tous les aspects de leur vie sont façonnés par la température et ses variations. En conséquence, la tolérance thermique (le degré de tolérance d'un individu aux extrêmes thermiques), en parallèle avec la plasticité de ce trait (la capacité d'ajuster la tolérance thermique par l'acclimatation), sont des prédicteurs importants de la répartition géographique et de la sensibilité des organismes au réchauffement climatique (Andersen et al., 2015; Buckley and Huey, 2016; García-Robledo et al., 2016; Hoffmann et al., 2013). De plus, la prise en compte de la variation de la tolérance à la température entre les populations au sein des espèces peut être utilisée comme indicateur de l'abondance et de la répartition potentielles en réponse au changement climatique (Sorte et al., 2011). En effet, la tolérance thermique, et les mécanismes physiologiques sous-jacents permettant l'adaptation aux changements de température et aux extrêmes, représentent des traits importants sous pression de sélection (Angilletta et al., 2002), en particulier pour les espèces invasives et à déplacement de distribution.

Outre le changement climatique, la pollution constitue un autre facteur de stress important d'origine anthropique (Secretariat of the Convention on Biological Diversity 2010). Parce que de nombreux pesticides (ou produits de leur transformation) peuvent être très persistants dans

l'environnement, ils sont répandus et peuvent également s'accumuler dans les organismes (Lushchak et al. 2018). De plus, l'exposition simultanée au stress thermique et chimique peut conduire à des interactions ayant des sorties synergiques, additives ou antagonistes, selon le type (température chaude/froide ou produits chimiques avec différents modes d'action ou voies), la durée et l'intensité du stress (Walker et al. 2012). De telles interactions peuvent avoir des effets néfastes en conduisant soit à une sensibilité aux toxiques induits par le climat, soit à une sensibilité climatique induite par les toxiques (Hooper et al. 2013).

2 Objectifs et hypothèses

L'objectif du chapitre 2 était d'étudier s'il existait un modèle de tolérance à la température après la période de colonisation de différentes populations. De plus, j'ai étudié s'il existait une association entre la localisation altitudinale des populations d'une espèce exotique et leur tolérance à la température. J'ai émis l'hypothèse que les animaux d'altitude croissante auraient une tolérance au froid plus élevée, et que les populations montrant une tolérance particulièrement forte au froid seraient moins tolérantes à la chaleur et vice versa. Enfin, J'ai émi émis l'hypothèse que nous trouverions une plage de tolérance de température plus large chez les individus collectés plus près du front d'invasion, dans le cadre d'un syndrome de dispersion.

Le chapitre 3 visait d'abord à décrire la tolérance d'*Alphitobius diaperinus* à une exposition soudaine à la chaleur ou au froid extrême en termes de capacité de récupération, et à mesurer le temps de récupération après différentes expositions. De plus, la capacité de différentes populations à résister à l'exposition aux pesticides en combinaison avec des pics de chaleur quotidiens, et les effets du traitement sur le métabolome, la reproduction et l'activité antioxydante des animaux ont été étudiés. De plus, la façon dont l'exposition aux pesticides et aux pics de chaleur quotidiens (séparément et simultanément) pourrait affecter la tolérance ultérieure des animaux aux températures extrêmes a été étudiée.

Le chapitre 4 portait sur la description de la résistance à la dessiccation chez *Alphitobius diaperinus*, et mon objectif était de découvrir comment l'exposition à des conditions sèches peut affecter la biologie de ce coléoptère en termes de longévité, d'activité, de teneur en eau, de profils métaboliques et de fécondité. J'ai émis l'hypothèse que les animaux dans des conditions sèches auraient une longévité plus courte. Je m'attendais en outre à ce que les animaux perdent de l'eau avec le temps, mais à un rythme très faible, et je m'attendais à ce que les animaux régulent à la hausse les sucres ou d'autres métabolites afin de résister à la

dessiccation. Je m'attendais à observer une diminution de l'activité des animaux gardés au sec, et une diminution de la fécondité suite à une exposition à une période sèche.

3. Chapitre 2

Dans ce chapitre, j'ai examiné la sensibilité thermique (au froid et à la chaleur) du carabe *M. soledadinus*, un coléoptère envahissant des îles sub-antarctiques Kerguelen. Dans l'archipel des Kerguelen, l'expansion progressive de l'aire de répartition de *M. soledadinus* a généré des gradients d'invasion, de temps de résidence, d'altitude et de bord de mer - intérieur des terres, permettant d'examiner si le processus d'invasion (syndrome de dispersion) et la colonisation des altitudes plus élevées et des régions intérieures sont associés à changements de sensibilité thermique.

Des adultes de *M. soledadinus* ont été collectés à la main dans neuf sites distincts de la partie orientale des îles Kerguelen. Port Couvreux (site d'introduction de *M. soledadinus* en 1913), Port Elizabeth (colonisé par *M. soledadinus* en 1939-1983), Cataractes (colonisé en 1983 – 1990), Saint Malo (colonisé en 1991-1995), (Anse de) Papous (colonisé en 1995-2000), Isthme Bas (colonisé en 2000-2005), Molloy (colonisé en 1995-2005), Val Studer (colonisé en 2014-2016) et Ratmanoff (colonisé en 2016). A Saint Malo et Molloy, des insectes ont été collectés le long d'un gradient altitudinal jusqu'à 250 m d'altitude (a.s.l.). A Papous, un transect horizontal bord de mer – intérieur a été réalisé, avec un échantillonnage à différentes distances de la côte.

3.1. Mesure de la durée de récupération des expositions au froid et à la chaleur

Les insectes de chaque population ont été exposés à une température donnée dans des incubateurs, et le nombre d'insectes dans le coma et le nombre d'insectes récupérés (capacité de marche restaurée) ont ensuite été notés pendant 30 min. Toutes les populations ont été exposées à -6, -7 ou 37 °C pendant 15 min. Pour tester l'effet d'une durée d'exposition prolongée sur la récupération ultérieure, d'autres essais menés à -6 °C ont été effectués avec une durée d'exposition de l'adulte *M. soledadinus* de 1 ou 2 h en plus des 15 min (0,25 h) traitement des populations des gradients altitudinal (Molloy, St Malo) et continental (Papous).

3.2. Récupération de *M. soledadinus* adultes échantillonnés le long d'un transect de gradient d'invasion

La dynamique de récupération de 20 *M. soledadinus* adultes de six populations différentes après une exposition à -7 °C ou 37 °C pendant 15 min est présentée à la figure 1A, B. Il y avait des différences significatives dans la dynamique de récupération et la tolérance à la température entre les insectes des différentes populations (-7 °C : P<0,0001 ; 37 °C : P=0,0018), mais ces différences n'étaient pas liées aux temps de séjour des différentes populations.

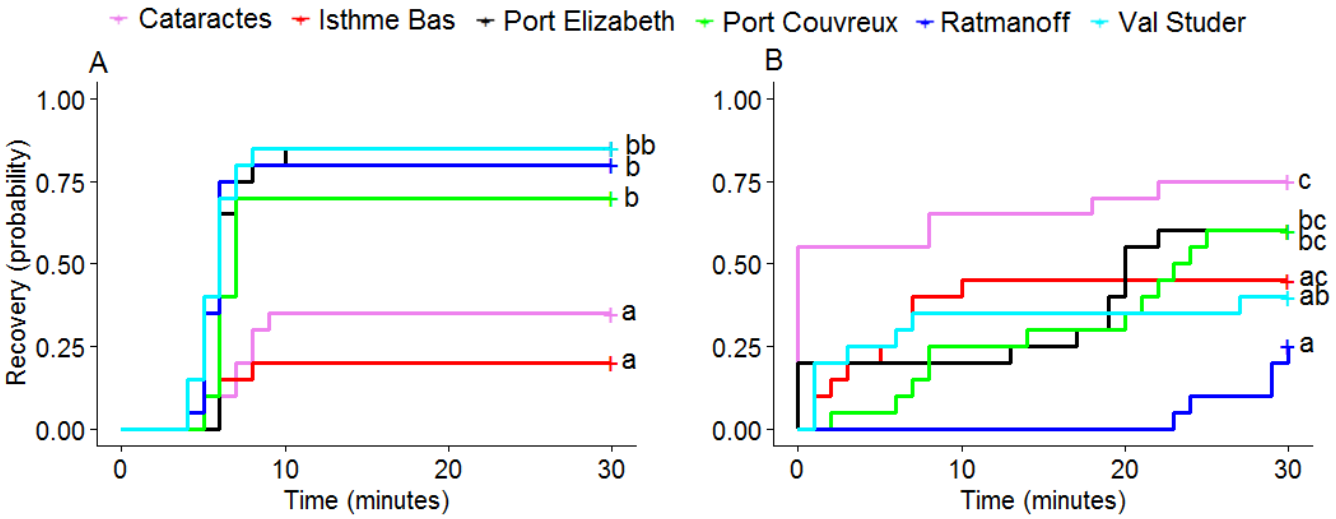


Figure 1 Courbes de récupération de *Merizodus soledadinus* adultes provenant de six populations différentes échantillonnées le long d'un gradient d'invasion aux îles Kerguelen après avoir été exposées à A) -7 °C pendant 15 minutes ou B) 37 °C pendant 15 minutes. Les courbes de récupération ont été estimées à l'aide de la méthode de Kaplan Meier ; *les graduations á la fin de chaque courbe représentent* les insectes qui n'ont pas récupéré à la fin de la période de récupération de 30 minutes (« données censurées à droite »). *Des lettres distinctes indiquent des dynamiques de récupération différentes parmi les populations (test du log rank avec des valeurs P ajustées (Benjamini & Hochberg) pour des comparaisons par paires.*

3.3. Récupération de *M. soledadinus* adultes échantillonnés le long d'un gradient altitudinal

La capacité à se remettre d'une exposition au froid ou à la chaleur chez les insectes échantillonnés le long d'un gradient altitudinal à Saint-Malo est présentée dans la figure 2. Toutes les populations collectées ont montré une capacité similaire à se remettre d'une exposition au froid ou à la chaleur (figure 2A, B) (-7 °C : P=0,056 ; 37 °C : P=0,17). Il n'y avait aucune différence significative dans la capacité de récupérer après une exposition à des températures extrêmement basses ou élevées dans aucune des populations de Molloy (-7 °C : P=0,13, 37 °C : P=0,063).

