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Individual differences in behaviour in social species – a multidisciplinary study with mice and ants.

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Individual differences in behaviour in social species – a multidisciplinary study with mice and ants.

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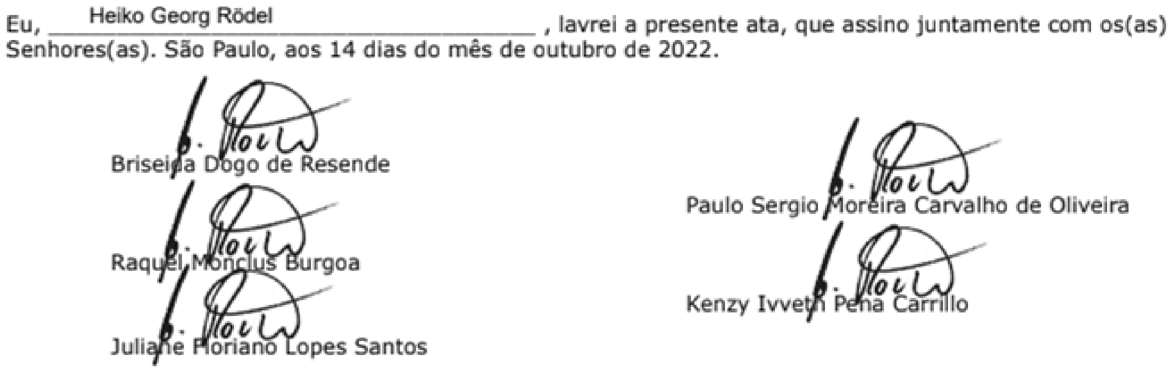

Eu, Heiko Georg Rödel, lavrei a presente ata, que assino juntamente com os(as) Senhores(as). São Paulo, aos 14 dias do mês de outubro de 2022.

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Raquel Monclus Burgoa

Juliane Floriano Lopes Santos

Paulo Sergio Moreira Carvalho de Oliveira

Kenzy Ivveth Pena Carrillo

"Don't let anyone rob you of your imagination, your creativity, or your curiosity. It is your place in the world; it is your life. Go on and do all you can with it and make it the life you want to live."

Mae Jemison

"What is research but a blind date with knowledge?"

Will Harvey

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DECLARATION OF AUTHORSHIP

I hereby declare that the thesis submitted, titled “Individual differences in behaviour in social species – a multidisciplinary study with mice and ants” is my own unaided work. All direct or indirect sources used are given as references. All contributions of co-authors are acknowledged.
This thesis has not been submitted to another university or institute for acquiring a higher degree.

Sao Paulo, Brazil, 25 August 2022

Veridiana A. Jardim

Veridiana Angeluzzi Jardim

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ABSTRACT

JARDIM, V. A. (2022) Individual behavioural differences in social species – a multidisciplinary study with mice and ants. (Doctorate thesis). Instituto de Psicologia, Universidade de São Paulo, São Paulo.

Interest in animal personality has been growing in recent decades, and with this we see the expansion of approaches, theories and methods in which animal personality is studied. More than that, what was previously an area very restricted to vertebrate species, now expands to many other taxonomic groups, such as invertebrates, adding even more to the discussion of the field. In this thesis I aimed to understand and integrate individual differences in behaviour in a theoretical and methodological approach, for species from two distinct taxonomic groups: a mouse species of wild origin, the mound building mouse (*Mus spicilegus)* and the ant *Holcoponera striatula*. I present in this thesis two main articles and one in the appendix, each article focused on different aspects, working in a complementary way discussing individual differences in behaviour. My work with mice is presented in two distinct articles. The first one (already published) focuses on the judgment bias test, a methodological tool that allowed us to integrate cognition and personality. The second one, presented in the appendix (already published), allowed a more physiological approach, connecting oxidative stress, ontogenesis, and personality. With ants, I seek to understand the inter-individual differences in behaviour in a multi-testing study over extensive repetition (article currently under review). In this thesis I show that, firstly, it is possible to find, discuss and integrate personality between vertebrates and invertebrate species. More specifically, I discuss the methodological limitations regarding the way we adapt, execute, and interpret behavioural tests, and how individual differences in behaviour influence these processes. Ontogenically, we also discuss how early parameters interact with personality resulting in different levels of oxidative stress expressed in the adult life. Despite the great differences and peculiarities in the way species perceive, process, and respond to their environment, and the various ways in this phenomenon can be studied, the future of personality research lies in the collaboration of the various fronts, approaches and species, adding and integrating knowledge for a more robust discussion of animal personality.

Keywords: Personality, Behavioural syndrome, *Mus spicilegus, Holcoponera striatula,*

RESUMO

O interesse pela personalidade animal vem crescendo nas últimas décadas, e com isso vemos a expansão das abordagens, teorias e métodos nos quais a personalidade animal é estudada. Mais do que isso, o que antes era uma área muito restrita às espécies de vertebrados, agora se expande para muitos outros grupos taxonômicos, como os invertebrados, agregando ainda mais à discussão do campo. Nesta tese pretendo compreender e integrar diferenças individuais de comportamento em uma abordagem teórica e metodológica, para espécies de dois grupos taxonômicos distintos: uma espécie de camundongo de origem selvagem (*Mus spicilegus*) e as formiga *Holcoponera striatula*. Apresento nesta tese dois artigos principais e um em apêndice, cada artigo focado em diferentes aspectos, trabalhando de forma complementar as diferenças individuais de comportamento. Meu trabalho com camundongos foi apresentado em dois artigos distintos. O primeiro (já publicado) teve como foco o teste de viés de julgamento, ferramenta metodológica que nos permitiu integrar cognição e personalidade. A segunda, apresentada no apêndice (já publicada), me permitiu uma abordagem mais fisiológica, conectando estresse oxidativo, ontogênese e personalidade. Com formigas, procuro entender as diferenças interindividuais de comportamento em um estudo utilizando multi-testes, repetindo-os extensamente (artigo atualmente em revisão). Nesta tese mostro que, em primeiro lugar, é possível encontrar, discutir e integrar a personalidade entre espécies de vertebrados e invertebrados. Mais especificamente, discuto as limitações metodológicas em relação à maneira como adaptamos, executamos e interpretamos os testes comportamentais e como as diferenças de comportamento dos indivíduos influenciam esses processos. Ontogenicamente, também discuto como os parâmetros experenciados no início da vida interagem com a personalidade resultando em diferentes níveis de estresse oxidativo expressos na vida adulta. Apesar das grandes diferenças e peculiaridades na forma como as espécies percebem, processam e respondem ao seu ambiente, e as várias maneiras pelas quais podemos estudar esse fenômeno, o futuro da pesquisa da personalidade está na colaboração das várias frentes, abordagens e espécies, agregando e integrando conhecimento para uma discussão mais robusta da personalidade animal.

Palavras-chave: Personalidade, Síndrome comportamental, *Mus spicilegus*, *Holcoponera striatula*

RESUME

L'intérêt porté à la personnalité animale par la communauté scientifique a augmenté au cours des dernières décennies, et avec cela, nous voyons l'expansion des approches, des théories et des méthodes pour l'étude de la personnalité animale. Plus que cela, ce qui était auparavant une zone très restreinte aux espèces de vertébrés, s'étend maintenant à de nombreux autres groupes taxonomiques, tels que les invertébrés, ajoutant encore plus à la discussion sur le terrain. Dans cette thèse j'ai eu pour objectif de comprendre et d'intégrer les différences individuelles de comportement dans une approche théorique et méthodologique, pour des espèces appartenant à deux groupes taxonomiques distincts : une espèce de souris d'origine sauvage, la souris monticuleuse (Mus spicilegus) et la fourmi Holcoponera striatula. Je présente dans cette thèse deux articles principaux et un en annexe, chaque article portant sur des aspects différents, travaillant de manière complémentaire sur les différences individuelles de comportement. Mon travail sur les souris est présenté dans deux articles distincts. Le premier (déjà publié) porte sur le test de biais de jugement, un outil méthodologique qui nous a permis d'intégrer cognition et personnalité. La seconde, présentée en annexe (déjà publiée), a permis une approche plus physiologique, reliant stress oxydatif, ontogénèse et personnalité. Avec les fourmis, je cherche à comprendre les différences interindividuelles de comportement dans une étude multi-tests sur répétition extensive (article en cours de révision). Dans cette thèse, je montre que, premièrement, il est possible de trouver, de discuter et d'intégrer la personnalité entre les vertébrés et les espèces d'invertébrés. Plus précisément, je discute des limites méthodologiques concernant la façon dont nous adaptons, exécutons et interprétons les tests comportementaux, et comment les différences individuelles de comportement influencent ces processus. Sur le plan ontogénique, nous discutons également de la manière dont les paramètres précoces interagissent avec la personnalité, entraînant différents niveaux de stress oxydatif exprimés dans la vie adulte. Malgré les grandes différences et les particularités dans la façon dont les espèces perçoivent, traitent et réagissent à leur environnement, et les différentes façons dont ce phénomène peut être étudié, l'avenir de la recherche sur la personnalité réside dans la collaboration des différents fronts, approches et espèces, ajoutant et l'intégration des connaissances pour une discussion plus solide de la personnalité animale.

Mots-clés: Personnalité, Syndrome comportemental, *Mus spicilegus*, *Holcoponera striatula*

TABLE OF CONTENT

CHAPTER 1: GENERAL INTRODUCTION

1.1 THESIS PRESENTATION

This thesis work aimed to investigate individual behavioural differences in social species. In its different models and specificities, I aimed to contribute to the understanding and integration of personality and cognition in a behavioural and physiological approach, contemplating theoretical and methodological discussions.

This doctoral thesis has undergone many changes throughout its completion, whether due to restrictions imposed by the COVID-19 pandemic, lack of time or methodological limitations. The initial idea, that finally was not carried out, was to work with transgenerational and maternal effects of social stress in personality, investigating how stressful situations experienced by mothers could pass along generations, potentially modifying personality traits of their brood. Our study experimentation was going to be carried out first in a species of mice of wild origin (which was going to be carried out in France), and after with a species of ants (that was going to be carried out in Brazil). The idea of working with such distinct groups was not by chance. It is undeniable that the choice was a to some extent based on the convenience that the two laboratories where the work was carried out already had the methodological facilities and experience in working with these specific groups, but most importantly, the model species and their characteristics allowed me to answer greater theoretical questions. Although the study of stress and transgenerational effects was not carried out, the studied species remained throughout the project.

The mouse species we chose (*Mus spicilegus*) could allow to interpret our results in a broader and comparative context, since species from the genus *Mus* (i.e. *Mus musculus musculus and Mus musculus domesticus)* are a standard species model when working with stress, cognition, and personality. Also, vertebrates' proximate pathways for behavioural expression are usually better understood than the ones in invertebrates, facilitating theoretical discussions. *Mus spicilegus* is social group-living species (Garza et al., 1997), as many species of the genus. In a similar way, ants (*Holcoponera striatula*) also life in social group-living, but in the extreme that entitles eusociality. Also, ants could provide us with a new approach when dealing with more complex social groups. Being a eusocial species, ants could allow us to research personality not only at the individual level, but at the group level as well.

Modifications of the original plan began to take place when, in 2020, with the limitations imposed by COVID-19, the transgenerational effects experiments in mice were not possible to be carried out. Due to the lack of time, we had to rethink our experimentation and we chose to carry out two distinct but complementary studies. With the males of the litter, we carried out a more methodological experiment that would question some models being used in judgment bias tests (article in chapter 2), discussing the personality and cognitive biases and their consequences in the interpretation of the test results. With the females of the litter, we carried out a study that would integrate personality and oxidative stress (article in the appendix), in line with the effects of stress and its metabolic pathways.

Meanwhile, in Brazil, we had thought out a similar test design to the one that we initially had planned to carry out in France on rodents, a study of transgenerational effects of social stress in personality of *H. striatula* queens. A study pilot was being carried out by an undergraduate student at the time, but due to the pandemic the experimentation had to be shut down. When I returned to Brazil, the need for a new experiment design took place, since at that time I had less time that I initially thought I would have, with the undergoing pandemic, to access to the lab and no pilot data to base any of my experiment on. On top of this, the transgenerational and maternal effect experiment with mice was not carried out, making it difficult to sustain a comparative study without the ant study counterpart. With that we went back to the clipboard and rethought all the experiments with ants so they could be carried out in a shorter period, during the undergoing COVID-19 pandemic, with limited access to the university and in accordance with was previously done on the mound-building mouse so we could keep a coherent thesis. The only possible solution that would allow me to continue with experimentation was to carry out the housing and experimentation with ants at my home, but with the imposition of a smaller scale. With the limited space, time, and material to carry out a more complex experimentation, unfortunately the transgenerational effect with ants also was impossible to be pursued. Alternatively, keeping in line with the previous work on mice, we carried out a multi personality essay, with ants undergoing several personality tests throughout extensive repetitions (article in chapter 3).

Considering what was originally thought for this thesis, and after all the modifications to how finally this thesis was carried out, I (together with my supervisors) decided to organize the chapters within the framework of the studied species. In this chapter I also chose to separate the theoretical introductions into two subtopics. Considering what was suggested in my qualification, I believe that considering the slightly divergent pathway which this thesis followed, to force the topics into a single introduction would be to miss the theoretical details of the different subjects dealt within each chapter. At the end of it, each article focused on different aspects and worked in a complementary way discussing individual differences in behaviour. Thus, I decided to present chronologically and topic oriented, the experiment of judgment bias test in mound building mice (*Mus spicilegus*) carried out in France in the second chapter and thereafter, in chapter 3, the experiments on the ant species *Holcoponera striatula* carried out in Brazil. To keep a more coherent structure throughout the thesis, I kept the oxidative stress paper on the appendix, but still presenting the topic in the general discussion of chapter 4. The oxidative stress article was a further extension of the general topic of my thesis, also carried out in the laboratory in France, soon after the end of the project on judgment bias testing. I am a co-author on this paper which discusses the effects of litter size, relative differences in body mass between pups and personality on oxidative status parameters during later life of *Mus spicilegus*.