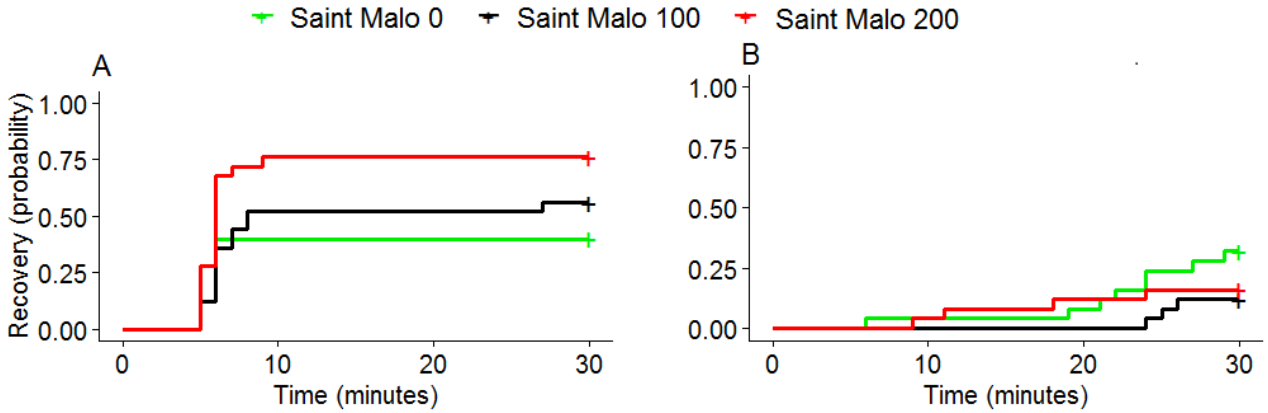


Figure 2 Courbes de récupération de Merizodus soledadinus adultes provenant de trois populations différentes échantillonnées le long d'un gradient altitudinal aux îles Kerguelen après que les insectes aient été exposés à A) -7 °C pendant 15 minutes (les courbes de récupération n'étaient pas significativement différentes, P = 0,056). B) 37 ° C pendant 15 minutes (les courbes de récupération n'étaient pas significativement différentes, P = 0,17). Les courbes de récupération ont été estimées à l'aide de la méthode de Kaplan Meier ; *les graduations á la fin de chaque courbe* représentent les insectes qui n'ont pas récupéré à la fin de la période de récupération de 30 minutes (« données censurées à droite »).

3.4. Durée de récupération des *M. soledadinus* adultes exposés pendant différentes périodes

Les temps de récupération de 25 individus des populations Molloy après avoir été exposés à -6 °C pendant 0,25 h, 1 h ou 2 h sont illustrés dans la figure 3.

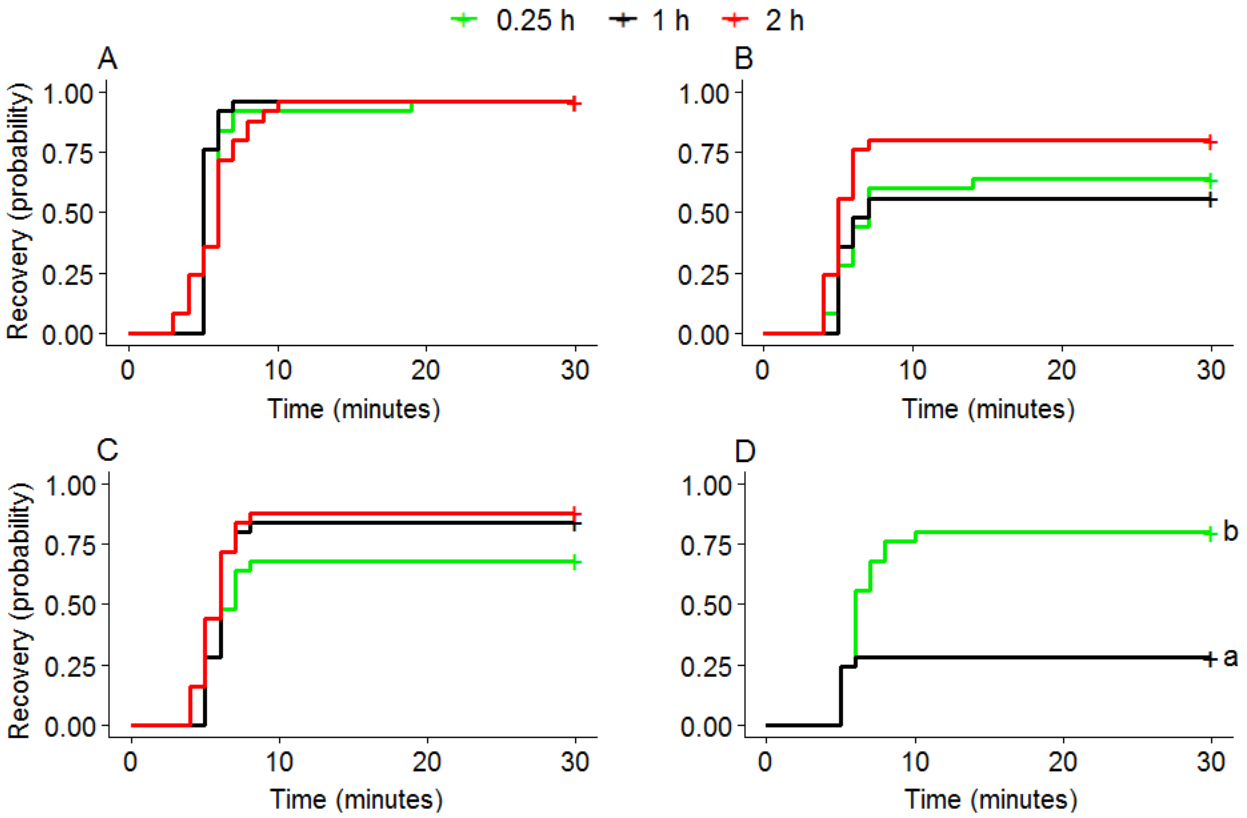


Figure 3 Courbes de récupération de *Merizodus soledadinus* adultes de quatre populations échantillonnées le long d'un gradient altitudinal à Molloy (îles Kerguelen) après avoir été exposées pendant des périodes croissantes (0,25 h, 1 h, 2 h) à -6 °C A) Individus échantillonnés à 0 m d'altitude ; les courbes de récupération n'étaient pas significativement différentes (P = 0,17). B) Individus échantillonnés à 125 m d'altitude dans un habitat rocheux; encore une fois, aucune différence significative entre les courbes de récupération n'est observée (P = 0,076). C) Individus échantillonnés à 125 m d'altitude dans un habitat végétalisé ; aucune différence significative entre les courbes de récupération n'est observée (P = 0,19). D) Individus échantillonnés à 250 m d'altitude ; des lettres distinctes indiquent des différences significatives entre les courbes de récupération (test du log rank avec des valeurs P ajustées par Benjamini et Hochberg pour des comparaisons par paires). Les courbes de récupération ont été estimées à l'aide de la méthode de Kaplan Meier ; les graduations *les graduations á la fin de chaque courbe* représentent les insectes qui n'ont pas récupéré à la fin de la période de récupération de 30 minutes (« données censurées à droite »).

3.5. Durée de récupération des *M. soledadinus* adultes échantillonnés le long d'un transect bord de mer - intérieur des terres

Les populations collectées à Papous ont été exposées à -7 °C (figure 4A) et 37 °C (figure 4B) pendant 15 min. Les insectes de la population Papous 800 étaient caractérisés par une récupération significativement plus élevée après une exposition à -7 °C pendant 15 min que ceux de Papous 200 (Figure 4A) (P = 0,02). Il n'y avait pas de différences significatives entre les courbes de récupération de différentes expositions à la chaleur (figure 4B) (P = 0,19).

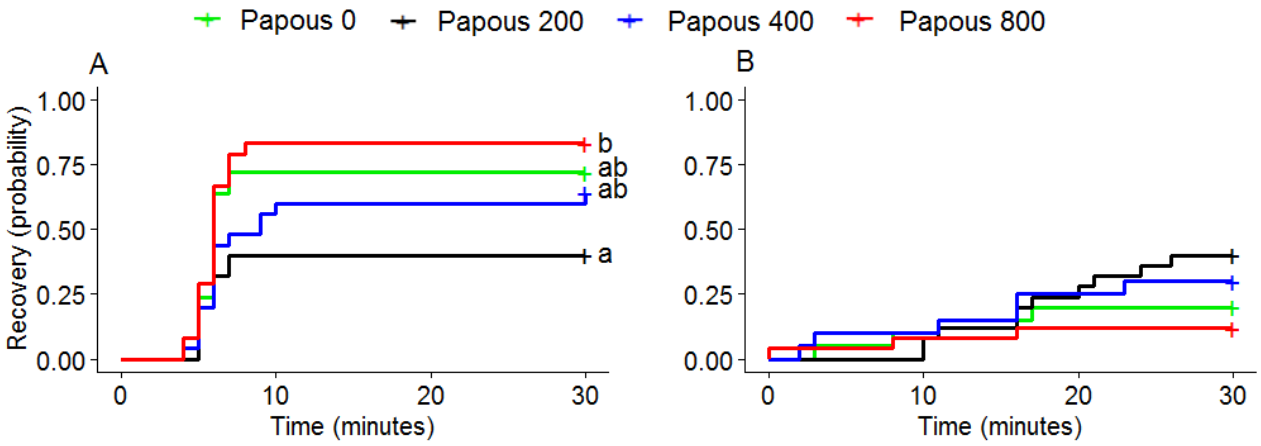


Figure 4 Courbes de récupération d'adultes de Merizodus soledadinus provenant de quatre populations différentes échantillonnées le long d'un transect bord de mer – intérieur des terres aux îles Kerguelen après avoir été exposées à A) -7 °C pendant 15 minutes. Des lettres distinctes indiquent des dynamiques de récupération différentes parmi les populations (test du log rank avec des valeurs P ajustées (Benjamini & Hochberg) pour des comparaisons par paires. B) 37 ° C pendant 15 minutes (les courbes de récupération n'étaient pas significativement différentes, P = 0,19). Les courbes ont été estimées à l'aide de la méthode de Kaplan Meier ; les graduations *les graduations á la fin de chaque courbe* représentent les insectes qui n'ont pas récupéré à la fin de la période de récupération de 30 minutes (« données censurées à droite »).

3.6. Conclusion

Dans cette étude, les carabes prélevés sur les populations de leur front d'invasion dans les îles Kerguelen étaient caractérisés par un haut niveau de tolérance au froid, mais c'était également le cas pour les populations ayant les temps de résidence les plus longs. A l'inverse, deux populations avec des temps de résidence intermédiaires présentent une tolérance au froid plus faible, confirmant que l'expression de ce trait n'est pas systématiquement renforcée le long du gradient d'invasion. Un modèle indiquant un compromis entre la tolérance au froid et à la chaleur a été observé pour de nombreuses populations testées collectées le long du gradient d'invasion. La tolérance à la température des insectes des deux transects altitudinaux n'était pas systématiquement améliorée avec l'altitude, illustrant que d'autres facteurs en plus de la température environnementale, y compris les ressources trophiques, déterminent la tolérance à la température des animaux. De même, la tolérance thermique variait parmi les insectes échantillonnés dans le transect horizontal littoral-intérieur des terres. Dans les études futures, l'influence de la qualité et de la quantité des ressources trophiques disponibles et des interactions biotiques sur la tolérance thermique de *M. soledadinus* adulte devrait être étudiée afin de mieux caractériser les moteurs de ce trait le long des gradients environnementaux. De plus, la conception expérimentale pourrait être modifiée en augmentant la durée

d'acclimatation en laboratoire afin de mieux démêler les modèles adaptatifs possibles des effets des conditions environnementales vécues par les insectes dans leurs habitats respectifs avant qu'ils ne soient échantillonnés. Enfin, les courbes de récupération similaires des insectes exposés à une plus longue durée d'exposition au froid suggèrent que tout dommage infligé par l'exposition au froid dans le traitement utilisé ici se produit dans les 15 premières minutes d'exposition chez cette espèce.

4. Chapitre 3

Afin d'étudier mes questions de recherche restantes, j'ai utilisé le coléoptère ténébrionide, *Alphitobius diaperinus* (Panzer) (Coleoptera : Tenebrionidae). Cet insecte nuisible dans de nombreux poulaillers dans le monde est un modèle particulièrement adapté pour les enquêtes sur les effets des pesticides sur les individus, car nous savons que plusieurs populations sont résistantes aux insecticides. La connaissance de la biologie d'*A. diaperinus* est d'une grande importance économique, car elle sert de réservoir potentiel pour les agents pathogènes aviaires (Casas, Harein, and Pomeroy 1972; Dinev 2013; McAllister et al. 1995) et peut causer des dommages physiques aux murs des poulaillers (Turner 1986).

Dans ce chapitre, nous nous sommes intéressés à comparer la tolérance thermique basale d'*A. diaperinus* adulte de quatre populations, à travers des évaluations de leur capacité à récupérer après différentes durées d'exposition au froid ou à la chaleur. Par la suite, nous nous sommes intéressés à comparer les effets de l'exposition à une dose écologiquement pertinente de l'insecticide cyfluthrine sur l'état des insectes maintenus à des températures constantes ou soumis à des pics de chaleur quotidiens. De plus, nous avons cherché à étudier les effets des pics de chaleur quotidiens et d'un traitement insecticide (le pyréthroïde cyfluthrine) sur la tolérance thermique d'*A. diaperinus* adulte.

De plus, nous avons mesuré le niveau de capacité antioxydante chez les insectes, car les piégeurs de ROS (Reactive Oxygen Species) (qui sont un groupe de substances antioxydantes) ont souvent été signalés en plus grande quantité dans plusieurs organismes manipulant des facteurs de stress, y compris les pesticides (Sulmon et al. 2015; Verheyen and Stoks 2019). De plus, la composition des métabolites chez les animaux des différents traitements expérimentaux a été déterminée, afin de voir si et comment les différents facteurs de stress, individuellement ou en combinaison, affectaient l'homéostasie des insectes.

Enfin, des adultes d'*A. diaperinus* ont également été utilisés pour déterminer si ces expositions au stress affectaient l'aptitude des coléoptères à long terme. Pour atteindre cet objectif, nous avons estimé les impacts en termes de reproduction, en mesurant le nombre de descendants produits par femelle.

Des adultes d'*A. diaperinus* ont été collectés dans trois élevages avicoles de Bretagne, France, ci-après dénommés FARM1, FARM2 et FARM3. De plus, des larves d'*A. diaperinus* ont été obtenues auprès de Proti-Farm via une animalerie, appelée population LAB.

4.1. Caractérisation de la tolérance thermique des insectes de différentes populations

Afin de comparer la tolérance thermique des insectes des quatre populations, des adultes ont été exposés à deux températures extrêmes, -6 ou +42,5°C, dans un cryothermostat. Chaque insecte a été exposé à l'une de ces deux températures extrêmes pendant une des six durées : 15 min, 30 min, 1h, 2h, 3h ou 4h, dans l'obscurité. À la fin du traitement thermique, des insectes ont été observés et la récupération des insectes a été notée en enregistrant les individus comme « Actif » ou « Immobile ».

La probabilité de rétablissement au fil du temps d'*A. diaperinus* adulte dépendait de la durée d'exposition à des températures extrêmes chaudes ou froides, ainsi que de la population (figures 5 et 6).