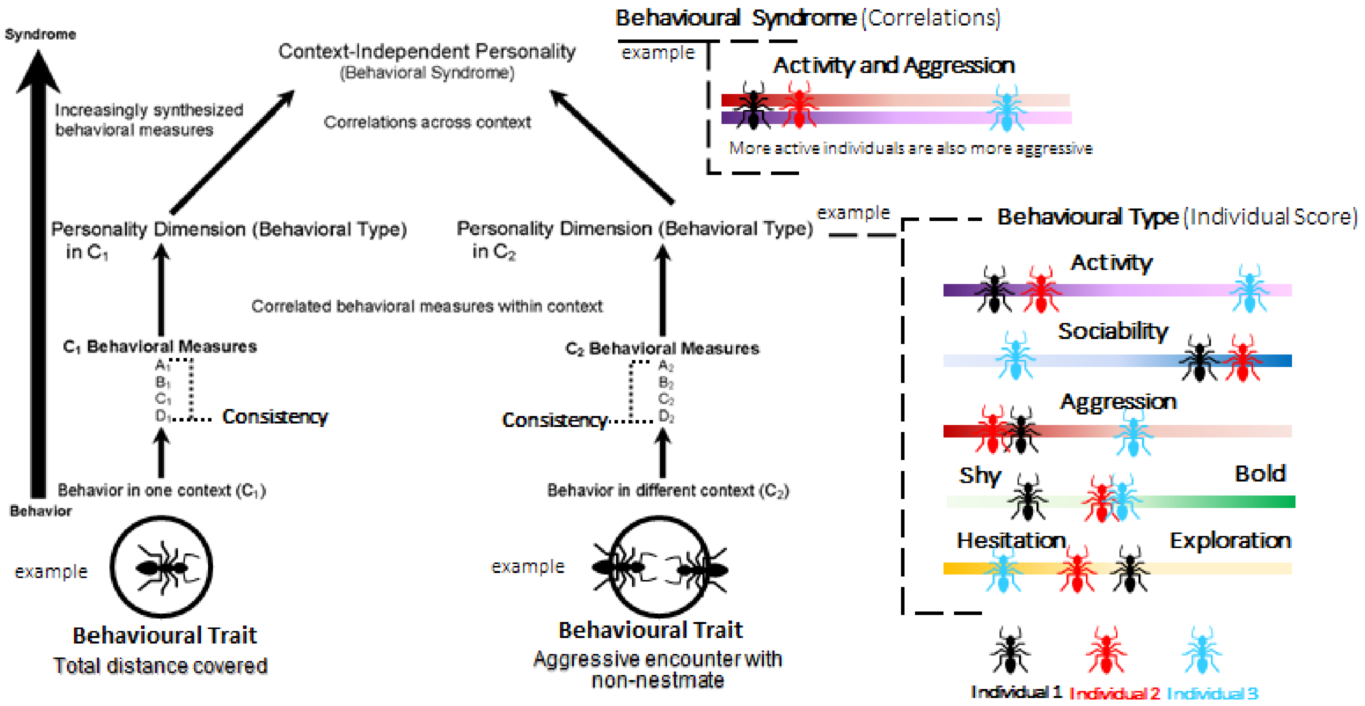
When conceiving the new projects, either on mice or ants, we focussed on the pillars issues when working with individual differences in behaviour. Both articles that are presented in chapter 2 and 3 discuss methodological and theoretical limitations on working with this theme, regardless of the studied species.

Great difficulties have been overcome throughout the thesis in the last two years. Unfortunately, not only in the scientific aspect, but specially in the lives of many people who have been directly and indirectly affected by undergoing pandemic. Despite the mishaps and overturns, I was able, supported by my supervisors, to change and adapt our protocols and methods without sacrificing the goals and questions that led me to start this doctorate in the first place. The theoretical fronts that were developed, perhaps not as well integrated as initially planned, are still guided by the same theoretical thread. I hope that my thesis can contribute to the various fields in which it operates, whether contributing to more thorough and accurate methods investigating cognition and personality in animal species or elucidating the role of early life parameters and personality on the individual's adult life.

1.2 TERMINOLOGY OF ANIMAL PERSONALITY

Throughout this thesis, some terminology referring to the study of animal personality is recurrently used. Thus, it is essential to define upfront some of the concepts applied here. Different terms are used today to describe individual variation in behaviour, such as personality traits, personality types, temperament, behavioural syndrome, coping styles, and many others (Koolhaas et al., 1999, Réale et al. 2007, Sih et al., 2004). Over the past decade, the lack of consistency in the terminology across this field has sparked a vigorous debate, not only in behavioural ecology, but also in other related disciplines (Réale et al. 2007). Smith & Blumstein (2008) proposed a hierarchical representation of behavioural measures that facilitates the comprehension of the levels of complexity involved in the study of observable behaviour, and this work will guide our further discussion of the topic (Figure 1).

Figure 1. The hierarchical representation of behavioural measures



Source: Modified from Smith & Blumstein (2008). Legend: Observable behaviours (A, B, C, D) are measured in 2 different contexts (C1 and C2). Behavioural traits exemplified by total distance covered and aggressive encounters with a non-nestmate. Correlated behavioural measurements within a single context represent a personality dimension or behavioural type (consistency over A_1, B_1, C_1, D_1 and A_2, B2, C_2, D_2). Individual behavioural types are exemplified by each individual ant over the behavioural axis. The 3 coloured individuals (black, red, and light blue) are displayed as scores on top of the 5 behavioural axes suggested by Réale et al. (2008). Scores are merely illustrative, representing possible results for other behavioural traits in different contexts. Correlations across contexts represent context-independent personality or a behavioural syndrome. Here exemplified by the correlation between Activity and Aggression, where more active individuals also have displayed more aggression.

A behavioural trait is defined as the first measurable aspect of an individual's personality. John & Gosling (2000) published one of the first papers to use the term "personality trait" in animal personality studies, and the authors defined it as the consistent patterns in the way individuals behave, feel, and think. Later studies maintained a similar concept, but applied it in a more methodologically objective framework, defining it as a specific aspect of a behavioural repertoire that can be quantified and that shows between-individual variation and within-individual consistency (Carter et al., 2013).

Applying the concepts in a more methodological approach, Réale et al. (2007) recommended using a 'simplified terminology' for the study of animal behavioural traits that has been widely adopted within animal personality research. The authors proposed five major continuum axes, into which behaviour traits can be categorized: boldness-shyness (reaction to any risky situation, e.g., predators in a non-novel situation), exploration-avoidance (reaction to a new situation, e.g., environment, food, or object), activity (the level of activity in a non-novel environment), Aggressiveness (aggressive reaction to a conspecific) and sociability (reaction of an animal to the presence or absence of a conspecific). The score in which a set of behavioural measures are expressed in each of these continuum axes is defined as behaviour types, for example, a more aggressive or a less active individual (Sih et al., 2004). It is important to note that the measured behavioural trait is expressed within a specific context and can only be defined as a behavioural type when behavioural measures are consistent within this specific context (Reále & Dingemanse, 2010). Also, when referring to individual behaviour types, it is important to note that this score is always given in relation to other individuals inside that population or sampled group (Groothuis & Trillmich, 2011). This means that when referring to a "more exploratory individual", this statement expresses that the exploration tendency of this particular individual is high in comparison to other individuals of this study group.

Behavioural types can correlate across axes in the studied population. That is, for example, individuals who are bolder are frequently also more aggressive or more exploratory (Evans et al., 2010, Rödel & Monclús, 2011). In fact, it is not uncommon to find recent studies that tested these behavioural correlations, many finding such associations (e.g., Bourne & Sammons, 2008, Chapman et al., 2011, Kortet & Hedrick, 2007, and the article on this chapter), but many others not (e.g., Way et al., 2016, and the article on chapter 3). Sih et al. (2004) define behavioural syndrome as a suite of correlated behaviours across multiple contexts, and in other study areas (such as animal welfare or stress physiology, Koolhaas et al., 1999, Blokhuis et al., 2000) we also find the term copying style. Behavioural syndrome can also be used to describe behavioural processes at different levels of organization, for instance, a population or species can exhibit a behavioural syndrome with each individual showing a behavioural type (e.g., bolder or shyer). Alternatively, a group of species can exhibit a syndrome with each species having a certain behavioural type (e.g. species that are bolder versus those that are shyer) (Réale et al., 2010; Stamps & Groothuis, 2010).

A choice that I made throughout this thesis was to use the term "personality" more consciously, on several occasions opting for the term "individual differences in behaviour". This choice was made since not every consistent individual difference in behaviour can be labelled as personality. As we defined earlier, to designate a group of behaviours as personality (even trait, type, or syndrome), it is required for the behaviour in question to fulfil a series of premises, such as temporal or contextual consistency. If in the study in question, for example, consistent individual differences are not found, one cannot use the term personality to describe it. In the same way, if the study failed to find correlations between consistent behaviours expressed by individuals in different contexts, we cannot describe behavioural syndromes. In the title of the thesis, for example, I opted for the broader definition, since in many parts of the thesis (especially in the article on ants in chapter 3, where we did not find behavioural consistency for many of the measured behaviours and no behavioural correlations across contexts) we will discuss various aspects of individual behaviour that do not necessarily fulfil the premises to define them as personality. Despite this, it is important to highlight that the term “personality” is still used on several occasions throughout the text, often to represent broader or untested theoretical ideas.

1.3 JUDGMENT BIAS AND PERSONALITY

Over the last few decades there has been an increasingly interest to understand how animals “feel” (Boissy et al., 2007, Whittaker & Marsh, 2019). There is still a great debate surrounding animals’ affective states, whether investigating what they consist of (Boissy et al., 2007), how to correctly access it (Ede et al., 2019) or all together the assumption that animals’ affective states are considered emotions. Many scientists are still refraining from naming animal affective states as 'emotions', as it still carries a great anthropomorphic connotation (Jirkof et al., 2019), and thus in this chapter I will refrain to use this formulation and instead apply a broader term. Affective states refer to the animal internal state in relation to subjective perceptions of internal or external stimuli, and are defined as multifaceted phenomena with neuronal, physiological, behavioural, cognitive, and subjective aspects. They can have different levels of arousal and either positive or negative valence (Desire et al., 2002, Jirkof et al., 2019, Spinka, 2012). This individual internal state will be a fundamental topic for the study of judgment bias.

In many studies “judgment bias” is often used as a synonym for “cognitive bias”, and although closely related, these terms define distinct processes. Here I will use the definitions presented in the glossary of the study of Bethell (2015), a “how-to” guide for designing judgement bias tests. Cognitive bias is often used in the field of animal welfare and cognition, and it describes the influence of affective states on cognition. More understood in humans, emotion–cognition interaction feeds into the conscious experience of feelings that underpin psychological wellbeing (Bethell, 2015). On the other hand, judgment bias is defined as the influence of affective states on the interpretation of, and response to an ambiguous stimulus. This response can vary greatly between individuals, since it is linked to processes unique to the ontogeny, reflecting on how each individual perceives, processes, and responds to an external stimulus. Furthermore, these states can be heavily affected by previous experiences of the individual (Ede et al., 2019, Walker et al., 2012). For example, humans that are in a negative affective state reveal a better memory of negative events, tend to focus their attention on the occurrence of negative events, and interpret ambiguous stimuli more negatively (negative judgement or interpretation bias) (Chan & Lovibond, 1996, Telzer et al., 2008,). The model presumes that positive affective states are reflected in more positive judgments about ambiguous stimuli (“glass half full”), whereas negative affective states are reflected in more negative judgments about ambiguous stimuli ("glass half empty"). Lastly, the judgement bias test (more details in the following article) is a task in which an individual, firstly, is trained in an associative learning task, to associate a cue with a reward. Individuals are thought to discriminate between two stimuli that differ along one dimension (e.g., size, colour, and location). One stimulus is rewarded, and the other cue is not rewarded or less rewarded. After learning to discriminate between the cues, during a judgement bias task, an ambiguous cue is presented (e.g., an intermediate location). Parameters such as speed, latency, frequency, or even go/no go responses, reflect whether the animal judges the intermediate cue to be more positive or negative (Bethell, 2015, Roelofs et al., 2016).

Like many of the methods used to study animal behaviour, the judgement bias test is a methodological tool based on and adapted from human studies (Schraw & Roedel, 1994). There is an extensive literature (Haselton et al., 2015, Kruglanski & Ajzen, 1983), showing that judgement bias tests were an imperative tool to understand how internal emotional states affect an individuals' choices by affecting its cognitive processes (Mathews & MacLeod, 1994). In a search to further understand how similar cognitive and affective states processes were structured in other species, it was hypothesized that similar biases could be measured in non-human animals (Paul et al., 2005).

In 2004, the first study to explore judgement bias in animals was made by Harding, Paul, and Mendl, proposing a new nonverbal method of accessing non-human animals' affective states. The judgement bias experimental paradigm formed the basis for most subsequent studies in the field (Mendl et al. 2009). Based on this proposition, judgement biases have been measured in a range of different laboratory animals (mice; genus *Mus*, as reviewed by Mendl et al., 2009, rats, genus *Rattus*, Barker et al, 2016, Brydges & Hall, 2017), domestic species (livestock review: Baciadonna & McElligott, 2015, sheep *Ovis aries*: Doyle et al., 2010, cattle *Bos taurus*: Daros et al., 2014, horses *Equus ferus caballus*: Löckener et al., 2016, domestic dogs *Canis lupus familiaris*: Burman, 2014, domestic chicken *Gallus domesticus*: Hymel & Sufka, 2012) and wild species (invertebrates such as ants *Camponotus aethiops*, d'Ettorre et al., 2017, honeybees *Apis mellifera*, Schluns et al., 2017, and different birds and mammals such as common starlings *Sturnus vulgaris*, Gott et al., 2019 and bottlenose dolphins, *Tursiops truncatus*, Clegg et al., 2017). By presenting a simplified approach that allows to widely explore several cognitive bias measures, and interpretations about the affective states of individuals, the judgement bias test became one of the most reliable methods to study animal welfare (see also Bethell et al., 2012; Burman et al., 2008).

In addition to affective states, other variables might influence judgement bias. As I mentioned previously, the process by which individuals perceive, process, and respond to the environment differs. Theoretical models suggest that between-individual state differences lead to consistent behavioural differences across time and contexts because animals adopt different behavioural strategies (Dingemanse & Wolf, 2010; Nettle, 2006). State differences can be morphological, physiological, or neurobiological (Dingemanse & Wolf, 2010). These consistent behavioural differences across time and contexts are precisely the definition of personality (Réale et al, 2007). These ideas suggest that personality is likely a key element in the decision-making processes (Barnard et al., 2018), and therefore also a key element when discussing judgement bias. Considering that individuals of different personalities also differ in the way they perceive, process, and respond to environmental cues, it should be also taken into account that individuals may be differentially influenced by the parameters of the test itself. In this sense, we must consider the plurality of possible effects of the test execution and the differential individual responses.