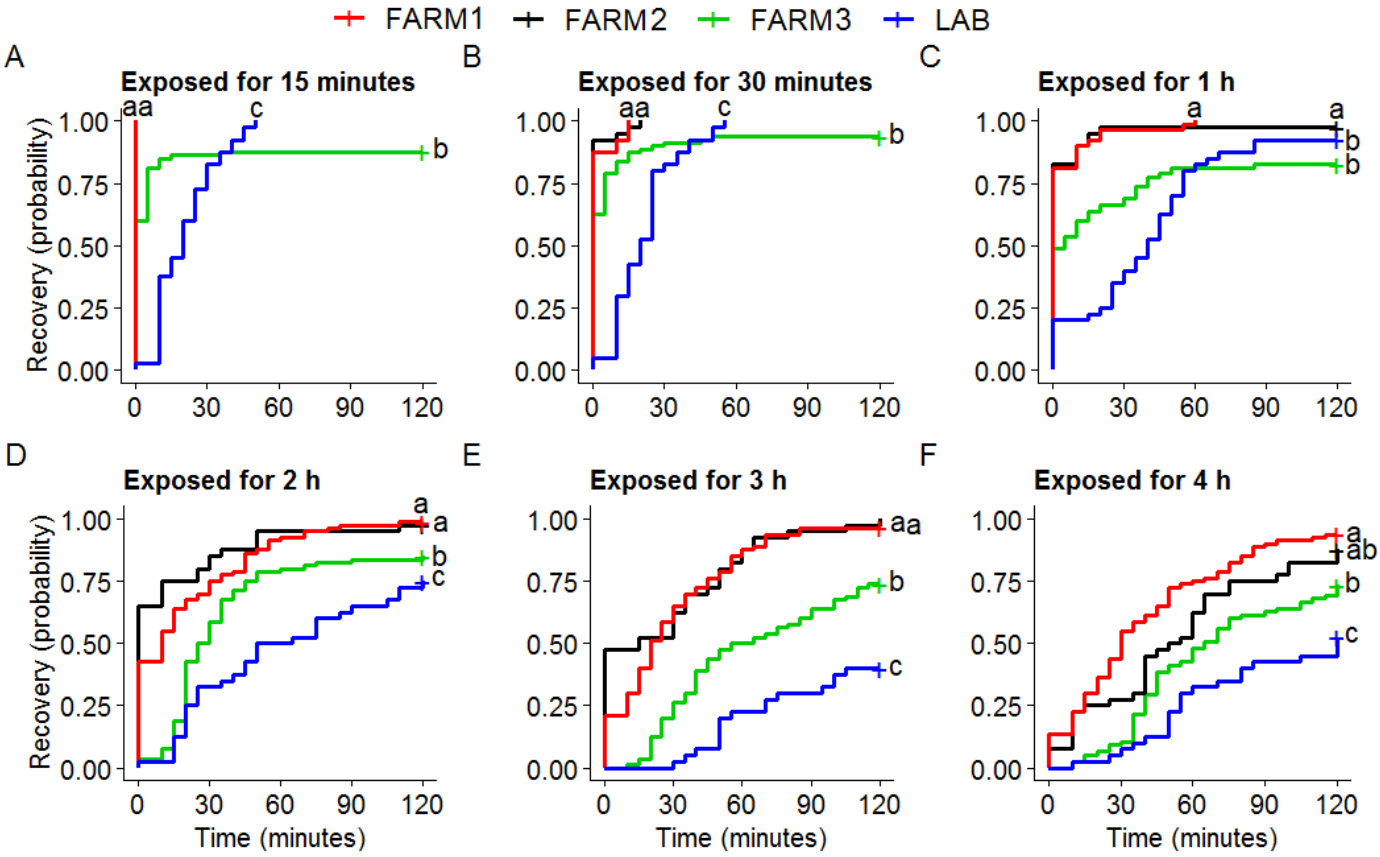


Figure 5 Probabilité de rétablissement d'individus de quatre populations différentes d'*A. diaperinus* exposés à 42,5 °C pendant six durées différentes : A) 0,25 heure (P global < 0,001) ; B) 0,5 heure (P global < 0,001) ; C) 1 heure (P global < 0,001) ; D) 2 heures (P global < 0,001) ; E) 3 heures (P global < 0,001) ; F) 4 heures (P global < 0,001). Les lignes rouges représentent les insectes de FARM1, les lignes noires représentent les insectes de FARM2, les lignes vertes représentent les insectes de FARM3, et les lignes bleues représentent les insectes de LAB. Des lettres différentes indiquent des différences entre les courbes de probabilité de récupération au sein de chaque durée.

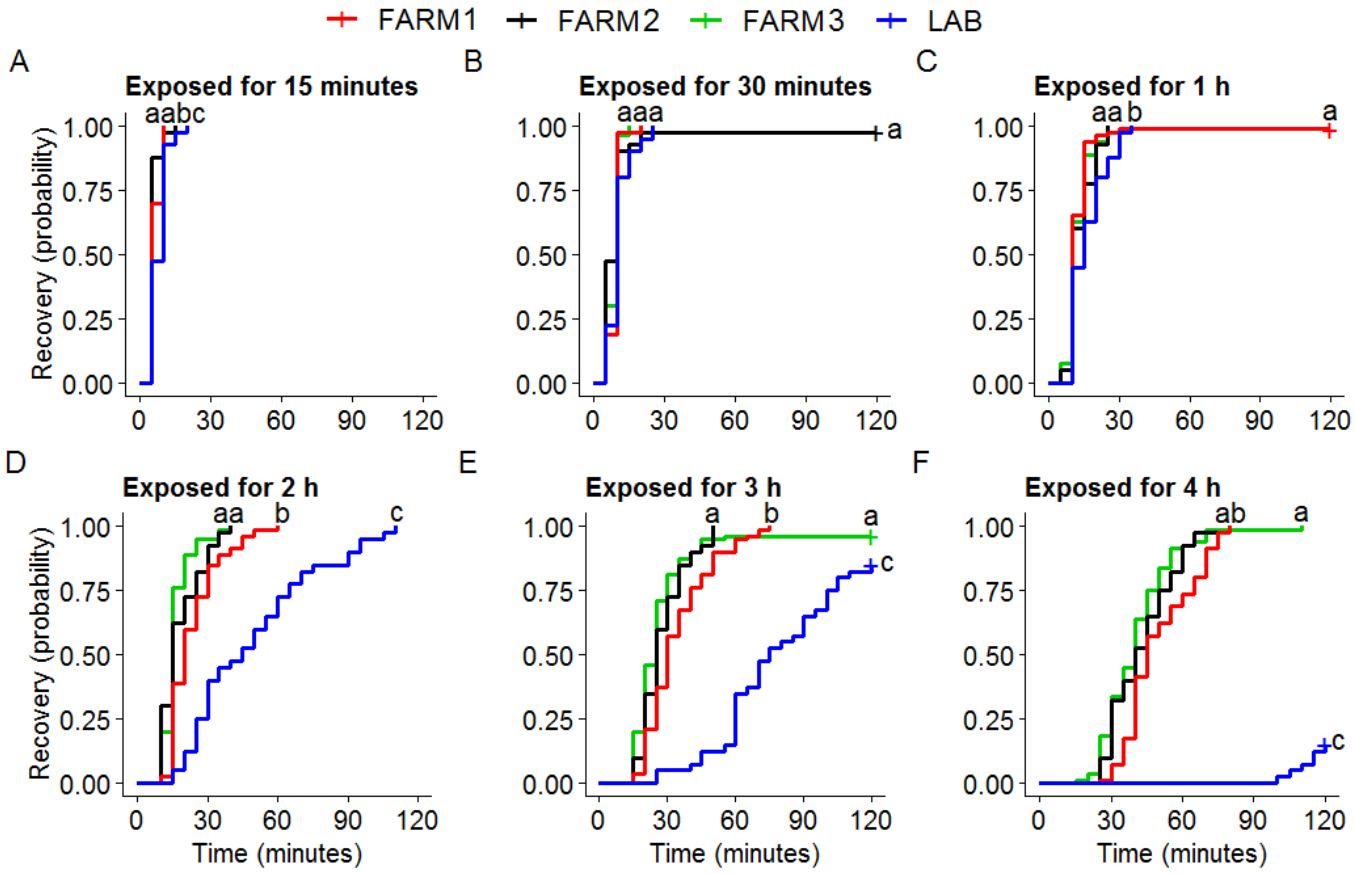


Figure 6 Probabilité de rétablissement d'individus de quatre populations différentes d'*A. diaperinus* exposés à -6 °C pendant six durées différentes : A) 0,25 heure (P global < 0,001) ; B) 0,5 heure (P = 0,074); C) 1 heure (P global < 0,01) ; D) 2 heures (P global < 0,001) ; E) 3 heures (P global < 0,001) ; F) 4 heures (P global < 0,001). Les lignes rouges représentent les insectes de FARM1, les lignes noires représentent les insectes de FARM2, les lignes vertes représentent les insectes de FARM3, et les lignes bleues représentent les insectes de LAB. Des lettres différentes indiquent des différences entre les courbes de probabilité de récupération au sein de chaque durée d'exposition.

4.2. Effet des pics de chaleur quotidiens et de l'exposition à la cyfluthrine

Ici, nous avons combiné deux stress abiotiques en exposant conjointement *A. diaperinus* adulte à des pics de chaleur modérément stressants et à un insecticide pendant huit jours, avant d'évaluer toute altération visible ou décès des individus exposés. Après huit jours, l'état de chaque individu a été noté : les insectes ont été considérés soit comme « Actifs » (pas d'effet apparent du traitement sur les activités locomotrices des insectes), soit « Altérés » (déficits locomoteurs : difficultés à bouger les membres, ou couché sur le dos en se déplaçant) ou « Immobile » (aucun mouvement observable).

Le nombre de petits vers de farine actifs était significativement réduit lorsque les insectes étaient exposés à la cyfluthrine à température constante (26 °C) par rapport aux insectes témoins (P < 0,05 (niveau de confiance à 95 %)) pour toutes les populations, à l'exception de FARM1 (Figure 7). De plus, l'insecticide cyfluthrine et les pics de chaleur quotidiens (18h à 26 °C suivis d'une période de 6h à 38 °C pendant 8 jours) ont significativement diminué la proportion d'insectes actifs par rapport aux conditions de contrôle et de traitement thermique (P < 0,05) pour tous populations. Pourtant, la proportion d'insectes classés comme actifs est restée proche de 100 % pour FARM1. Il n'y avait aucune interaction significative entre les pics de chaleur quotidiens et la cyfluthrine pour aucune des populations (P = 0,28 ou plus).

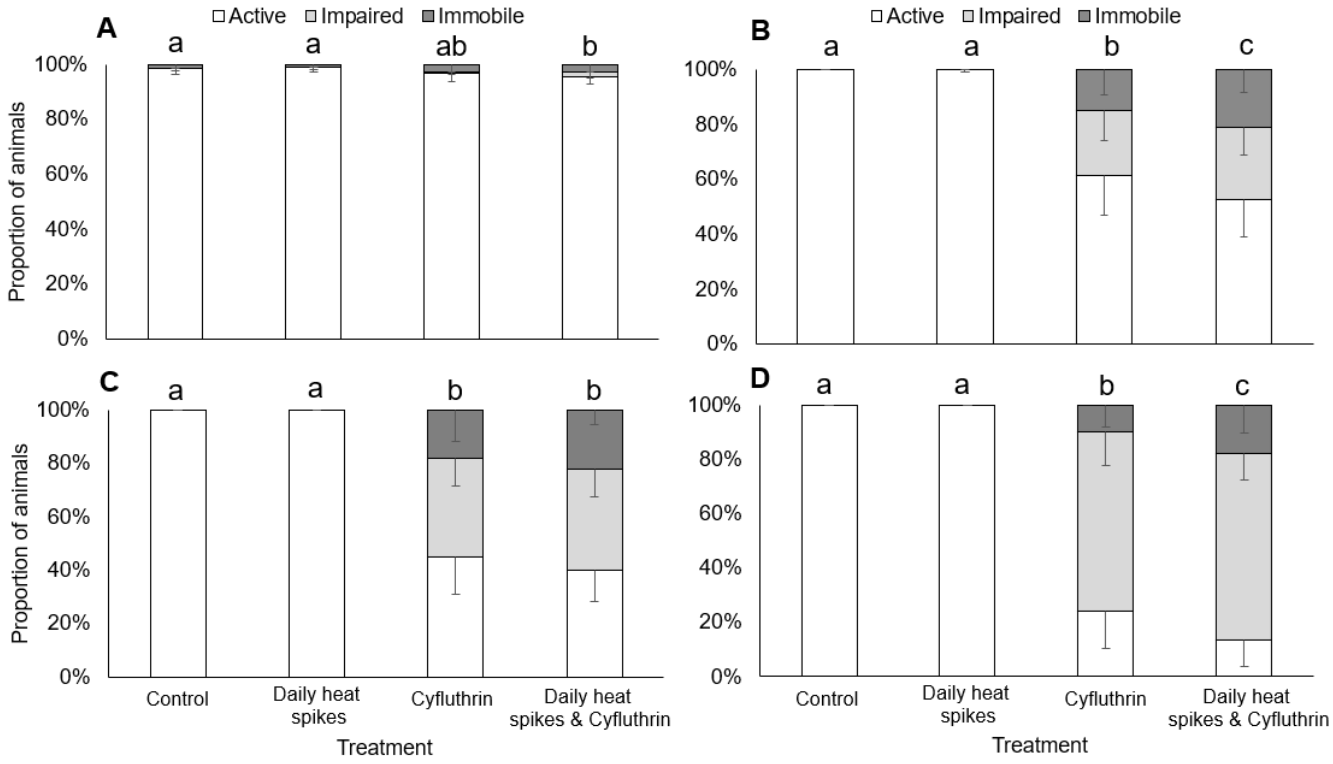


Figure 7 Statut des *A. diaperinus* adultes des populations A) FARM1, B) FARM2, C) FARM3 après avoir été exposés pendant huit jours à des conditions témoins (constante 26 °C), des pics de chaleur quotidiens (26 °C pendant 18h, 38 °C pendant 6h), cyfluthrine (20 mg/m2) ou pics de chaleur quotidiens et cyfluthrine et D) LAB après huit jours de mêmes traitements d'exposition, mais avec une concentration de cyfluthrine de 0,5 mg/m2. Des lettres différentes indiquent des différences entre le nombre d'insectes actifs au sein de chaque population.

4.3. Effet des pics de chaleur quotidiens et de l'exposition à la cyfluthrine sur la récupération après une exposition à des températures extrêmes

Nous avons mesuré les capacités d'*A. diaperinus* adulte à se remettre de chocs thermiques ou de froid après avoir été exposés à un insecticide et/ou à des pics de chaleur quotidiens pendant huit jours. L'exposition à des températures extrêmes a été réalisée comme décrit ci-dessus.

La probabilité de récupération d'*A. diaperinus* adulte de FARM1 diminuait avec l'augmentation des doses de cyfluthrine après que les insectes aient été maintenus pendant huit jours sous différents régimes de température et concentrations de cyfluthrine avant une exposition à des températures extrêmes ($P < 0,001$), en particulier les insectes exposés à une chaleur extrême (figure 8). La pré-exposition à un pic de chaleur quotidien pendant huit jours (Figure 8, BC) a accéléré la récupération des insectes après qu'ils aient été exposés à 42,5°C pendant 2h, par rapport aux animaux exposés à 26°C constant (Figure 8, A). A l'inverse, les courbes de récupération sont restées comparables pour les insectes exposés à un froid extrême (-6 °C pendant 2h ; Figure 8, D-F), même s'ils ont été préalablement soumis à différents pics de chaleur pendant 8 jours.

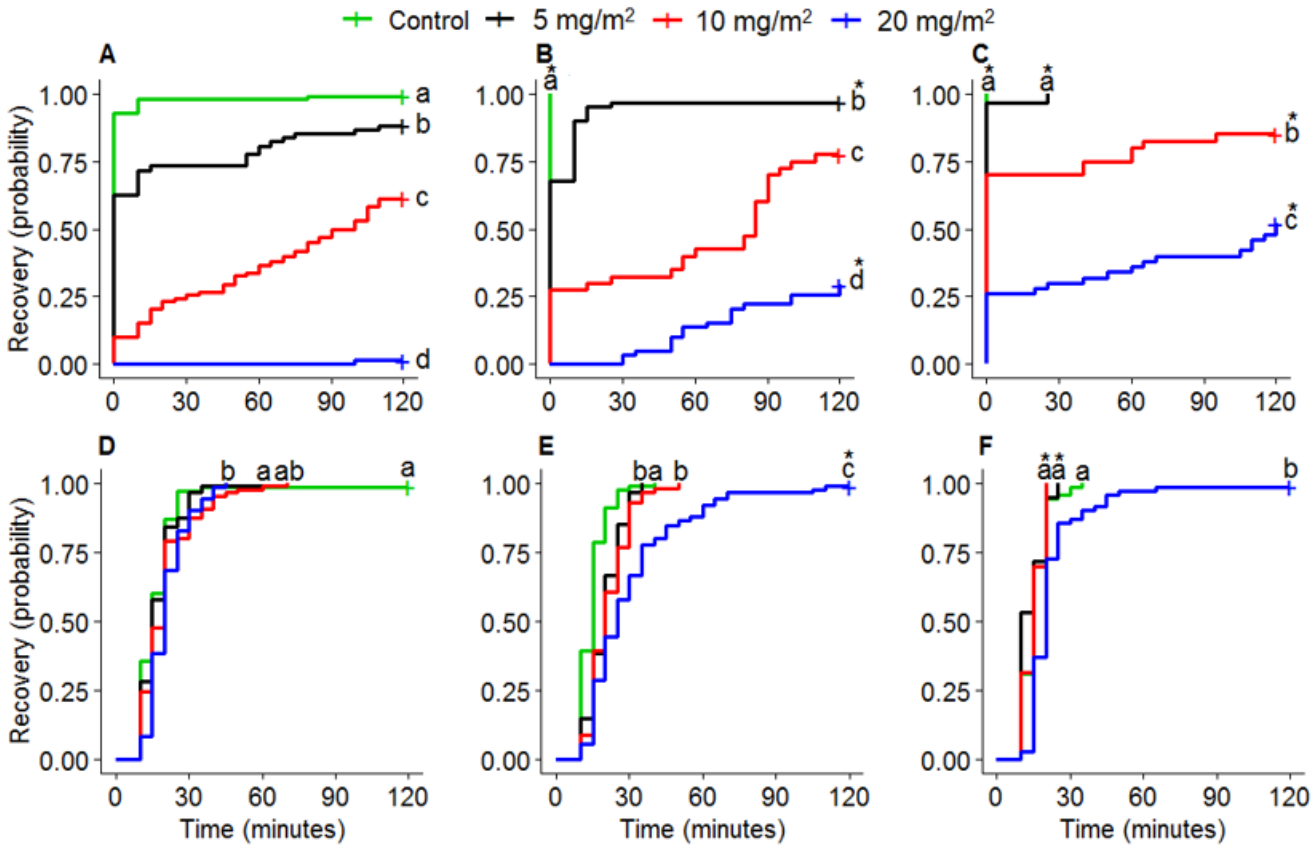


Figure 8 Probabilité de récupération d'*A. diaperinus* de FARM1 observée toutes les 5 minutes après une exposition de 2 heures à 42,5 °C (A, B et C) ou -6 °C (D, E et F). Avant le test, les insectes ont été exposés à différents régimes de température : (A) et (D) constante 26 °C ; (B) et (E) 26 °C par jour interrompus de 6h à 32 °C ; (C) et (F) 26 °C par jour interrompus de 6h à 38 °C. Pour chaque régime de température, les insectes ont été exposés à quatre concentrations différentes de l'insecticide cyfluthrine : 0 (témoin), 5 (lignes noires), 10 (lignes rouges) ou 20 (lignes bleues) mg de

cyfluthrine/m2. Les étoiles (*) représentent des différences significatives entre la courbe marquée et la courbe de la même couleur dessinée sur la figure 4A (régime de température constante utilisé comme probabilité de récupération de référence). Des lettres différentes indiquent des différences entre les courbes de probabilité de récupération au sein de chaque groupe de prétraitement de température.

4.4. Analyses physiologiques

Après 1,4 ou 8 jours d'exposition à l'insecticide et/ou à la chaleur (voir figure 9), les animaux FARM 2 ont été notés comme actifs ou non, et retirés de l'exposition aux pesticides et/ou à la chaleur, les individus actifs de chaque condition ont été utilisés pour échantillonnage pour la capacité antioxydante et les tests métabolomiques.