In the study of animal behaviour, when designing and running a behavioural test, or choosing behavioural variables, and many other factors underling the behavioural research, one must always consider and choose ways to minimize the most the number of confounding variables in an experimental setting (Bateson, 2016). A topic that ends up being neglected in behavioural studies, especially those that rely on assembled apparatus and laboratory tests, is that the test itself may not be as innocuous for the individuals as previously anticipated, possibly adding great result variation. For example, light intensity or condition of animal handling prior to the test can affect behaviour in an open field (Walsh &Cummins, 1976). Furthermore, it has been shown that complex objects can elicit more exploration than less complex objects (Mettke-Hoffmann et al., 2006).

Koolhaas et al. (1999) in their pioneering study in the field of animal personality, already discussed the idea that individuals differ, not only in the expression of their behavior, but also in the way they observe and process elements of their environment. In this work, for example, they describe proactive and reactive coping styles, in which proactive individuals would be less aware of environmental cues, being more prone to routine formation, while reactive individuals seemed to be more adaptive and flexible, as they are more aware of their environment. Processes associated with running tests, such as manipulation, handling, exposure to new locations, objects and environmental stimuli to which individuals are not used to, can be perceived as a notable stressor (Batson, 2016, Squibb et al., 2018). Other works have already shown and discussed that the event of testing can generate behavioural and physiological stress responses, especially when the animals have not been previously habituated to the environment or handling (Visser et al., 2002, Martin & Réale, 2008, Squibb et al., 2018). Considering this, an animal may therefore show different responses to a test setting, simply because of the specific environmental conditions prior to or during the test, and not solely based on the behaviour the test originally was designed to measure (Henderson, 1990).

There is still an ongoing discussion in the field about how to deflect this novelty effect issue. On the one hand, a possible way to circumvent this is to repeat the test several times with each individual and to estimate interindividual variation and behavioural individual consistency (Henderson, 1990, Dingemanse et al., 2002). Others have simply habituated the individuals to the test settings and/or to the handling procedure prior to the test itself (Richter et al., 2012, Burman & Mendl, 2018;). In any way, all ideas revolve around reducing the behaviour variation linked to the stress the animals perceive when performing the test. A further issue is that it must be consider that individuals may differ, not only in their original response to novelty, but also in the speed with which they habituate to it (Martin & Réale, 2008). Despite the potentially confounding effects of environmental variation and habituation in their reaction to novel stimuli, only few studies have yet tackled the issue regarding the methodological bias in behavioural studies. In this sense, I hope that the articles and discussions presented in my thesis will contribute to the improvement of methods related to the study of animal personality.

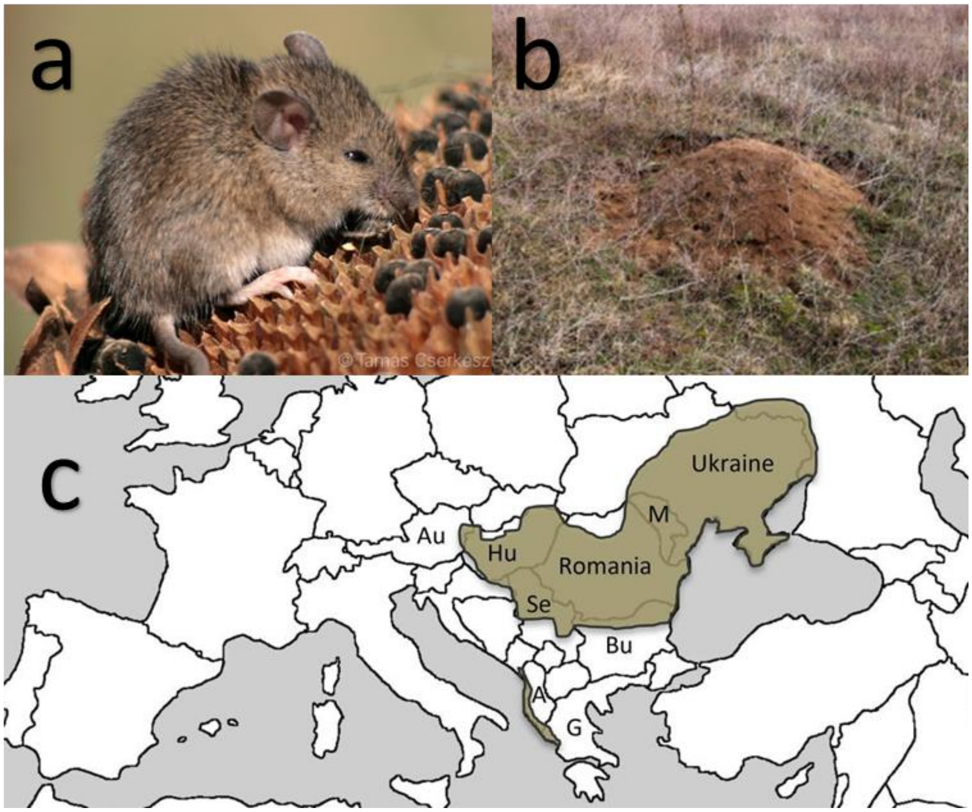
1.3.1 THE MOUND BUILDING-MOUSE

The mound-building mouse (*Mus spicilegus*) is a rather nondescript, brown mouse that looks just like its close relative, the commensal house mouse (*Mus musculus*), and in many localities being sympatric to this species (Tong & Hoekstra, 2012). They are distributed across eastern and central European grasslands (Figure 2), hence they are also known as ‘steppe mouse’ and are currently listed as least concern by the IUCN Red List of Threatened Species (Coroiu et al., 2016).

A distinctive character of this species is its group grain-hoarding activity. Unlike the house mouse, the mound-building mouse lives outdoors, and constructs its own overwintering structures (Szenczi et al., 2011) Mound-building mice pile up soil and up to 50 litres of vegetation, which when completed are about 40,000 times the volume of a single mouse (Tong & Hoekstra, 2012). Construction of mounds occurs as a collective, from mid-August to mid-November with peak construction in mid- to late-September (Unterholzner et al., 2000). These complexes mounds are built in late summer/early autumn, and are essential to encompass winter (Muntyanu, 1990). Adults do not live on through the winter and because that there are two distinct cohorts of reproducing animals (Ponteaux et al., 2008): 'long life' animals, born the previous year, and which experienced long winter cohabitation within mounds, and 'short life' individuals, born from first spring reproduction, and which reproduce in summer. Juveniles are born in spring/summer and spend the cold period in nest chambers within the mounds, in large groups often encompassing several litters (Gouat et al., 2003). In spring, now aged at least 6 months, individuals leave the mound and disperse (Milishnikov et al, 1998).

Mus spicilegus is not a cooperative breeder and females are highly aggressive towards each other (Tong & Hoekstra, 2012). They are socially monogamous, with strong mating pair-bonds and significant paternal care, with stable pair bond between partners demonstrated both in the laboratory and in semi-natural enclosures (Patris & Baudoin, 1998). Curiously, *Mus spicilegus* also have the largest testicles relative to body mass of any *Mus* species, and as large testicle size often is an indicator of high sperm competition, these mice are probably not strictly monogamous as previously thought (Gomendio et al., 2006). The described peculiarities of *Mus spicilegus* make it an interesting object of study (also considering the divergences from other species of the same genus), but still maintaining the characteristics that make species of the genus *Mus* excellent to work with and test in the laboratory.

Figure 2. The *Mus spicilegus* mouse and its geographic distribution



Source: (a) Tamás Cserkész (Nov, 2022) (b) Szenczi et al. (2012) (c) Coroiu et al. (Nov, 2022), Modified from Smith & Blumstein (2008). Legend: (a) Picture of a mound of mound-building mouse, (b) the mounts, overwintering structures made up from pile up soil and vegetation, and (c) The geographic distribution of the mound-building mouse .

1.4 PERSONALITY STUDIES OF EUSOCIAL INVERTEBRATES

The study of personality in invertebrates emerged relatively recently within the field of animal personality, which was always much more restricted to Vertebrates, more specifically to the vertebrate group (Gosling, 2001). But this scenario has been changing, especially with the increasingly number of publications on the field that highlights and describes, for example, complex processes of affective states, cognition, task execution and complex learning, and many others in several invertebrate species. A few recent studies, for example, described affective pain in octopus (*Octopus bocki*, Crook, 2021), visual and colour learning in bumblebees (*Bombus terrestris*, Wolf & Chittka, 2016), reviewed cognitive processes in invertebrates through attention gaze (Winsor et al., 2021), or as a whole tried keeping invertebrate research ethical in a landscape of shifting public opinion (Drinkwater et al., 2019).

Although the study of invertebrate personality is still very much focused on the class Insecta, a review of Kralj-Fišer and Schuett (2014) points out that inter-individual differences in behaviour that would fit the concept of personality, have already been described for more than 19 invertebrate genera with the majority of these (15 genera) within Arthropoda. After the publication of this particular review, more invertebrate genera were described as displaying personality (e.g. crickets from the genus *Teleogryllus*, Rudin et al., 2017, crabs from the genus *Carcinus,* Fürtbauer, 2015, spiders from the genus *Portia,* Chang et al., 2018, and the genus *Latrodectus,* Golobinek et al., 2021, butterflies from the genus *Pararge,* Kaiser, 2020, beetles from the genus *Nebria*, Labaude et al., 2018, snails from the genus *Cornu*, Dahirel et al., 2017, isopods from the genus *Porcellio,* Golobinek et al., 2021, and others).

There are many interesting and compelling aspects in working with invertebrates in the study of animal personality. These aspects will also be discussed in the following article in this chapter. In conjunction with what is already known about personality in vertebrates, understanding how this phenomenon is expressed in invertebrates would allow comparative studies of numerous personality aspects (Kralj-Fišer, & Schuett, 2014), from the importance of genetics, developmental processes, to social interactions and many more. This comparison offers new avenues in personality research, especially because invertebrates exhibit a range of aspects regarding their life histories, such as various social and sexual behaviours that are extremely rare or absent in vertebrates (Jandt & Gordon, 2016, Jeanson, 2019, Jeanson et al., 2012, Kralj-Fišer, & Schuett, 2014). For example, clonal species or species with great genetic similarity, like eusocial species such as ants and bees, can further elucidate the role of genetics and ontogenesis in the expression of consistent individual differences in behaviour (Bernadou et al., 2018, Liedtke et al., 2015). Another developmental and ontogenetic example that still not quite yet understood is the effect of metamorphosis from larval to adult stages, an aspect that could add to the understanding of consistency of behavioural traits throughout the life stages of the individual (Mather & Logue, 2013). Studies on this matter showed that, while consistency in personality traits has been proven for several insect species (see review from Amat et al., 2018), whether or not individual personality traits correlate between adult and juvenile life stages depends strongly on the metamorphosis degree that individuals go through. For holometabolous species (complete metamorphosis, involving a complete reorganization of the organism), consistency of personality traits throughout the individual's life seems to be lower than heterometabolous insect species (‘incomplete’ metamorphosis, successive molts until adult) (Amat et al., 2008). These findings evoke questions about how the expression of personality relies in a complex interaction between physiological/ neuronal mechanisms (individual neuronal restructuring can be one of the causes of lack of personality consistency from larva to adult, Consoulas et al., 2000) and external factors experience throughout individual ontogeny (niche differentiation from larvae to adults in holometabolous species can also modulates individual personality, Sih & Bell, 2004). This example highlights how investigating specific aspects of invertebrates’ species can help us understand from how individual differences in behaviour are constituted and expressed in the individual, to the existence and maintenance of personality variation throughout evolutionary time (Kralj-Fišer, & Schuett, 2014).

In addition to such specific aspects of each group, there are numerous methodological practicalities when working with invertebrates (Mather & Logue, 2013). Many invertebrates are easy to rear, maintain and manipulate, including various species that can be obtained in large numbers from urban or easy to access areas, or even specialized stores (Kralj-Fišer, & Schuett, 2014). Besides, many invertebrates have fast life cycles in comparison to vertebrates, with typically high reproductive output (Kralj-Fiser & Schneider, 2012, Kralj-Fišer & Schuett, 2014, Schuett et al., 2011), which allow us to study aspects such as transgenerational effects or longitudinal comparisons that encompass the different life stages of the animal. The study of eusocial species can also be very enriching for us to understand many aspects of how highly social groups interact with individual aspects of personality (Gartland et al., 2022).

The study of personality is typically linked to the individual level. However, to the extent that cooperative groups differ from one another in their collective behaviours, some authors defend the idea that these groups can also be thought of having collective behavioural types (Japyassú et al., 2021, Pinter-Wollman., 2012). In eusocial insects, for example, some studies assume that, besides individual level, differences in behavioural types can be observed at the species level (e.g. more aggressive species outcompete and displace subordinate species; Davidson, 1998, Suarez & Holway, 1999), at the colony level (e.g. number of ant workers outside the colony, high and low; Pinter-Wollman, 2012) and caste levels (e.g. ant soldiers are more aggressive than other workers; Jandt et al., 2014). Although being a common and widespread idea in the field, the use of the concept of personality on a level higher than the individual generates questioning; can we state that any consistent difference in behaviour patterns means personality?

From an evolutionary perspective, eusocial colonies differ from other animals living in social groups because they function as a superorganism (Hölldobler & Wilson, 2009), meaning that natural selection can act both at the level of the individual and at the level of the colony. Some authors defending the idea that more so in the latest (Pinter-Wollman, 2012).

Although we use the term personality in eusocial taxa for both the individual and the group level (for colonies, for example), it is important to note that the way in which personality is structured is clearly different in each case. When looking at the group level, for example when discussing colony level personality, it is referring to the consistent individual differences among colonies in how they behave across different situations. This variation is not in how much of each task a colony performs but in how a colony performs and regulates these tasks compared to others (Pinter-Wollman, 2012).