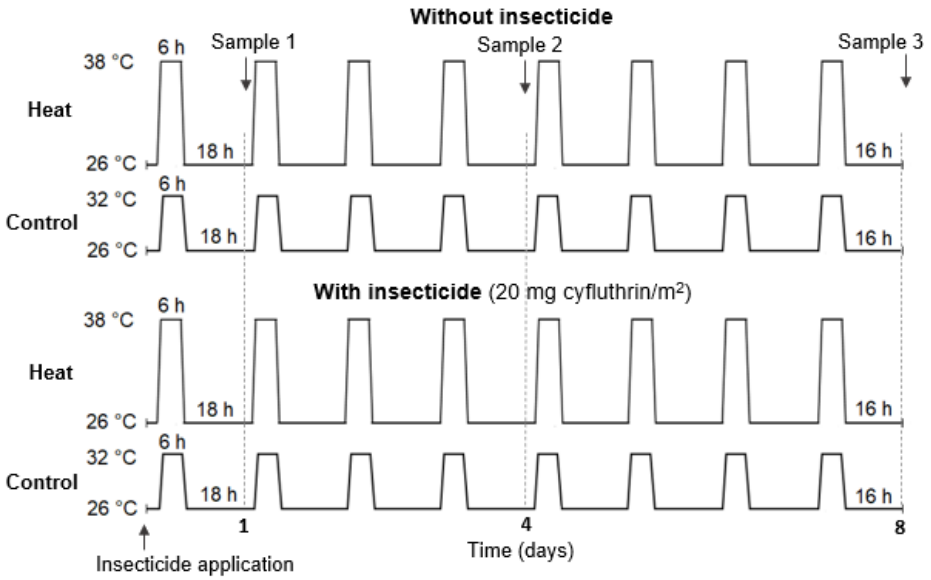


Figure 9 Illustration des températures d'exposition des différents traitements et des temps de prélèvement. Pour les insectes de la FERME 2, les temps d'échantillonnage 1 à 3 représentent les moments où les insectes ont été observés et notés comme actifs, mobiles ou immobiles, puis immédiatement échantillonnés pour des analyses physiologiques. Après 8 jours d'exposition, ils ont été retirés de toute exposition et mis à récupérer pendant 4 jours dans une armoire de contrôle avant d'être prélevés pour des analyses physiologiques.

Les échantillons ont d'abord été traités comme décrit dans le kit de dosage antioxydant fourni par Cayman. À la suite de dosages d'antioxydants, l'analyse des protéines a été réalisée en utilisant la technique de Bradford (dosage colorimétrique, longueur d'onde 595 nm), après quoi la chromatographie en phase gazeuse/spectrométrie de masse (GC-MS) a été utilisée pour

comparer les profils métaboliques des coléoptères à la fin de leur exposition et après 4 jours de récupération.

Sur la base des résultats présentés dans la figure 10, il n'y avait pas de différences significatives entre la capacité antioxydante des animaux de différents traitements au fil du temps. De même, aucun changement dans la composition des métabolites n'a été trouvé.

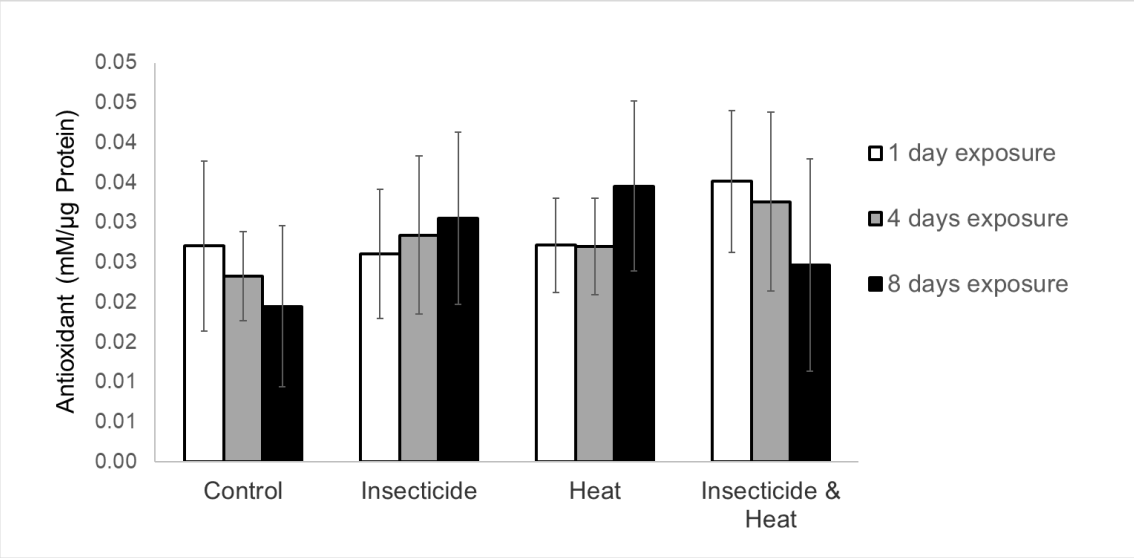


Figure 10 Capacité antioxydante moyenne +/- SE chez *A. diaperinus* après 1 (blanc), 4 (gris) ou 8 (noir) jours d'exposition à 4 groupes de traitement différents (témoins, pesticides exposés (20 mg/m2, cyfluthrine) exposés à la chaleur (6 h à 38°C) ou Pesticide & Chaleur Aucune différence significative dans la capacité antioxydante n'a été observée entre les différents groupes de traitement.

4.5. La fécondité

Pour tous les groupes de traitement, après les 8 jours, les individus FARM 1 du même traitement ont été placés ensemble dans des boîtes en plastique pendant sept jours, avant que les adultes ne soient retirés. Les adultes ont ensuite été déterminés selon le sexe. Après environ 7 semaines, le nombre de descendants dans chaque boîte a été compté. Le nombre de descendants était significativement augmenté pour les animaux connaissant un pic de chaleur quotidien, par rapport aux témoins. Néanmoins,animaux connaissant des pics de chaleur quotidiens tout en étant exposés à un insecticide avaient significativement moins de descendants par rapport aux animaux ayant connu des pics de chaleur quotidiens sans autre stress (Figure 11). Cependant, l'exposition aux pesticides à elle seule n'a pas réduit de manière significative le nombre de descendants par femelle.

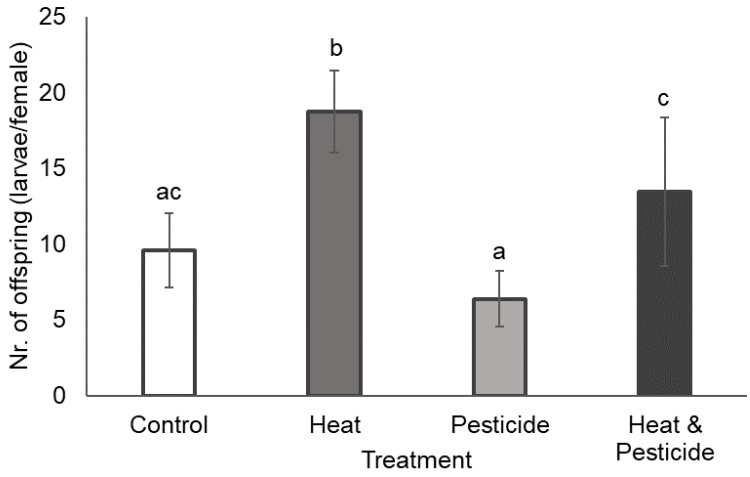


Figure 11 Représente le nombre total moyen de descendants par femelle (+/- SE) après 8 jours d'exposition à l'un des quatre groupes de traitement différents (Témoins, Pesticides exposés (0,02 g/m2, Cyfluthrine) exposés à la chaleur (6h à 38°C) ou Pesticide & Exposition à la chaleur, suivie de 7 jours de reproduction et de 30 jours de développement des larves sans la présence d'adultes.

4.6. Conclusion

Le présent travail rapporte que les populations de petits vers de farine ont des capacités distinctes à se remettre du stress thermique et insecticide, et de leurs interactions. Ce constat souligne l'importance de concevoir des expérimentations incluant plusieurs populations pour évaluer la sensibilité d'une espèce donnée aux facteurs environnementaux, et plus particulièrement lorsque ces données alimentent des modèles visant à prévoir l'avenir de la biodiversité. Aucune interaction synergique n'a été trouvée lorsque les petits vers de farine ont été soumis conjointement à des pics de chaleur quotidiens et à la cyfluthrine; pourtant, ce type de procédure expérimentale, qui simule les conditions qui pourraient être vécues par des organismes en liberté dans des environnements naturels et anthropiques, augmente le réalisme écologique de l'étude. C'est encore plus vrai dans un contexte de réchauffement climatique, car les pics de chaleur quotidiens devraient devenir plus fréquents dans plusieurs habitats. Ici, nous avons également constaté l'importance de travailler avec des insectes collectés sur le terrain. La tolérance thermique et la sensibilité aux insecticides de la population de laboratoire étaient très différentes de celles des échantillons de terrain, ce qui signifie que des conclusions différentes seraient tirées si l'on travaillait avec la première population uniquement. Enfin, et conformément à des recherches antérieures, notre étude a souligné l'importance de considérer les effets de multiples facteurs de stress et les effets durables qu'ils peuvent avoir sur d'autres traits des insectes.

5. Chapitre 4

Dans le contexte du changement climatique, le réchauffement et la fréquence croissante des périodes de sécheresse menacent la biodiversité terrestre en augmentant la probabilité de dessèchement des individus (IPCC 2021; Trenberth 2011). En particulier, les petits animaux tels que les insectes, qui ont un rapport surface/volume élevé, sont particulièrement sensibles à l'évapotranspiration et à l'exposition à des conditions de déshydratation (Hadley 1994). Par conséquent, ce chapitre visait à découvrir comment l'exposition à des conditions sèches peut affecter la biologie du coléoptère nuisible envahissant *Alphitobius diaperinus* en termes de longévité, d'activité, de teneur en eau, de profils métaboliques et de fécondité.

5.1. Survie des insectes exposés à des conditions de dessiccation

Les capacités de survie d'*A. diaperinus* adultes continuellement exposés à des conditions de dessiccation ont été comparées à des témoins. Tous les insectes ont été privés de nourriture pendant toute la durée de l'expérience pour éviter les différences de survie qui se seraient inévitablement produites entre les insectes stressés tombant progressivement dans le coma et les témoins. Au cours de l'expérience, l'humidité relative était de 14 ± 2 et 60 ± 5 % dans des conditions d'humidité relative et optimale, respectivement. La dessiccation a affecté de manière significative la durée de survie des coléoptères ($P < 0,001$), avec environ 50 % des individus étant morts après 30 jours d'expérimentation, alors que presque aucune mortalité n'a été enregistrée chez les témoins au même moment (Figure 12). Mais il a fallu au total 50 jours avant que tous les insectes exposés à des conditions sèches soient morts.

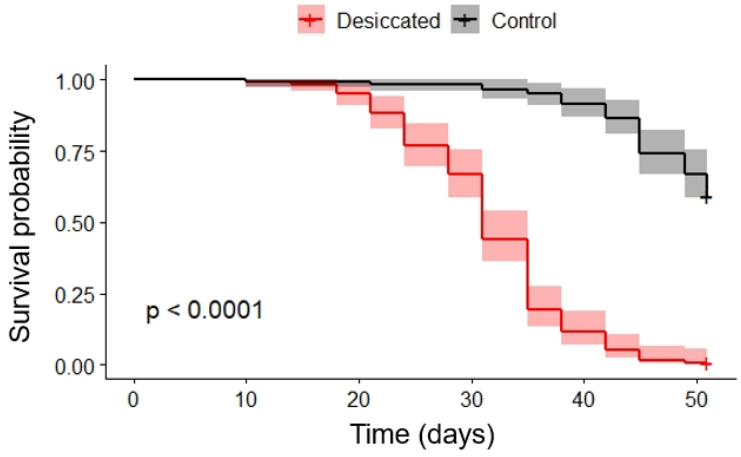


Figure 12 Temps de survie des animaux privés de nourriture maintenus à 14% ± 2 R.H. (dessiccation, ligne grise) par rapport aux animaux maintenus à environ 60% R.H. (ligne noire). Valeur p résultant du test du log rank comparant les deux graphiques de Kaplan Meier, tracée avec des intervalles de confiance à 95 %. Données censurées marquées par +.

5.2. Mesures de la teneur corporelle en eau, de la teneur en sucre et de la composition en métabolites

Les changements dans la teneur en eau, en sucre et la composition en métabolites ont été surveillés par un échantillonnage hebdomadaire à partir de la configuration ci-dessus. La teneur en eau a été significativement réduite chez les animaux traités par dessiccation après 2 semaines de dessiccation (valeurs P par rapport à la semaine 0), puis s'est stabilisée (Figure 13, A). La teneur en eau des témoins n'a jamais changé de manière significative par rapport à la teneur en eau des animaux avant le début de l'expérimentation (Figure 13, A). La teneur en sucre chez les animaux a été fortement réduite au cours de la première semaine expérimentale, après quoi elle s'est stabilisée pour les deux traitements (Figure 13, B).

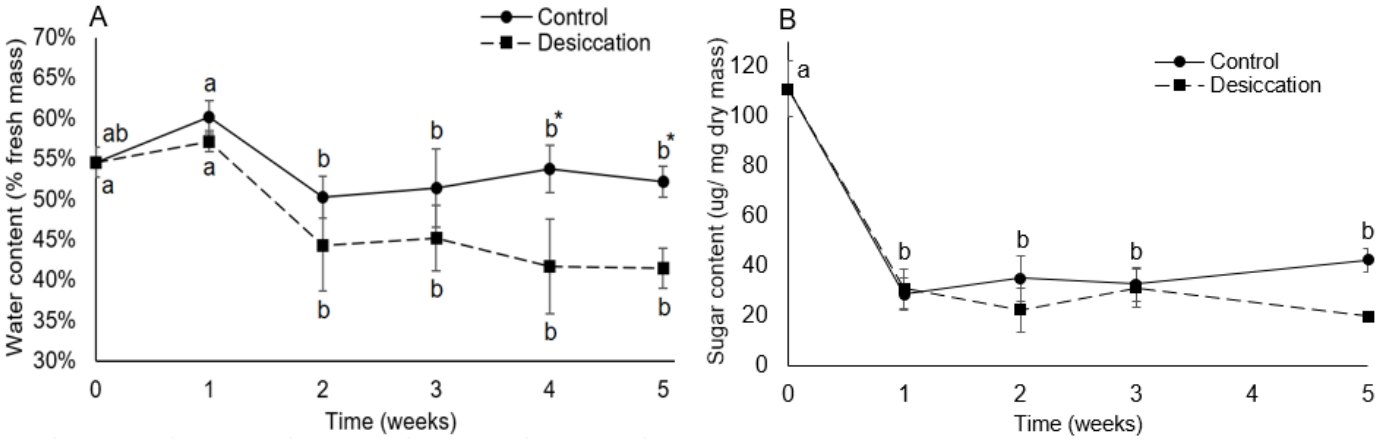


Figure 13 Teneur en eau (A) et teneur en sucre (B) des animaux privés de nourriture maintenus à 14 % ± 2 H.R. (dessiccation, ligne pointillée) par rapport aux animaux maintenus à environ 60 % H.R. (ligne continue). Les lettres indiquent des différences significatives au sein des groupes de traitement. Les étoiles (*) indiquent une différence significative entre les groupes de traitement à ce moment-là en utilisant une ANOVA à deux facteurs

La composition des métabolites était significativement différente (intervalles de confiance à 95 %) après 1 semaine, par rapport aux semaines restantes, mais aucune différence n'a été observée entre les groupes de traitement (Figure 14).

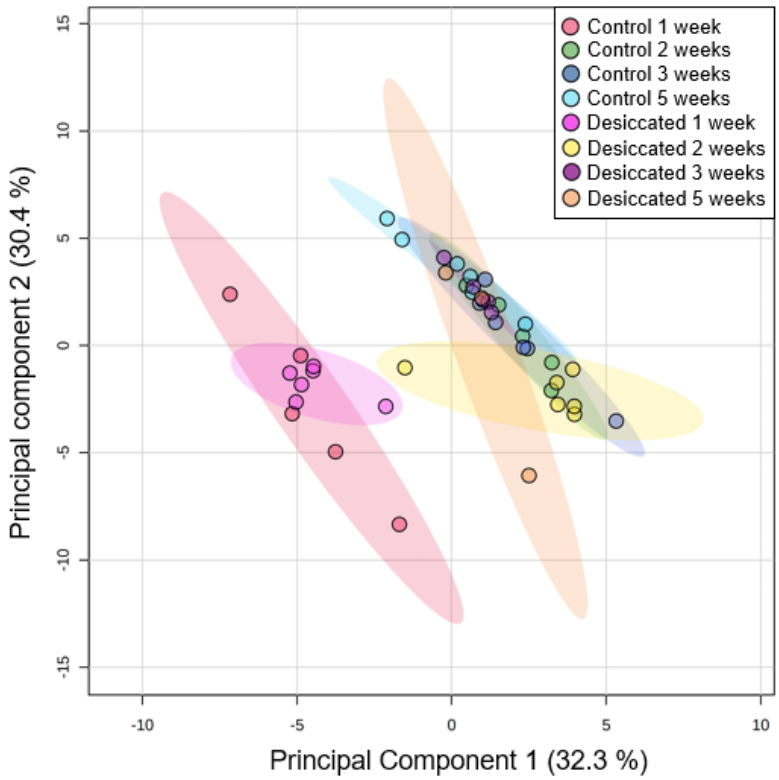


Figure 14 Diagramme représentant l'analyse en composantes principales effectuée à l'aide de Metaboanalyst v 5.0. Une analyse en composantes principales représentant le traitement de dessiccation et la séparation du groupe témoin sur la base des deux composantes les plus significatives. Les cercles colorés représentent des intervalles de confiance à 95 %. Les points colorés représentent des échantillons individuels.

5.3. Effets des conditions de dessiccation sur l'activité et les capacités de reproduction des insectes

Pour chaque traitement expérimental (témoin, dessiccation), l'activité du coléoptère individuel a été mesurée une fois au début de l'expérience, et après trois semaines d'expérience. Enfin, nous avons cherché à évaluer les effets à long terme que des conditions de dessiccation pourraient avoir sur la fitness des coléoptères en mesurant leurs capacités de reproduction après 1 semaine en conditions sèches.

L'activité des animaux a augmenté de manière significative après 2 semaines de traitement expérimental ($P < 0,001$) mais le niveau d'activité pour les deux groupes de traitement est resté similaire (Figure 15). Les animaux ayant subi une semaine de dessèchement avaient significativement moins de descendants après une semaine de reproduction et 30 jours de développement de la progéniture, par rapport aux animaux témoins ($P < 0,001$) (Figure 16).