The behaviour of social groups is influenced by the personalities of the individuals comprising them (Crosland, 1990, Pinter-Wollman, 2012, Watters & Sih, 2005). In the same way, in eusocial species, colony personality emerges from the aggregation of the workers' personalities that comprise it. There are several non-exclusive theories that explain how individual personalities make up group-level personality, and in their work, Pinter-Wollman (2012) describes a few of these: Firstly, colonies may vary in their average worker personality (different average, similar distribution), meaning that colonies do not vary in the distribution of worker personalities, but in the mean worker personality within each colony. For example, a colony composed mostly of active workers might respond faster to changes in its environment than a colony composed of workers that are on average less active. Secondly, colonies may vary in worker personality distribution (similar average, different variance), meaning that distribution of worker personalities varies, but not the average of their worker personality. For example, colonies show overlapping ranges and average worker personalities, but they vary in the frequency of individuals, or representation, resulting in different colony level personalities. Lastly, colony personality may not emerge from worker personality but from variation in local environment (external effects), meaning that colonies do not vary in the average or in the distribution of the worker personalities comprising them, but from variation from local environment. For example, if one colony entrance is placed in a more shaded area than another colony entrance, it is possible that variation of luminosity and temperature influences the activity of foragers, resulting in one nest being considered more "active" than the other (Pinter-Wollman, 2012). This example also raises the question that if every observable difference in behaviour can be considered a difference between personality traits. It is important to emphasize that although the examples given refer to the colonial level, the theory broadly refers to different levels of social groups, in this way, the same logic can be applied, for example, to division of labour, referring to a group of individuals from the same caste or even between castes.

We can find studies that discuss collective behaviour at many hierarchical levels, such as at the level of the colony, sub-colony, caste etc (e.g. Gandra et al., 2021, Kolay et al., 2020, and the following article). Studies of individual variation in behaviour among social insect workers often focus on difference between individuals that perform only one task, also called specialists, and those that perform many tasks, called elites (Robson & Traniello, 1999).

There is still great debate on what determines how each individual worker behaves and varies among each order (Pinter-Wollman, 2012), and more so when considering these processes at a greater scale, such as at the colony level. Some mechanisms are already better understood, even more so because many of these processes are not restricted to invertebrates, or social insects, but are general processes that have also been explored in many other taxonomic groups (Bengston & Jandt, 2014, Stamps & Groothuis, 2010). Differences in the way individuals behave come from a synergic and complex interaction that includes genetic, physiological, and developmental processes (Stamps & Groothuis, 2010), and many reviews and studies discuss these aspects more specifically for eusocial insects (see Chapman et al., 2011, Horna-Lowell et al., 2021, Jandt et al., 2014, Jandt & Gordon, 2016, Pinter-Wollman, 2012). These studies highlight the importance of various factors, such as whether the colony has one or multiple queens, with how many males the queens reproduced, how the colony is structured, the size and worker movement in the colony, and culminating in aspects of genetic closeness between workers. With respect to development, further aspects may be considered, such as polymorphism among workers, or age polyethism, the quality and quantity of food the larvae are fed, in which environmental conditions they develop, previous individual experience, distribution and type of workers, among many others.

As I mentioned previously, ants provide a unique system in which to study the feedback between causes and consequences of personality (Horna-Lowell et al., 2021), especially because they allow us not only to explore the individual, but also understand the mechanisms in which individuals make up, affect, and are affected by social groups. I believe that future works on ants and the pluralities of approaches that the group allows (e.g., individual, caste or colony level), will benefit the broad field of animal personality.

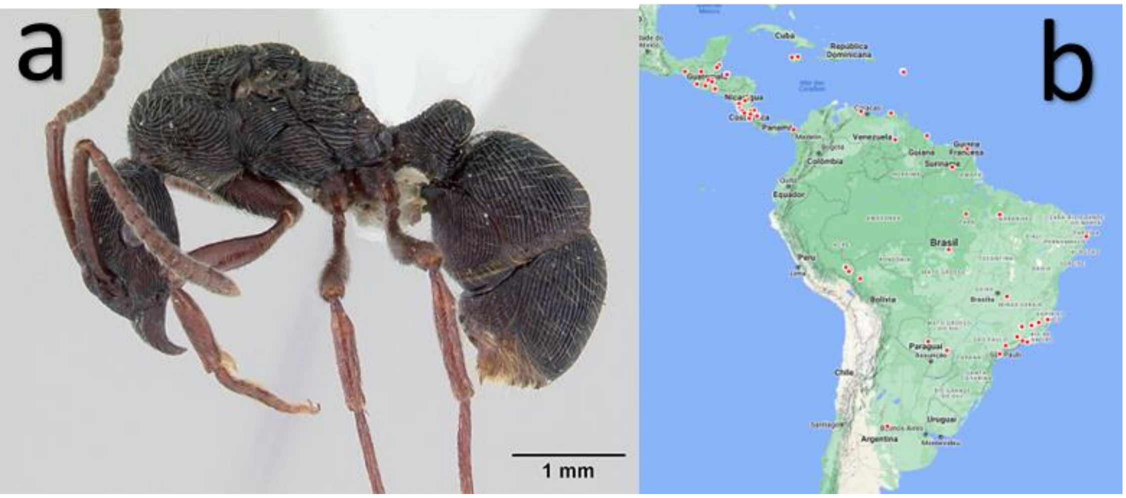
1.4.1 THE ECTATOMMINAE ANT *Holcoponera striatula*

A member of the *striatula* subgroup, *Holcoponera striatula*, formerly known as *Gnamptogenys striatula*, is an ant from the Ectatomminae subfamily. This common species is widespread, and can be found in the mainland, such as Argentina, Brazil, and French Guiana up to Costa Rica, or in insular areas such as Jamaica, all throughout Central and South America (Giraud et al., 2000) (Figure 3). The species has a generalist foraging habit, it is usually found nesting on branches, litter, and ground, mainly in native forest habitats (Silva et al., 2021), but can also be easily found in urban and suburban areas, which was, in the case of this thesis, the place of colony collection.

In this species, colonies are polygynous, which means that several queens can coexist and reproduce in the colony in the same time (Giraud et al., 2000). Differentiated queens are slightly larger than workers (Blatrix & Jaisson 2000), but under natural condition many workers are potentially able to mate, and some colonies contain gamergates (mated and fertile workers, sensu Peeters & Crewe, 1984) instead of queens. Colonies can contain up to 1,200 workers, a large number for ants of this subfamily, and up to 60 queens, resulting in one of the highest degrees of polygyny in ants (Blatrix & Jaisson, 2001).

Adult females of *H. striatula* ants, as common seen in other polygynous species, often mate within or close to the mother colony, being new colonies founded by division of existing ones, e.g. budding (Rosengren & Pamilo,1983), limiting gene flow and dispersion in bigger scales (Giraud et al., 2000). Even though, the relatedness between the queens and their mates, which is highly variable, caused by a different gene pool, may indicate long-distance migration mating (Giraud et al., 2000). In this sense, the peculiarities of this specialty, such as polygyny, the existence of gamergates, make this species an interesting subject for laboratory experimentation, as colonies are easy to reproduce and to be maintained (Blatrix & Jaisson 2000).

Figure 3. The *Holcoponera striatula* ant and its geographic distribution



Source: AntWeb (Nov, 2022). Legend: (a) Worker of *H. striatula* ant and its (b) Geographic distribution, occurring throughout South America.

1.5 OUTLINE OF THE FOLLOWING CHAPTERS

This thesis consists of six chapters, two regarding the main articles of this thesis (chapter 2 and 3) which are complemented by this general introduction (chapter 1), a general discussion (chapter 4), and an additional article in chapter 6 (appendix) in which I am a co-author.

In the *second chapter*, I investigate how individual differences in behaviour affect the way adult individuals from *Mus spicilegus* respond to a judgment bias test. It is important to note that individual differences do not only exist with respect to the expression of certain behaviours but may also be apparent regarding the way in which individuals perceive and process environmental stimuli (Koolhaas et al., 1999, Réale et al., 2008). Considering this fact, we discuss in this chapter how tests that seek to measure animal behaviour should take into account the differential ways in which individuals can interpret the apparatus and the test itself, in addition to the task which the test was initially designed to measure. This chapter highlights and discusses many of the methodological limitations regarding the way animal behaviour is studied, especially when creating and executing behavioural tests, and the caution we must have when interpreting and analysing the results from these.

In the *third chapter*, I investigated how individual differences in behaviour are expressed across many contexts (tested throughout many behavioural tests) and across time, in an extended repetitions essay, for foragers of the ant *Holcoponera striatula*. In this chapter, I attempt not only to understand how the behaviour individual differences of this species is structured, in the sense of how repeatable and correlated are behavioural traits (since there are still no records of the existence of personality in the genus *Holcoponera*), but also to discuss limitations of working with animal personality, especially in invertebrates. I also discuss how many of the animal personality studies are still limited by few (if not only one) contexts and with low (if none) repetitions over time. I also discuss that when working with invertebrates and adapting tests and variables that were previously established in vertebrates' studies (which historically have been much more focused on), it must be taken into account that these may not be necessarily biologically relevant to invertebrate species. In the same line with the previous chapter, I discuss the necessity to take particular caution when designing methods and interpreting results of behavioural tests.

In the *fourth chapter*, I summarise the novel insights achieved in the context of this thesis. More specifically, in this chapter, I attempt to unite and discuss the articles here presented, not only focussing on study species itself, but rather on greater theoretical and methodological questions when studying animal personality. Of course, to discuss individual differences in behaviour more generally does not imply that the aspects and specificities of each species are neglected, but rather they enrich the discussion from different standing points. Here, I also include some of the aspects that were dealt with in the article presented in the appendix, which also explores personality, but as an aspect that interacts with many others in the development of the individual.

In the *sixth chapter*, in the appendix, as mentioned above, we also explore individual differences in behaviour, but from perspective slightly different to the one of the previous chapters. Here, personality differences are used as an aspect underlying the way how individuals differentially interpret, and process stressful events experienced early in life. Individual differences in behaviour may also affect physiological pathways by which stress is perceived and processed. The findings of this article contribute to enlighten the complex interplay between early life conditions, individual differences in behaviour and oxidative stress.

CHAPTER 2: JUDGMENT BIAS AND PERSONALITY IN MICE

Is there a bias in spatial maze judgment bias tests? Individual differences in subjects' novelty response can affect test results

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Highlights

- Judgment bias tests are useful tools for the assessment of internal states.
- Spatial settings linking ambiguity with novelty may be biased by subjects' personality.
- High explorers showed longer latencies to the ambiguous cue as they explored more.
- Longer latencies were possibly driven by a motivational conflict in high explorers.
- However, these longer latencies did not mirror subjects' pessimist judgment bias.

Abstract

Judgment bias tests have become an important tool in the assessment of animals' affective states. Subjects are first trained to discriminate between two cues associated with a positive and a less-positive outcome. After successful training, they are confronted with an ambiguous cue, and responses are used for judgment bias assessment. In spatial settings, ambiguous cue presentation is typically linked with novelty, i.e. to yet unexplored areas or areas to which the animal has a low degree of habituation. We hypothesized that in such settings, responses to ambiguity might be biased by the animals' perception of novelty. We conducted judgment bias tests in mound-building mice phenotyped for their exploration tendency. After subjects had learned to distinguish between the positively and less-positively rewarded arms of a maze, a new ambiguous middle-arm was introduced. During the first test trial, more exploratory, less neophobic individuals displayed higher bidirectional locomotion in the ambiguous arm, indicating intensive exploration. Although this resulted in longer latencies to the reward in more exploratory animals, we conclude that this did not reflect a 'more pessimistic judgment of ambiguity'. Indeed, during the following two trials, with increasing habituation to the ambiguous arm, the direction of the association was inversed compared to the first trial, as more exploratory individuals showed relatively shorter approach latencies. We suggest that in spatial test settings associating the ambiguous cue to novel areas, results can be confounded by subjects' personality-dependent motivational conflict between exploration and reaching the reward. Findings obtained under such conditions should be interpreted with care.

Keywords: Ambiguous cue; Cognitive bias test; Exploration tendency; Mound-building mouse; *Mus spicilegus*; Personality

CHAPTER 2

2.1 INTRODUCTION

The judgment bias test (or cognitive bias test) is an effective tool to assess whether an individual evaluates an ambiguous situation rather positively or negatively (Harding et al., 2004; Lagisz et al., 2020; Mendl et al., 2009). During the last decade, this test, in its multiple designs, has been increasingly used in a variety of taxa (Burman et al., 2009; d'Ettorre et al., 2017; Doyle et al., 2011; Rygula et al., 2015). The results obtained by this test can provide valuable insights into an animal's emotional (affective) state, with various applications in the fields of animal behaviour and cognition, including in studies on welfare. For example, it has been shown that animals in a poorer welfare state tend to judge ambiguous stimuli more negatively (Baciadonna & McElligott, 2015; Bethell, 2015; Clegg, 2018; Matheson et al., 2008).

Judgment bias tests usually consist of two consecutive experimental phases. During (a) the learning phase, animals are trained to discriminate between two highly distinctive rewarded stimuli, one associated with a positive and the other with a negative or less-positive outcome. This is followed by (b) the test phase, i.e. the judgment bias task itself, during which a new, intermediate, and thus ambiguous cue is presented (Bethell, 2015; Harding et al., 2004). The recorded responses are assumed to reflect the individual's affective state. A more negative affective state is expected to generate a response to the ambiguous cue more similar to the one displayed when the individual is confronted with a negatively or less-positively reinforced cue. In contrast, ambiguous cue responses more similar to the ones to the positively reinforced cue are taken as indication of an animal's more positive affective state (Bateson, 2016; Mendl et al., 2009; Roelofs et al., 2016). These states are often termed as "pessimist" or "optimist" bias, respectively (Bateson, 2016).

A recent review of judgment bias studies has shown that variation in the responses to ambiguous cue presentation is not exclusively related to individuals' affective states, but can be modulated by factors such as the nature of the task, training conditions and sex (Lagisz et al., 2020). Furthermore, personality differences, i.e. consistent individual differences over time and across contexts in the way animals behave (Briffa & Weiss, 2010; Koolhaas et al., 1999; Réale et al., 2007; Stamps & Groothuis, 2010), have the potential to affect the test outcome. This is exemplified by studies of different mammal species, suggesting an association between judgment bias and personality traits, since more social (domestic dogs *Canis familiaris,* Barnard et al., 2018), common bottlenose dolphins *Tursiops truncates*, Clegg et al., 2017), extraverted (humans, Marshall et al., 1992), proactive (domestic pigs *Sus scrofa,* Asher et al., 2016), and less exploratory individuals (house mice *Mus musculus,* Verjat et al., 2021) have been reported to judge an ambiguous situation more positively.

But apart from such personality trait-dependent tendencies to judge ambiguity differentially, we suggest that personality differences may influence how an individual perceives basic aspects of the test setting itself. As a consequence, personality differences may possibly compromise the test setting's efficiency in judgment bias discrimination. In the judgment bias test framework, the ambiguous cue is frequently introduced as a novel stimulus into the previously learned setting, although the degree of novelty it carries may vary across the different test settings. For example, several (spatial) experimental settings make use of a multiple arms maze, thus segregating the ambiguous cue in a distinct area (i.e., by its presentation in the distal part of one or several of the middle arms). This area becomes accessible only after the training period during which the animal has learned to associate the peripheral (reference) arms of the maze to positive and less-positive (or negative) cues (Briefer & McElligott, 2013; Burman & Mendl, 2018; Burman et al., 2009; Kloke et al., 2014; Novak et al., 2015; Richter et al., 2012). In such settings, the animal's response to the ambiguous cue (e.g., its latency to reach the reward) is inevitably linked to the necessity of passing through this new area. Depending on the methods applied, the ambiguous arm(s) is either novel to the animal, or animals may have been briefly habituated to it, but typically to a lesser extent than to the positive and the less-positive (or negative) reference arms. Thus, it might be expected that in such a test setting, an individual's either faster or slower (or missed) response to the ambiguous cue may not necessarily reflect its positive or negative judgment of an ambiguous situation ("optimist" or "pessimist" bias, Bateson, 2016). However, it may rather constitute a personality-related response to aspects of the setting, depending on the way the individual perceives and responds to the novel environment related to the presentation of the ambiguous cue.

Especially for highly exploratory personality types the association between ambiguous cue presentation and the necessity to pass through a novel environment might create a conflict between the motivation to explore this environment (Réale et al., 2007) and the motivation to seek, approach and consume the reward (approach motivation, reviewed in Barker et al., 1999). As a consequence, this motivational conflict could affect the subject's latency to approach the ambiguous cue when presented in a novel environment, and thus might compromise the use of this behavioural parameter to assess individual judgment bias. This led us to hypothesize that consistent individual differences in behaviour might potentially bias or even mislead researchers about the interpretation of judgment bias tests, an aspect that has not yet attracted much attention (cf. discussion in Verjat et al., 2021).

To test this general hypothesis, we carried out judgment bias tests using a three-arm maze in which the ambiguous cue was presented in the middle arm. Prior to judgement bias testing, we behaviourally phenotyped the study animals to determine individual differences in exploration tendency, a main behavioural ('personality') trait frequently used to phenotype mammals and birds (Carere & Maestripieri, 2013; Dingemanse et al., 2004; Réale et al., 2007; Rödel et al., 2015). Exploration (as opposed to avoidance) can be broadly defined as an individual's response to a new situation or its activity in an unfamiliar environment, considering neophilia (as opposed to neophobia) as part of this trait (Réale et al., 2007; Sih et al., 2004). As study animals, we used male mound-building mice (*Mus spicilegus*), since individuals of this species show clear and consistent individual differences in exploration tendency in repeated open field and novel object tests (Duparcq et al., 2019; Rangassamy et al., 2016; Rangassamy et al., 2015).

More specifically, in our study, we tested how the latency to approach and consume the reward therein were affected by individual differences in exploration tendency. We hypothesized that more exploratory individuals may engage in more exploratory behaviour once inside the ambiguous arm, as they are more motivated to explore this novel environment than approaching and consuming the reward therein. This could finally lead to an overall longer latency before more exploratory individuals reach and consume the reward, an indicator usually used to determine whether an animal judges an ambiguous situation more positively or more negatively. If, as we predict, the test outcome rather reflects a personality-driven 'perception bias' of the test setting, we would expect that a possible link between exploration tendency and judgment bias test outcome should only occur when the ambiguous arm is novel to the animals. Thus, we re-tested our animals during the following two days, i.e. when they became increasingly habituated to this arm. As a consequence, we expected that with increasing habituation to the ambiguous arm, the above-described association linking a higher exploration tendency and a longer latency to the reward would disappear.

2.2 MATERIAL AND METHODS

2.2.1. Study animals

The study was conducted on mound-building mice of wild origin, descending from animals caught in Hungary in 1999 and bred at the animal facilities of the Laboratoire d'Ethologie Expérimentale et Comparée at the University Sorbonne Paris Nord, France. Additional individuals were captured at the same Hungarian collection sites every 4-5 years and integrated into our breeding stock to maintain genetic variation (details in Duparcq et al., 2019).

Animals were kept under laboratory conditions with a temperature of around 20 ± 1 °C and a 14/10 light/dark cycle (red light on at 12:30 pm). They were housed in polycarbonate cages (32.5 × 16.5 cm and 14.2 cm high, Plexx, Elst, The Netherlands) with wood shavings, two cardboard rolls as enrichment (length: 10 cm, diameter: 6 cm) and three cotton balls per individual as material for building the nest. Food (rodent standard diet; Special Diet Services type M20, Witham, Essex, UK) and water were provided ad libitum.

For the present study, we used males originating from 15 litters (1 to 6 males per litter), making up a sample of $N = 45$ individuals. Animals were sexed (by external genital inspection) and weaned on postnatal day 28. They were kept in mixed-sex sibling groups of 6 individuals until day 55, shortly before reaching maturity (Busquet et al., 2009), until when they were kept in same-sex litter sibling groups. To allow individual identification, subjects were marked on postnatal day 28 with different symbols using a black permanent non-toxic hair dye (Prodye, Weaver Leather Livestock, Ohio, USA; see details Rangassamy et al., 2015).

2.2.2. Behavioural phenotyping

To assess individual difference s in exploration tendency, including the animals' responses to novelty (Réale et al., 2007), individuals repeatedly underwent two consecutive behavioural tests, an open field followed by a novel object test, in that order, on postnatal day 32 (T_1) and day 43 (T_2). Testing was carried out during the animals' activity period during the dark phase (red light conditions). Behaviours were recorded with a video camera (FDR AX-100 4K, Sony, UK) fixed 140 cm above the centre of the test arena and the data were stored for later analysis. The apparatus was made of white polyethylene and was used for both the open field and the novel object test. It consisted of a circular open field arena (diameter: 60 cm) with a central circular area (diameter: 20 cm, division was not visible to the animal), surrounded by walls (height: 65 cm).

The experimenter was never present in the room during the experiments, only before and after each trial to clean the apparatus with hot water and non-perfumed soap (L'Arbre Vert, Novamex, France), and to prepare the next trial.

2.2.2.1. Open field test

Individuals were placed singly in a defined peripheral position at the edge of the arena, released and let to explore for 5 min. The total distance covered and the latency to reach the centre of the arena were quantified using the software Ethovision, version XT10 (Noldus Information Technology, Wageningen, The Netherlands).

2.2.2.2. Novel object test

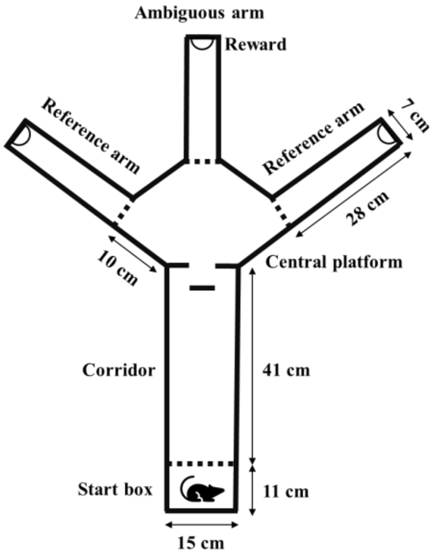
Directly after the open field test, the individual was caught and kept in a squared transparent plastic box (diameter: 8.0 cm × 8.0 cm, height: 17.3 cm) at the same peripheral position (as described above) for around 20 s, while a novel object was introduced into the centre of the arena. The individual was then released again from the plastic box into the test arena. After 5 min of testing, animals were caught and returned into their home cage. During the first test on postnatal day 32, a small transparent, round glass pot (outer diameter: 6.5 cm, inner diameter: 6 cm, height: 4.5 cm), and during the second test on postnatal day 43, a rounded kidney-shaped metallic box with smooth, varnished surface and with a slightly convex top (length: 9.5 cm, height: 2.2–2.7 cm, width: 5–6 cm) were used as 'novel objects'. We did not randomize the presentation of the two different novel objects across the two tests, as our preliminary studies had shown that animals generally stayed longer (and explored, sniffed more) on top of the (slightly larger) metal box than on the glass pot. These differences in population-level exploration time of the two objects across trials were accounted for in our statistical analysis, since we were scaling all behavioural variables within trials, i.e. we subtracted the mean per trial from each variable and divided it by the standard deviation (see below). Videos were analysed using the software Boris, version 7.9.8 (Friard & Gamba, 2016). The behavioural variables recorded were (a) the latency to touch the novel object and (b) the time spent exploring the object by climbing and moving while being on top of it.

2.2.3. Judgment bias test

Animals were tested after reaching maturity, at an age of 6 to 10 months, and experimentation for each individual lasted 14 days once started. Individual differences in the age of testing were due to methodological limitations that made it impossible to carry out the experimentation (i.e. including the long training phase) of all individuals in parallel. But see below that possible age effects on the outcome of the judgment bias test were always included in our models but were not statistically significant.

The judgment bias test procedure was based on and adapted from a setting proposed by our group (Verjat et al., 2021). Tests were carried out in a "Y" form apparatus with an additional middle arm (three-arm maze), made of opaque white PVC (0.3 cm thick, 50 cm high; Figure 6). The start box was connected to a straight corridor that led to a semi-circular central platform, which was only accessible through an opening (width: 4 cm, height: 5 cm) in the corridor. To avoid that subjects could see which of the three arms was opened before entering the central platform, a free-standing wall was placed 5 cm in front of the exit of the corridor, with rectangular openings (4 × 4 cm) at each side (Figure 4). The central platform was connected to the three arms (outer arms: left and right, plus a central arm), accessible by a single opening (width: 4 cm, height: 5 cm, only one arm opened by trial), and at the end of the arms a fixed reward (see below) was placed for experimentation (Figure 4). Two video cameras (FDR AX-100 4K, Sony, UK) were used in parallel, one filming the whole apparatus from above, mounted to a height of 200 cm, and the other one mounted 140 cm over the central platform and over the arm in focus (reference or middle arm, see details below). The test consisted of two consecutive phases, first a training phase of 11 days, followed by a test phase of 3 days.

Figure 4. Schema of the apparatus used for judgment bias testing



Source: Jardim et al., 2021. Legend: Modified after (Verjat et al., 2021). Dotted lines represent removable walls. See text for a detailed description of the different parts.

2.2.3.1. Habituation to sugar water

Prior to the training phase, individuals were offered around 2 ml of sugar water (10% of pure sucrose 99%, after Verjat et al., 2021), presented in a plastic Petri dish (diameter: 4 cm) inside their home cage for three consecutive days. This was done to habituate the animals to this new food item, which was used as a reward in the positive arm later on.

2.2.3.2. Training phase

During this phase, individuals were trained to associate an arm with the presentation of a specific cue. This learning association was carried out only with respect to the peripheral, reference arms (left and right; Figure1). One of them (left or right) was defined as the “positive arm”, always including a Petri dish with a drop of sugar water (same concentration as used for habituation, see above), and the other one was defined as the “less-positive arm” containing a Petri dish with a drop of plain water. Petri dishes containing sugar water or plain water (hereafter referred to as “positive reward” and “less-positive reward”, respectively) were fixed to the wall at the end of the corridor, at a height of 2 cm (Figure 4). Sugar water and plain water (also for habituation) contained blue odourless and tasteless food colouring (Vahiné, France; 3 drops into 10 ml), to facilitate later discrimination of reward consumption during the analysis of videos recorded under red light conditions. The position of the positive reward was (randomly) predefined for each individual over all trials, i.e. the animals either found the positive reward always in the right or always in the left reference arm. The middle arm was always closed and thus never accessible to the animals during the training phase.

During each of the 11 training days, subjects underwent four consecutive trials, consisting in two positive and two less-positive trials. Only one arm, either the positive or the less-positive arm, was open (accessible for the subject) at each trial. The order of these four different trials per day was randomized.

Before the trials started, individuals were put into the start box for 3 min. Then, to start the trial, the experimenter opened the removable wall (via a rope) connecting the start box to the corridor (Figure 4). The trial was stopped as soon as the animals consumed the sugar water (positive arm) or the plain water (less-positive arm). In cases when the animals had entered the open arm and did not consume the plain or sugar water, the trial was stopped after 3 min. In few cases, when the animals did not enter the open arm at all, the trial was stopped after 5 min. After the trial had ended, individuals were carefully guided back to the start box by the experimenter's hand, without touching the animal. This was then closed again and the animal remained there for 3 min before the next trial was started. At the end of the four trials, individuals were returned to their home cage and the apparatus was cleaned. The same procedure was carried out during the test phase (see below).

For each trial (4 trials/day), we calculated the latency to approach and consume the reward after the animal entered the central platform, i.e. after the animals passed their head through the opening separating the corridor from the central platform (Figure 4). In cases when the animals did not consume the plain or sugar water, we assigned the maximum time of 3 min (see above) to this trial. Then, for each training day, we calculated the mean latencies to approach and consume the reward during the positive and less-positive trials (two trials per day, respectively; see above).

2.2.3.3. Learning criterion

We defined a learning criterion that the individual needed to reach in order to be considered a 'learner' , and thus to be included in further analysis. According to this criterion, learners had to show shorter daily latencies (daily values were averages over the different trials per day, see above) to approach and consume the positive reward than the less-positive reward during (at least) each of the last four consecutive days of training. Four individuals did not fall into this strict criterion but were also considered as learners, as they had shown shorter latencies to the positive reward compared to the less-positive one during four out of the last five days of training. Our decision to consider these individuals as learners was finally confirmed by the fact that all of them showed shorter latencies to the positive reward compared to the less-positive one during the three days of the test phase (see below) following the training phase. In total, $N = 33$ out of the initial sample size of 45 individuals (73%) reached the learning criterion, and thus were considered for the next part of the test. The probability that an individual was considered a learner was not significantly associated with its individual exploration score, see results section.

2.2.3.4. Test phase

After the training phase, animals were tested for their responses to a new, ambiguous situation, i.e. the accessibility of the middle (ambiguous) arm. During this phase, individuals went through three trials per day, first a positive (only the positive arm opened) followed by a less-positive (only the less-positive arm opened), and then an ambiguous trial, during which the centrally-located arm (hereafter: “the ambiguous arm”) was introduced (i.e. its access was open to the animals), while the entrances to the two reference arms (left and right arms) remained closed. This phase lasted three days (days 1, 2 and 3; see Figures 3 and 4).