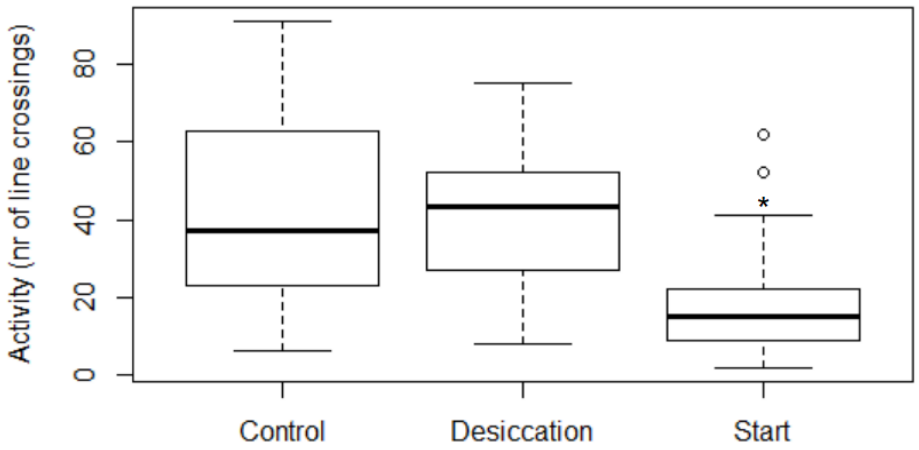


Figure 15 Activité des animaux privés de nourriture maintenus à 14% ± 2 R.H. (dessiccation) par rapport aux animaux maintenus à environ 60% R.H. L'activité a été mesurée comme le nombre de fois qu'un individu a franchi une ligne dans une boîte de Pétri avec une grille de lignes au fond. L'étoile (*) indique une différence significative par rapport aux autres groupes trouvée par les tests t par paires (P < 0,001) suite à une ANOVA à sens unique.

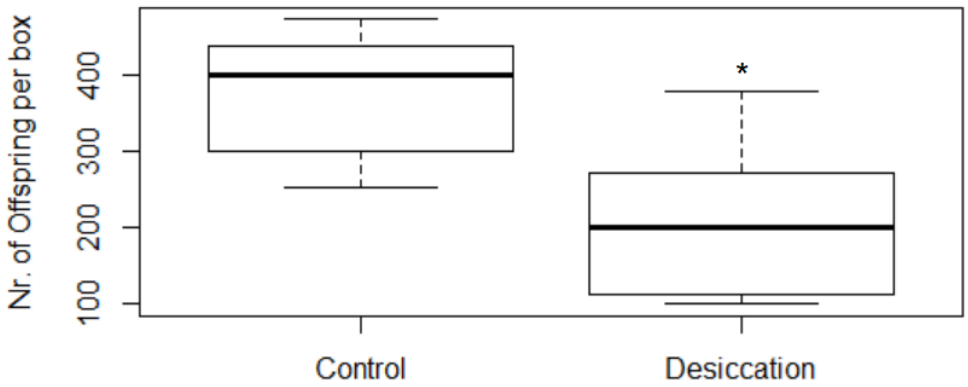


Figure 16 Nombre de descendants dans chaque réplicat d'animaux après avoir été maintenus à environ 14% R.H. (dessiccation) par rapport aux animaux maintenus à environ 60% R.H. pendant une semaine, suivie d'une semaine de reproduction et de 30 jours de développement de la progéniture. Les étoiles (*) indiquent une différence significative montrée par une ANOVA à un facteur (P < 0,001).

5.4. Conclusion

Nous avons trouvé qu'*A. diaperinus* est très résistant à un environnement desséchant, ce qui peut expliquer son succès en tant que ravageur dans les habitats très secs. Nous ne voyons aucune preuve que la méthode de tolérance à la dessiccation pour cette espèce implique une régulation à la hausse des osmolytes compatibles ou une réduction de l'activité. Ces résultats nous amènent à émettre l'hypothèse que les animaux dépendent de leur cuticule hautement imperméable et de l'utilisation de la cavité élytrale pour la respiration, ce qui leur permet d'obtenir une perte d'eau respiratoire et une transpiration cuticulaire très faibles. Malgré une tolérance élevée à la dessiccation, le nombre de descendants d'adultes se reproduisant pendant une semaine après huit jours de conditions de dessiccation était significativement inférieur à celui des témoins, indiquant que des conditions sèches peuvent toujours avoir un effet néfaste sur les populations d'*A. diaperinus*.

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UNIVERSITÉ DE RENNES 1

Titre : Conséquences écophysiologiques et écotoxicologiques des changement globaux chez les insectes terrestres

Mots clés : Température, Tolérance, Distribution, Pesticide, Multi-stress, Desiccation

Résumé : Les insectes terrestres sont affectés par de nombreux facteurs environnementaux qui varient dans le temps et dans l'espace à macro- et micro-échelles. Le changement climatique affecte par ailleurs la manière dont ces facteurs varient, avec de potentielles conséquences pour les individus, les populations et les communautés d'insectes. Au sein de ce travail de thèse, les effets de facteurs abiotiques sur différents traits biologiques et physiologiques des insectes ont été étudiés, en employant des coléoptères comme modèles biologiques. Dans une première partie, l'impact de l'expansion géographique sur la plasticité thermique a été étudiée, e en se focalisant sur le coléoptère Merizodus soledadinus envahissant une île subantarctique. Différentes populations ont été collectées, et ce travail a permis de metter en évidence un schéma général selon lequel la plupart des populations présentaient de fortes aptitudes à tolérer le froid ou le chaud. Ces résultats indiquent qu'il existe des compromis entre ces deux traits avec d'autres caractères biologiques, les insectes. Au sein de ce travail de thèse, les niveaux de tolérance et les réponses des insectes aux thermiques et chimiques (cyfluthrine) ont été étudiés. L'objectif était de déterminer si les réponses mises en place pour faire face au stress thermique pouvaient affecter la sensibilité au stress chimique et vice versa. Dans ce but, différentes populations du coléoptère ravageur envahissant Alphitobius diaperinus ont été employées : une population élevée en conditions contrôlées, et trois populations collectées dans différents élevages avicoles.

Toutes les populations d'A. diaperinus testées possèdent une large tolérance thermique. Cependant, les populations collectées dans les élevages avicoles présentent une tolérance thermique plus importante que la population élevée en conditions contrôlées. Ces recherches démontrent le rôle des environnements variables comme moteur d'une plasticité thermique accrue. Le prétraitement des populations par exposition quotidienne à des pics de chaleur réduit le temps de récupération des insectes qui sont exposés à une chaleur extrême (mais pas au froid). A l'inverse, l'exposition aux pesticides avant le stress thermique a augmenté les temps de récupération. Ces recherches fournissent des preuves des interactions entre insecticide et tolérance à la chaleur, ce qui n'avait pas été démontré auparavant chez les insectes terrestres adultes. Suite à une exposition au stress sous forme de pics de chaleur quotidiens, avec ou sans exposition à des insecticides, aucune altérationdu métabolome ou l'activité antioxydante des insectes n'a été observée. Cependant, l'exposition à la chaleur a entraîné une augmentation du nombre de descendants, tandis que le traitement insecticide avait tendance à réduire ce nombre. En d'autres termes, les insectes ont démontré une tolérance élevée aux pesticides, mais cela se traduit par un contre coût en termes de reproduction. Pour finir, la tolérance et les réponses d'A. diaperinus aux stress hydriques ont été prises en compte. Cela a permis de mettre en évidence les fortes capacités de cet insecte à faire face à des épisodes de sécheresse, sans que cela n'affecte la composition en métabolites. Cependant, après une période dans un environnement très sec, le succès reproducteur des insectes a ici aussi été diminué.

Title : Ecophysiological and ecotoxicological consequences of global changes in terrestrial insects

Keywords : Temperature, Tolerance, Distribution, Pesticide, Multi-stress, Desiccation

Abstract : Terrestrial insects are affected by many environmental factors that vary temporally and spatially at micro- and macroscales. Climate change is moreover affecting the variations of these factors in several ways, with potential cascading effects for insect individuals, populations and communities. This thesis addresses how certain environmental abiotic factors can affect the physiological ecology of insects, with beetles as model insects. Firstly, I examined how geographic expansion could affect the thermal tolerance of invasive insects. For that purpose, different populations from the beetle Merizodus soledadinus invading a sub-Antarctic Island were used. This investigation illustrated a general pattern that most populations were best at coping with either cold or heat, and a few did not excel at recovering from either, compared to other populations of the species. This indicates that there are trade-offs between insects having high tolerance to hot or cold temperatures, and other un-investigated traits. Further, researches were conducted to elucidate tolerance levels and responses of insects to temperature and insecticide (cyfluthrin) stress, and investigate how thermal tolerance can be affected by pesticides. Different populations of the invasive pest beetle Alphitobius diaperinus, i.e. one laboratory-reared population and three populations collected from different farms were used. The investigations revelaed that all populations of A. diaperinus had a large thermal tolerance breadth.

However, farm collected populations had a wider temperature tolerance than the investigated laboratory reared population. It evidences how variable environments experienced by farm-collected populations can increase tolerance as compared with the individuals reared at constant temperature. In addition, pre-treatment with daily heat spikes decreased the recovery time of the insects to subsequent extreme heat exposure (but not cold), whereas pesticide exposure prior to temperature stress increased recovery times. This provided evidence of interactions between insecticide and heat tolerance that has not been shown in adult terrestrial insects before. Following stress exposure in the form of daily heat spikes, with or without insecticide exposure, no changes in the animals´ metabolome or antioxidant activity was reported. However, heat exposure resulted in increased numbers of offspring, while insecticide treatment had a tendency to reduce these numbers. In other words, the insects demonstrated a high tolerance to pesticide, but at a reproductive cost. Finally, tolerance and responses of A. diaperinus to dry conditions was tested. This study showed that the insects were highly desiccation resistant, but there were no changes in metabolite composition that could be associated with active upregulation of osmolytes in response to desiccating conditions. However, after a period in a very dry environment, the insects' reproductive output decreased, indicating that drought could have detrimental effects, even on populations of this desiccation resistant species.