Generally, when repeated tests over time are carried out, as it has been done in our study for conceptional reasons, the animals may learn that the ambiguous probe is not rewarded. This could affect their latency time or their probability to approach the ambiguous probe, as it has been shown in studies on different species (reviewed in Bethell, 2015). In order to avoid such effects, we provided a drop of plain water (as it was provided in the less-positive arm) in the ambiguous arm as well (see Clegg et al., 2017 for a similar setting). The latencies to approach and consume the water were calculated during the three days of testing in the same way as it was done for the positive and the less-positive arm. And indeed, by our setting, the latency by which the animals approached and consumed the plain water cue inside the ambiguous arm did not change significantly between the first and second day of testing (slight increase in the mean latency by +9.3 sec, $CI_{95\%}$ = [–17.8 sec, +36.5 sec]; LMM: $\chi^2_1 = 1.701$, $P = 0.192$) and between the first and third day of testing (slight decrease in the mean latency by –15.4 sec, $CI_{95\%}$ = [–46.1 sec, +15.2 sec]; $\chi^2_1 = 1.021$, $P = 0.312$).

2.2.3.5. Latency index to approach the ambiguous cue

For each learner, we calculated an index based on the relative latency to approach and to consume the reward in the ambiguous arm, in relation to the latencies quantified in the positive and less-positive arms. This was done separately for each test day, resulting in three latency index values per individual. The calculation followed the formula proposed by Verjat and co-workers ((Verjat et al., 2021), modified after Mendl et al., 2010), therein termed as "judgment bias index". In the formula below, L represents the latency to approach and consume the reward. The mean values of L_{positive} and $L_{\text{less-positive}}$ were calculated based on the mean latencies quantified during the three days of testing (one trial per day, respectively; see details above), and $L_{\text{ambiguous}}$ is the latency to approach and consume the ambiguous reward on each test day (one trial per day; see details above).

$$\text{Latency Index} = 1 - ((L_{\text{ambiguous}} - \text{mean } L_{\text{positive}}) / (\text{mean } L_{\text{less-positive}} - \text{mean } L_{\text{positive}}))$$

For each of the three days of testing, we obtained a latency index ranging from 0 to 1. An index of 0 indicates that the latency to approach and consume the ambiguous reward is equal to or larger than the latency to approach and consume the less-positive one, and an index of 1 indicates that the latency to the ambiguous reward is equal to or smaller than the latency to the positive one. That is, when an individual approached and consumed the ambiguous cue relatively faster than the positive cue (resulting in values of > 1), the index was rounded to 1, and for cases in which an individual was slower to approach and to consume the ambiguous cue than the less-positive one, the index was rounded to 0. Such cases exceeding the limits of [0, 1] were rare, and an alternative analysis based on non-rounded index values led to the same principal result as given in Figure 6 (significant interactive effects with a positive slope during day 1, but negative slopes during the following days, $P < 0.05$).

2.2.3.6. Behaviour with respect to the ambiguous arm

A further fine scale analysis was carried out for the following key behaviours with respect to the ambiguous arm.

2.2.3.6.1. Latency to enter the ambiguous arm.

The time (in sec) it took the individual, once inside the central platform, to enter the ambiguous arm (Figure 6). The individual was considered to be inside the central platform or inside the ambiguous arm once its head passed the opening leading to the central platform or the opening of the ambiguous arm, respectively.

2.2.3.6.2. Indicators of exploration activity inside the ambiguous arm

Locomotor activity: % Time spent inside the ambiguous arm in movement (walking and running) directed to the reward (i.e. directed to the distal part of the arm) or away from it (see below). Since the arm was designed as a narrow corridor (7 cm wide, Figure 6) that did not allow extensive sideways movement, animals predominantly showed locomotor activity only along one spatial axis.

Reversed locomotor activity: We calculated the mean frequency (N per 10 s) by which the animals-initiated locomotor activity to the opposite direction of the reward, i.e. directed to the central platform (Figure 4).

These variables were used to test whether individuals previously phenotyped as more exploratory engaged in more exploration activities once inside the ambiguous arm.

2.2.4. Ethics note

Animals were kept and treated according to accepted international standards (Vitale et al., 2018) and to the ethics and animal care guidelines of France, where the experiments were carried out. Experimental procedures were approved by the French Ethics Committee for Animal Experimentation ‘Charles Darwin’ (APAFIS#17922-2018112916198301 v8) and by the ethics committee of our institution (SBEA-LEEC-USPN). Animals (45 males) were bred especially for this study and the remaining female siblings were used for other experiments in our work group. After the animals underwent the judgment bias test, they were euthanised during the following few days.

2.2.5. Statistical analysis

Statistical analyses were done with the program R, version 4.0.3 (R Core Team, 2021). First, we tested for individual-level repeatabilities (R_{ICC}) over time of the four behavioural variables collected during the repeated open field and the novel object tests (postnatal day 33: T_1, postnatal day 43: T_2). This analysis, based on $N = 45$ individuals, was done by linear mixed model (LMM)-based intra-class correlations (R package *rptR* Stoffel et al., 2017) with individual identity as a random factor. *P*-values were calculated by 10,000 Monte Carlo permutations.

Furthermore, we assessed individual-based associations between these 4 different behavioural variables (averages from T_1, T_2) by a multivariate LMM, using the R package *MCMCglmm* (Hadfield, 2010), including litter identity as a random factor. Based on the among-individual variance matrix provided by this multivariate model, we calculated pair-wise correlation coefficients between the four behavioural variables. If 95% credibility intervals did not include zero, the pair-wise associations tested were considered statistically significant (Houslay & Wilson, 2017).

We ran principal component analyses PCA, based on these four different behavioural variables, (a) the total distance covered and the (b) latency to approach the centre of the open field, (c) the latency to touch the novel object and (d) the time spent exploring it. Two PCA were run separately for the behavioural data collected at T_1 and T_2. The aim of this analysis was to express these four different variables by a single score. The variables were scaled for analysis within T_1 and T_2. Furthermore, we log[*x*] transformed the latency to touch the novel object and the time spend climbing and exploring it, and we square-root[x] transformed the latency to reach the central area of the open field, as these variables showed a strong right-skewed distribution. In doing so, the resulting PCA scores were well adjusted to a normal distribution, facilitating the use of parametric statistics (see below). The first axes of each of both PCA (T_1, T_2), which showed highly similar loadings in terms of the direction of how the input variables were associated to it, were averaged for later analyses, hereafter referred to as ‘exploration score’. Before averaging, we verified that this ‘exploration score’ was consistent, i.e. significantly repeatable over time over time (T_1, T_2) by an LMM-based intra-class correlation, with individual identity as a random factor (see above for details on this method).

By a generalized linear mixed-effects model GLMM for binomial data (with a logit link, R package *lme4* Bates et al., 2015), we tested for the possible association between the exploration score (covariate; score values averaged over T_1 and T_2, also for all later analyses) and whether or not an animal fulfilled the learning criterion (binomial response variable) during the 11 days of training ($N = 33$ ‘learners’, $N = 22$ ‘non-learners’). The model included each individual’s cage identity as a random factor to account for potential effects of the animals' shared cage environment (16 different cages, 2 to 6 males per cage, see above for details).

The following analyses were based on 98 measurements (repeated measurements over 3 days) from 33 individuals who had fulfilled the learning criterion (details above). One measurement was not available for analysis due to a failure during video recording of one animal on the second day of the test phase. For the three days of testing (factor with 3 levels), we analysed the effects of exploration score (covariate) on the different behavioural (response) variables recorded. These response variables were (a) the latency time until the animals entered the ambiguous arm, (b) the percentage time of locomotor activity inside the ambiguous arm (for analysis: proportional values between 0 and 1), and (c) the frequency of reversed, backward-directed locomotion (away from the reward) inside the ambiguous arm. Furthermore, we analysed (d) the effects of testing day and exploration score on the latency index (values between 0 and 1, see above). These analyses were done by linear mixed-effects models LMM for the response variables (a) and (c), and by GLMM for proportional data (with a logit link) for the response variables (b) and (d). LMM and GLMM were carried out by using the R package *lme4* (Bates et al., 2015). The models included the cage identity (animals were housed in 14 different cages) and individual identity as (intercept) random factors, the latter to account for repeated measurements over the three consecutive days of testing (one trial per day; details above). We also included the age of the animals at testing (as a covariate), as for methodological reasons these values varied considerably, between 194 and 307 days (see above). However, the effects of this covariate were never statistically significant (all $P > 0.10$), thus indicating that the variation in subjects' age at testing did not affect the results obtained. We always started the analyses by testing the interaction between the two predictor variables, the day of testing and exploration score, to check whether potential associations of the different response variables with the animals' exploration score were day-specific. In cases when this interaction was statistically significant (or in one case, when the interaction showed a statistical trend of $P = 0.066$), we tested separately for the associations between exploration score and the different response variables at the daily basis, again using LMM or GLMM, respectively (see Figures 3 and 4). For these day-specific analyses, we did not apply a correction of the alpha level (Nakagawa, 2004), since significant interactions (in one case a statistical tendency) had already revealed that the regression lines during the different days of testing crossed due to the opposing directions of their slopes.

For GLMM for proportional data, we verified that the models were not overdispersed by checking the ratio of the residual deviance and the degree of freedom. LMMs were checked for normal distribution of model residuals by normal probability plots, and we verified that variances were homogeneous by plotting residuals versus fitted values (Faraway, 2006). If not stated otherwise, P-values for GLMM and for LMM were calculated by likelihood ratio tests. All statistical tests reported in this study are two-tailed.

2.3. RESULTS

2.3.1. Consistent individual differences in exploration tendency

The four behavioural variables quantified during repeated behavioural tests on postnatal days 32 (T_1) and 43 (T_2) were significantly repeatable over time: the distance covered in the open field (LMM-based intra-class correlation with permutation test: $R_{\text{ICC}} = 0.288$, $P = 0.027$), the latency to reach the central area of this test arena ($R_{\text{ICC}} = 0.259$, $P = 0.047$), and the latency to touch ($R_{\text{ICC}} = 0.533$, $P < 0.001$) and the time spent climbing and exploring the novel object ($R_{\text{ICC}} = 0.259$, $P = 0.036$). That is, animals showed consistent inter-individual differences across time with respect to these behaviours.

Furthermore, a multivariate LMM revealed significant associations between these four different variables. Individuals which covered a higher distance in the open field were also quicker in reaching the central area of this arena ($R = –0.352$, $P < 0.05$), whilst animals showing such shorter latencies to the central area of the open field were significantly quicker in approaching and touching in the novel object ($R = 0.333$, $P < 0.05$) and climbed and explored the novel object significantly longer ($R = –0.597$, $P < 0.05$). Accordingly, there was also a significant, negative association between the approach latency and the time spent climbing and exploring the novel object ($R = –0.504$, $P < 0.05$). However, there were no significant associations between the distance covered in the open field and the latency to touch the novel object ($R = –0.159$, $P > 0.05$) or the time spent climbing and exploring it ($R = 0.034$, $P > 0.05$).

Two PCA carried out separately for T_1 and for T_2, each based on these four behavioural variables, revealed first axes explaining 47.4% (T_1) and 53.7% (T_2) of the variance of the data, respectively (eigenvalues T_1: 1.90, T_2: 2.15). All other axes had eigenvalues < 1 and thus were not considered for further analyses. Higher scores of these first axes were associated with a higher distance covered during the open field test (loadings T_1: +0.329, T_2: +0.438), a shorter latency to reach the central area of the open field arena (loadings T_1: –0.552, T_2: –0.373), a shorter latency to touch (T_1: –0.551, T_2: –0.587) and a longer period of time spent climbing and exploring the novel object (T_1: –0.533, T_2: +0.568). Score values of the first axis were significantly repeatable (LMM-based intra-class correlation with permutation test: $R_{\mathrm{ICC}} = 0.563$, $P < 0.001$), indicating individual consistencies over time (T_1, T_2) with respect to this score. For later analysis, the individual scores of T_1 and T_2 ($N = 45$ animals) were averaged and the resulting variable is hereafter referred to as 'exploration score'.

2.3.2. Training phase during judgment bias test

Out of a sample of 45 males, 33 individuals (73%) reached the learning criterion during the 11 days of training (see methods for details). For these 33 animals ("learners"), we recorded and analysed the responses to the presentation of the ambiguous cue and calculated the latency index, separately for each of the three days of testing (one trial/day during the following three days after the training phase). There was no significant association between the individual exploration score and the probability that an animal was categorized as a successful learner (GLMM for binomial data: $\chi^2_1 = 1.551$, $\beta = 0.438 \pm 0.355$ SE, $P = 0.213$).

2.3.3. Fine scale behavioural analysis during ambiguous trial

2.3.3.1. Latency to enter the ambiguous arm

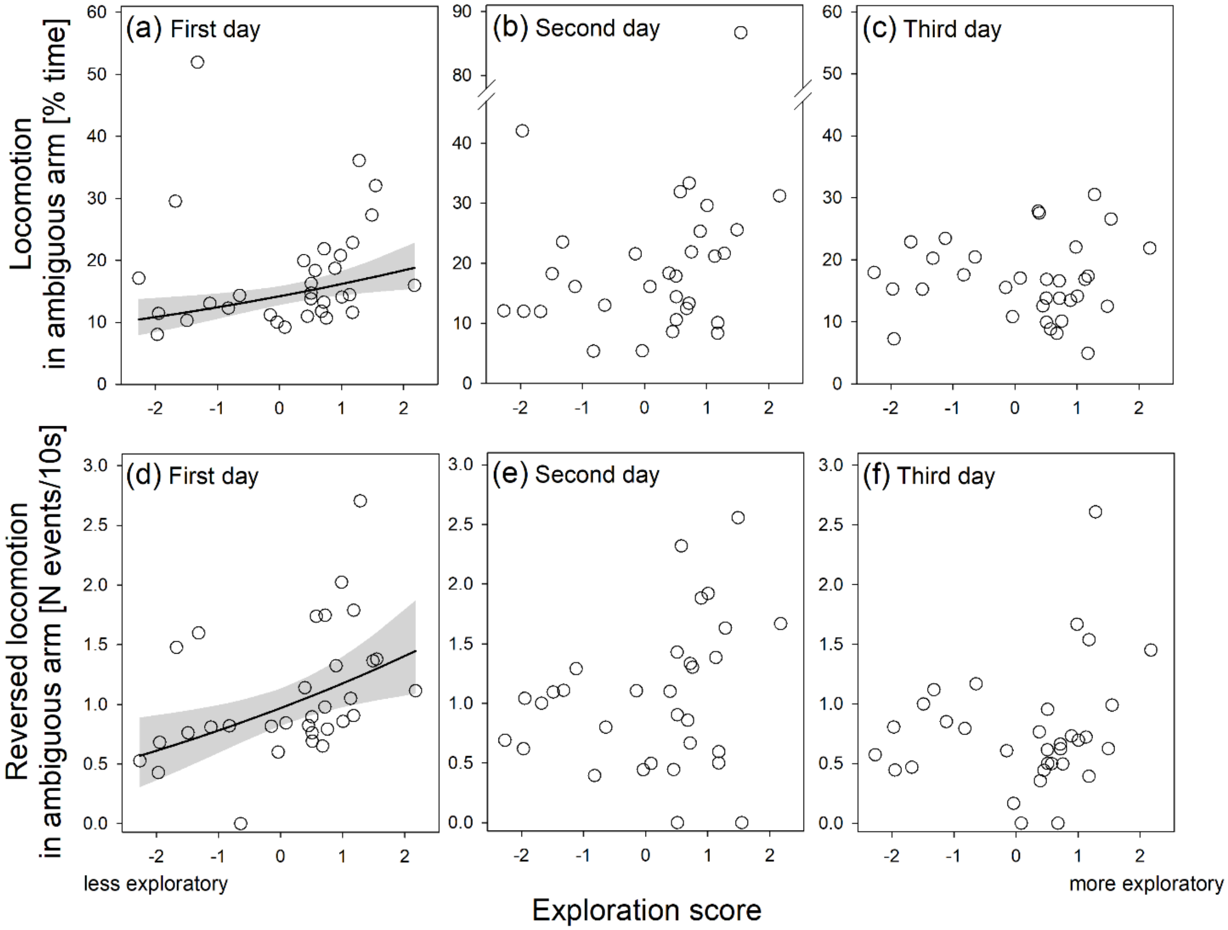
There were no significant associations between the latency to enter the ambiguous arm and the animals' exploration score, neither on the first (LMM: $\chi^2_1 = 0.337$, $\beta = –0.052 \pm 0.091$ SE, $P = 0.562$), second ($\chi^2_1 = 0.505$, $\beta = 0.567 \pm 0.452$ SE, $P = 0.477$) nor third day of testing ($\chi^2_1 = 1.537$, $\beta = –0.117 \pm 0.085$ SE, $P = 0.215$). The lack of any day-specific differences in the association between the latency to enter the ambiguous arm and the animals' exploration score was also evident by the non-significant interaction of day × exploration score ($\chi^2_2 = 2.118$, $P = 0.347$).

2.3.3.2. Exploratory activity inside the ambiguous arm

However, there was a day-specific association between the animals' exploration score and the percentage of time they spent in overall locomotion activity inside the ambiguous arm, as indicated by the significant interaction between exploration score and the day of testing (GLMM for proportional data: χ^2_2 = 8.001, P = 0.018). Accordingly, post hoc comparisons showed a significant and positive association between the exploration score and the overall locomotion activity during test day 1 (χ^2_1 = 5.721, β = 0.139 ± 0.085 SE, P = 0.016; Figure 5a), but there were no significant associations between these two variables on test day 2 (χ^2_1 = 0.127, β = –0.101 ± 0.087 SE, P = 0.721; Figure 5b) or test day 3 (χ^2_1 = 0.272, β = –0.009 ± 0.084 SE, P = 0.602; Figure 5c).

Such an interaction between exploration score and day of testing was also indicated with respect to the frequency of reversed locomotion, although missing the level of significance (LMM: χ^2_2 = 5.148, P = 0.066). During the first day of testing, the frequency of reversed locomotion in the ambiguous arm was significantly increased in animals with higher exploration scores (χ^2_1 = 7.841, β = 0.091 ± 0.035 SE, P = 0.005; Figure 5d), whilst there were no significant associations between both variables on the second (χ^2_1 = 0.498, β = 0.011 ± 0.043 SE, P = 0.480; Figure 5e) and on the third day of testing (χ^2_1 = 0.173, β = 0.007 ± 0.041 SE, P = 0.678; Figure 5f).

Figure 5. Associations between exploration tendency, locomotion activity and reversed locomotion during three consecutive days



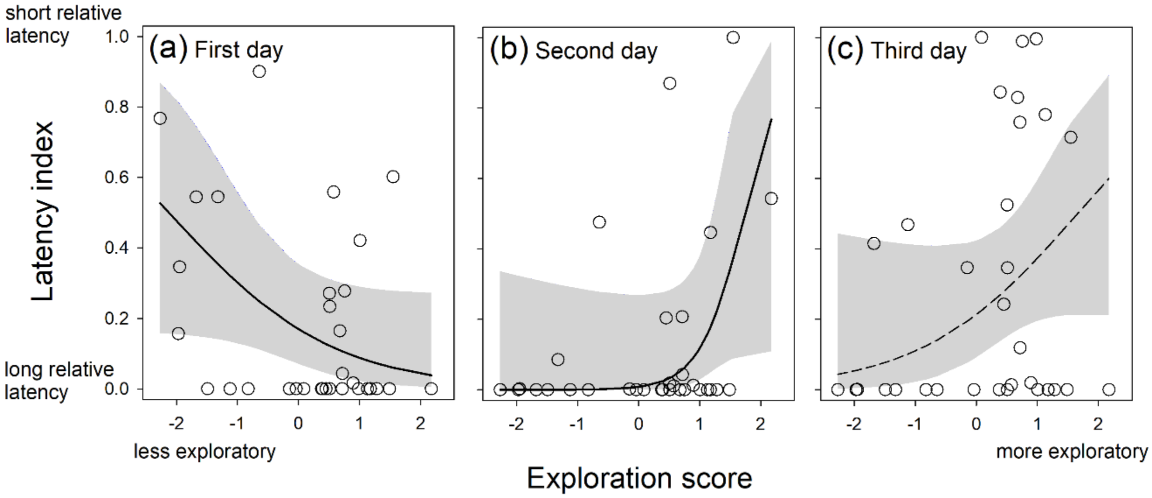
Source: Jardim et al., 2021. Legend: (a-c) the % time the animals spent in total locomotion activity, (d-f) the frequency they spent in reversed locomotion (directed away from the reward) inside the ambiguous (middle) arm of the apparatus (*N* = 33 males). Tests were carried out during three consecutive days, with one trial per day. For all significant effects, regression lines and 95% confidence intervals (grey areas) based on parameter estimates of (a) GLMM for proportional data and (d) LMM are shown; see text for details.

2.3.4. Associations between exploration tendency and latency index

Differences in the latency index were significantly explained by the interaction between the exploration score and the day of testing (GLMM for proportional data: χ^2_2 = 12.964, P = 0.001), indicating that the direction of the association between an individual's exploration tendency and its latency index was day-specific (Figure 6).

Post-hoc comparisons revealed that there was a significant and negative association between exploration score and the latency index during the first day of testing (χ^2_1 = 4.357, β = –0.991 ± 0.509 SE, P = 0.037; Figure 6a). That is, more exploratory individuals showed a longer relative latency to approach and consume the reward in the ambiguous arm. However, the direction of this association changed on the second day of testing (i.e. during the second trial), when the exploration score and the latency index were significantly and positively associated (χ_1^2 = 6.391, β = 1.182 ± 1.757 SE, P = 0.011; Figure 6b). One day later, during the third day of testing, there was also a trend of a positive association between the two variables, barely missing the level of significance (χ_1^2 = 3.794, β = 0.948 ± 0.566 SE, P = 0.051; Figure 6c).

Figure 6. Associations between exploration tendency and latency index during three consecutive days (one trial per day)



Source: Jardim et al., 2021. Legend: Associations between exploration tendency and latency index, expressing the relative latency to approach and consume the reward, during three consecutive days (one trial per day) of ambiguous cue presentation. A latency index of 1 indicates that the latency to the reward in the ambiguous arm equals to the latency to the reward in the positive arm; an index of 0 indicates that the latency related with the ambiguous cue equals to or is higher than the one related with the less-positive cue. Note that the latency index is equivalent to a judgment bias index (e.g., Verjat et al., 2021), according to which higher score values (close to 1) are typically interpreted to express an "optimist" judgment bias and lower values a "pessimist" bias. The analysis (GLMMs for proportional da ta) is based on repeated measurements from N = 33 males; regression lines and 95% confidence intervals (grey areas) are shown. The positive association depicted in (a) and the negative association in (b) are statistically significant, and the negative association in (c) is a statistical trend of P = 0.051, see text for details.

2.4. DISCUSSION

2.4.1. Summary of key results

According to our hypothesis, more exploratory (and thus less neophobic) individuals displayed greater arm exploration when confronted with ambiguity during the first test trial, as exemplified by the higher percentage of time spent in total locomotion and the higher frequency of reversed locomotor activity (i.e. locomotion directed away from the reward) in the ambiguous, novel arm. We suggest that this personality type-specific difference in ambiguous arm exploration was the main reason finally leading to longer relative latencies to the ambiguous cue (i.e. lower latency index scores, see Figure 6a) in more exploratory individuals. However, during the following two test days (one trial per day), along with subjects' increasing habituation to the ambiguous arm, the direction of the association turned around, and more exploratory individuals were relatively faster than less exploratory ones in approaching and consuming the ambiguous cue.

2.4.2. Behavioural phenotyping, training success, and test design

Our study broadly confirms the existence of consistent individual differences in exploration tendency in the mound-building mouse, also demonstrating the usefulness of repeated open field and novel object tests in assessing this personality trait (Duparcq et al., 2019). These findings are in accordance with studies in other rodents, in which similar test settings were used (Dammhahn et al., 2020; Ferrari et al., 2013; Yuen et al., 2015).

By the 11-day long training phase, prior to judgment bias testing, we achieved a reasonably high proportion of learners (73%), indicating that the apparatus including the procedure used (after Verjat et al., 2021) were adequate for training the animals to discriminate between two stimuli with positive and less-positive outcome. Although different studies report a link between exploration tendency and learning speed in either direction (Light et al., 2011; Rivera-Gutierrez et al., 2017; Sih & Del Guidice, 2012), we did not find a significant association between individual exploration scores and the probability that an animal reached the learning criterion. The lack of such an association confirms that our final sample of learners (33 out of 45) used for judgment bias testing did not constitute a subset of particularly high or low-exploratory individuals.

Furthermore, although our study design included repeated testing, which has been frequently reported to lead to a decrease in subjects' motivation to approach the (unrewarded) ambiguous probe (reviewed in Bethell, 2015), we did not observe notable differences in latency during the first and the subsequent two days of testing (see methods section). We suggest that this may possibly be attributed to the fact that in our setting, we always presented the less-positive reward (plain water) inside the ambiguous arm (cf. Clegg et al., 2017 for a similar test design).

2.4.3. Association between personality and judgment bias test outcome

There were no indications that the animals' exploration tendency affected their latency to enter the ambiguous arm when it was introduced to them during the first day of testing (or during the following two test days). However, when encountering the ambiguous arm during the first test day, more exploratory individuals showed a higher proportion of time spent in locomotion inside this arm compared to less exploratory ones. This reflects the typical response mode of exploratory personality types, characterised by an intrinsically higher motivation to explore and to show locomotor activity in novel environments, or in environments to which they have a low degree of habituation (Jansen et al., 2009; Luo et al., 2019). Intense exploration can be motivated by curiosity and the motivation to gather information, or may be interpreted as an effort to reduce uncomfortable states due to environmental uncertainty (Berlyne, 1960; Litman & Jimerson, 2004). We suggest that in our study, such an attempt of more exploratory, less neophobic individuals to obtain information was particularly evident by their higher proportion and frequency of reversed locomotion, resulting in a higher frequency of back-and-forth movements inside the ambiguous arm. This strongly suggests a motivational conflict in such individuals, with the motivation of gathering information about the novel environment outweighing the motivation of seeking and consuming the reward during the first ambiguous trial (Barker et al., 1999; Sih & Del Guidice, 2012), and finally leading to their longer relative latencies to approach and consume the ambiguous reward (i.e., lower latency index, Figure 6a). We cannot fully exclude that a generally higher activity, which can be considered as an independent personality trait (Kelley, 1993; Réale et al., 2007), may have contributed to the increased exploratory activity of some of the study animals inside the ambiguous arm. And indeed, frequently a higher exploration tendency is positively linked with a generally higher activity (Dosmann et al., 2015; Wilson & Godin, 2009). However, inter-individual differences in activity alone could not explain the observed, inversed association between behavioural type and latency to the ambiguous cue along the 3 days of testing (see Figure 5). The latter finding strongly suggests habituation effects on the animals' exploration of novelty as the key mechanism underlying the observed changes over times.

Comparisons of animals' relative latencies to the ambiguous cue (i.e. relative to the latencies to the positive and the less-positive cue) are frequently used to assess their judgment bias of ambiguity (Bethell, 2015; Lagisz et al., 2020). That is, individuals with a shorter latency are typically interpreted to show a positive (“optimist”) judgment, whilst individuals with a longer latency are scored to have a more negative (“pessimist”) judgment of an ambiguous situation (Bateson, 2016). However, based on the arguments outlined above, we conclude that in our study the interpretation of the observed longer relative approach latencies (i.e. lower latency indices; Figure 6a) to the ambiguous reward in animals with higher exploration scores as a “negative judgment bias in more exploratory individuals” would be misleading and most likely flawed.

Our argumentation is further supported by the change in the direction of the association between exploration score and the relative latency to the ambiguous reward (i.e. the latency index) during the first and following test days (Figure 6b,c). As we had hypothesised, during these subsequent trials on test days 2 and 3, more exploratory animals showed shorter relative latencies (i.e. higher latency indices) compared to the relatively longer ones observed during the first day of testing. And accordingly, during the two following test days, there was no more apparent association between the animals’ exploration tendency and their locomotor activity inside the ambiguous arm (see Figure 5). This could be explained by effects of habituation along with repeated exposures, corroborating the findings of studies in birds showing that exploratory behaviour declines with the number of test replicates (Dingemanse et al., 2002; Mettke-Hofmann et al., 2006; van Oers et al., 2005).

In fact, some judgment bias studies using a multiple arms maze made an effort to habituate the animals to the ambiguous arm(s) prior to the training phase by letting them explore the whole test central platform including all arms (e.g., (Briefer & McElligott, 2013; Burman & Mendl, 2018; Richter et al., 2012). This might be an effective way to reduce differential personality-related responses to arm novelty. However, due to the necessity of an intensive training prior to judgment bias testing, there might be still notable differences in the degree of habituation to the reference arms (which are the ones the animals learn to discriminate) compared to the middle, ambiguous arm(s) only accessible to the animals during the final judgment bias test trial(s). Future studies directly comparing judgment bias test responses of different groups of animals which have or have not been previously habituated to the ambiguous areas or arms of a maze, and how personality in these different groups is linked to their judgment bias, may provide further insights into potential methodological pitfalls related to this test paradigm.

Nevertheless, our study provides some insights into the possible link between personality and judgment of ambiguity. Even though we conclude that the results obtained during the first test day (i.e. during the first trial, Figure 6a) were confounded by personality-related differences in novelty response and thus uninterpretable in the context of judgment bias testing, we speculate that such a personality-dependent perception bias of novelty would not play any notable role during the following days due to the increased habituation of subjects to the test setting. When discarding the first day of testing, the remaining results obtained during the second and third test days showed a consistent pattern (see Figure 6b,c). Thus, this positive association between individual differences in exploration and the latency index may be interpreted to reflect a more positive (“optimistic”) judgment of ambiguity in more exploratory individuals. Such a finding of a more positive judgment of ambiguity in more exploratory individuals goes in line with a previous study in domestic pigs (Asher et al., 2016). In contrast, a recent study in house mice found relatively longer ambiguous cue latencies in more exploratory animals, indicating a more negative judgment of ambiguity in such individuals, Verjat et al., 2021); but see alternative discussion points therein).

2.4.4. Conclusions and recommendations

Although in the majority of judgment bias studies the possible effects of personality differences may not be part of the research question itself, we suggest that our findings have some general implications. Animals can differ consistently in the way they assess and respond to novelty and may even differ in the speed they habituate to it (Augustsson & Meyerson, 2004; Koolhaas et al., 1999; Martin & Réale, 2008). Most importantly, such different individuals might potentially be unevenly distributed across the different treatment groups the experimenter aims to compare. And when sample sizes are moderate or small, even few individuals with extreme personalities or coping styles may become especially influential. Under such conditions, personality-biased novelty responses during judgment bias assessment may lead to misinterpretations of group differences or treatment effects. In conclusion, our study highlights the importance to consider personality-related inter-individual variation in response to novel elements, in judgment bias test settings but possibly also in other test designs aiming to assess subjects’ affective states.

Declaration of Competing Interest

None.

CRediT authorship contribution statement

Veridiana Jardim: Conceptualization, Project administration, Funding acquisition, Methodology, Investigation, Data Curation, Formal analysis, Visualization, Writing - Original Draft. **Aurélie Verjat:** Methodology, Writing - Review & Editing. **Christophe Féron:** Methodology, Resources, Writing - Review & Editing. **Nicolas Châline:** Writing - Review & Editing. **Heiko G. Rödel:** Conceptualization, Project administration, Supervision, Formal analysis, Visualization, Writing - Review & Editing.

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CHAPTER 3: PERSONALITY IN ANTS

Temporal consistency of behavioural traits of an Ectatominae ant in a multiple test set-up

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Abstract

To date, a large and still increasing body of evidence has been published on the existence of animal personality in a wide range of taxa. Surprisingly, studies on invertebrates are still underrepresented in personality research, although this taxonomic group presents many unique characteristics that are not seen in vertebrates. In our study on adult foragers of *Holcoponera striatula* ants, we investigate whether behavioural traits are consistent across ecologically relevant contexts, and study their changes over time, and possible correlations among them. We performed four behavioural tests (open field, shelter, aggressiveness, and social test) which we repeated three to five times with 54 individuals. Only two out of seven behavioural variables showed significant, although low repeatability across time: the distance covered in the open field (R = 0.176) suggesting consistent individual differences in locomotor activity, and the latency to exit a shelter (R = 0.109) as an indication of boldness. Such low repeatabilities are not uncommon in personality studies. Behavioural correlations across tests reflecting different contexts, although seemingly reinforced in animal personality studies, are not a necessary requirement, since personality axes can function independently. Furthermore, we found a general tendency for behavioural responses during the first test to be different than subsequent ones, probably due to habituation and novelty effects. These findings highlight the complexity of personality testing, especially due to the methodological limitation of performing extensive repetitions across time and the importance of designing tests and choosing variables that are relevant for the studied species.

Keywords: animal personality, eusocial insects, open field, behaviour syndrome

3.1. INTRODUCTION

Animal personality usually defined as individual differences in behaviour consistent across time and/or across contexts, has been extensively studied in recent decades (Sih et al., 2004, Réale, 2008, Stamps & Groothuis, 2010). Now described and characterised in a large variety of taxa (for example, in mammals: Freeman & Gosling 2010, Gartner & Weiss 2013, Koski 2014, birds: Exnerová, et al., 2012, fish: Toms et al., 2010, invertebrates: Kralj-Fišer & Schuett, 2014), the study of animal personality had started and continued for an extensive period of time limited to vertebrate species. Only recent studies have focused more intensely on other taxa, such as invertebrates (Carter et al., 2013, Gosling, 2001, Kralj-Fišer & Schuett, 2014, Kolay et al., 2020).

In the past, the gap on invertebrate studies in the field of animal personality may have been rooted in the erroneous idea that these species do not present sufficiently interesting behavioural complexity for the study of personality. Invertebrates were often seen almost as "robots", with limited and stereotypical behavioural repertoires based only in responding directly to external stimuli with no or low individual differences in behaviour (as discussed in Bell et al., 2009, Carter et al., 2013, Brembs 2013, Kralj-Fišer and Schuett 2014). Only recently this scenario started to shift, and although most personality studies on invertebrates have been conducted in arthropods (mainly Insecta, Carere & Mather, 2019, Gherardi et al., 2012, Kralj-Fišer & Schuett, 2014, Mather et al., 2012), studies now report consistent behavioural differences across many invertebrate taxa, and numbers continue growing (Mather & Logue, 2013). A recent review of Kralj-Fišer and Schuett 2014, points out that personality has already been described for more than 19 invertebrate genera with the majority of these (15 genera) within Arthropoda, and since then numbers continue on growing (e.g. genre *Teleogryllu*, Rudin et al., 2016, the genus *Carcinus*, Fürtbauer, 2015, the genus *Portia*, Chang et al., 2018, the genus *Pararge*, Kaiser, 2020, the genus *Nebria*, Labaude et al., 2018, the genus *Cornu*, Dahirel et al., 2017, the genus *Latrodectus*, Golobinek et al., 2021, and others).

At first sight it may appear like studies on invertebrate personality entail some limitations, for example due to the generally lower repeatability found in studies on invertebrates compared to those on vertebrates (Bell et al., 2009). A further difficulty relies on the adaptation relevant behavioural tests and variables that were often initially developed for vertebrate species (Carter et al., 2013). More specifically, for eusocial species, extensive experimentation may be more difficult to carry out due to social grouping. Discrimination of individuals can be particularly hard to maintain for long periods of time since individual markings, for example in insects, can be easily lost (Hagler and Charles 2001)Nevertheless, these difficulties are not impediments to the study of social species in animal personality, and these limitations are being consistently worked around with the growth of scientific body in this area, mainly because findings on invertebrates have much to enrich the discussion of the field (Kralj-Fišer & Schuett, 2014).

Using invertebrates as model species for personality studies can provide great experimental advantages because they are usually easy to rear, maintain and manipulate, and have shorter life cycles allowing repetitions that encompass a large part of an individual's average life span (Kralj-Fišer and Schuett 2014). In addition to such methodological advantages, the mere proof of personality across invertebrates demystifies many of the speciesism ideas about the behaviour complexity of these species, expanding ethological, ecological, and evolutionary concepts (Kralj-Fišer & Schuett, 2014), thus contributing to the improvement of theories in the field. Also, importantly, invertebrates exhibit a range of characteristics in their life histories, social and sexual behaviours that are extremely rare or absent in vertebrates and can offer new avenues for personality research (Carere et al., 2018, Gartland et al., 2022, Kralj-Fišer & Schuett, 2014, Maák et al., 2020). Thinking more specifically about eusocial species, such as ants, research on the group can add a new layer of complexity to the concept of personality, since natural selection can act not only at the individual level but at the group level as well, since colonies can function as a superorganism (Dall et al., 2012, Kralj-Fišer & Schuett, 2014), opening new discussions on the mechanisms and structure of personality in social groups.

Animal personalities are mostly phenotyped through behavioural studies in the laboratory and/or in the field, most commonly by repeated, individual-based experimental tests (reviews: Carter et al., 2013, Kelleher et al., 2018, O'Malley et al., 2019, Vazire et al., 2007). As discussed previously, it is very common that protocols and tests used in invertebrate studies are those that were created and adapted from vertebrate species (e.g., open field test, applied in vertebrate studies since the beginning of the 20th century, Walsh & Cummins, 1976). One of the biggest challenges in personality research is finding behavioural tests that are biologically relevant for a given species (Carter et al., 2013). This appears particularly critical in invertebrates, since individuals have different physiology, cognitive pathways, communication canals and social structures (e.g., acoustic, tactile, chemical, and visual communication, social organisation in castes and subcastes, polygyny). Although it is known that a test for one taxon/species or even age class is not necessarily appropriate as a test for another (Weiss & Adams, 2013) some tests such as open field test, novel object test or emergence test (shelter test) amongst others are considered classic, well-founded, and relevant to a very extensive number of taxa (Carter et al., 2013). The open field test, for example, has attained the status of one of the most widely used instruments in animal psychology (Walsh & Cummins, 1976). It is used to measure distinct behavioural traits (depending on the species and the experimental question), from gregariousness to exploration (Walsh & Cummins, 1976, Carter et al., 2013), but more commonly to measure activity, and it is now a widespread tool in the field of animal personality, frequently and successfully applied across many invertebrate studies (e.g., d'Ettorre et al., 2017, Kralj-Fišer & Schuett 2014, Maák et al., 2020, Royauté, et al., 2015). Many other tests were designed to measure specific personality traits, such as aggressive encounters, testing intra or interspecific aggression, or social tests in its many forms, focusing on an individual's social interactions (see review by Chapman et al., 2001, Carter et al., 2013, Gartland et al., 2022). These tests are designed to target and categorise specific behavioural traits, seeking to access the same type of response in each test repetition, aiming to find behavioural repeatability

In addition to consistency over time, another aspect of animal personality is consistency across contexts (Koolhaas et al., 1999, Réale et al., 2007). To understand how traits interact with each other we must observe individual behaviour being expressed in different contexts, a concept that formulate the idea of behaviour syndromes. We define behaviour syndromes as a suite of correlated behaviours reflecting between individual consistency in behaviour across multiple (two or more) situations (Sih et al., 2004). Although many studies measure several different behavioural variables in their tests, they are often limited to one or two contexts (as discussed in Highcock and Carter 2014, Kralj-Fišer & Schuett 2014, Sih et al., 2004). Even less studies take in consideration the premise of extensive repetition in time since most are often limited to two behavioural measurements per individual (reviewed by Bell et al., 2009). On top of that, the number of repetitions can vary greatly between studies, depending heavily on the animals' life history and the methodological approaches (Bell et al., 2009). Thus, reliability and biological relevance of repeatability studies for assessing personality traits are still under great debate (Bell et al., 2009, Kralj-Fišer & Schuett, 2014). Exploring how behaviours change or not over time can also be an important step especially when working with behavioural tests, since the behavioural response throughout repetitions can lead to processes such as habituation and/or sensitization (Greenberg & Mettke-Hofmann, 2001), that can bias the behaviour response we are seeking to understand.

To contribute further on the study of personality traits in invertebrates, focusing on eusocial insects, we designed an experimental protocol that includie extensive repeated testing in different contexts. We used Ectatomminae ants, *Holcoponera striatula* (Mayr, 1884) as study species, a generalist, polygyne, easy to rear and widespead ant. More specifically, we aimed to investigate, for *H. striatula* adult foragers, (a) the repeatability of their behavioural responses in different contexts (as reflected by our different test settings) (b) and whether and how these behaviour responses changed across time. If repeatable, (c) how did these behaviours correlate with each other within and across contexts, possibly reflecting a behavioural syndrome for the studied species. We predicted to find behavioural temporal consistency, especially for aggressive behaviour, which in previous studies have shown to be a class of behaviour with high repeatability in many vertebrate and invertebrate species (reviewed by Bell et al., 2009). We also predicted that behavioural responses would correlate with each other, more often within the same context than across contexts, since behaviours measured in specific personality tests often are categorised in the same personality axis (Réale et al., 2007). Furthermore, we explored whether nestmates behaved more similarly to each other than non-nestmates, thus leading to colony-level repeatabilities in their behavioural responses. Finally, we expected that behaviour response would change over time, since, in experimental designs of repeated measurements, processes such as habituation or sensibilization are common to subjects naïve to the experimental setting (Bejder et al., 2009, Rudeck et al., 2019).

3.2. MATERIAL AND METHODS

3.2.1 Study Species, Field collection and Housing of Colonies

Holcoponera striatula Roger (Hymenoptera: Formicidae: Ectatomminae) ants occur throughout South and Central America (Lattke, 1995). Colonies can have either one or several queens, or more commonly, several gamergates (Blatrix & Jaisson, 2001). Like most species of the genus *Holcoponera* they are generalist predators (Camacho, 2013). Nests collection was carried out at the University of São Paulo campus, São Paulo, Brazil, in October 2020.

Nests were kept away from the sun and near a humidifier, in an attempt to keep temperature and humidity as constant as possible. Water and food (apple with honey and larvae) were available *ad libitum*. In total, two nests were collected at the field and from them six experimental sub colonies were assembled, each one with the start combination of 250 workers, 20 larvae, 20 pupae and 20 eggs. Sub-Colonies 1, 2 and 6 were assembled from nest 1, and subcolonies 3, 4 and 5 from nest 2.

3.2.2 Personality tests

Only foragers underwent the personality tests, and were selected through behavioural observation, in which all individuals outside the colony (the foraging area) were individually marked with a dot of ink on the gaster (UniPaint Marker, Mitsubishi™), and then checked at the following day to see if they were found again outside the nest. Individuals that were found again received another dot of ink, on the thorax this time, allowing individual discrimination. Since we could not finish testing all individuals in one day, foragers were divided into two groups and tested throughout two days for each repetition. Repetitions were scheduled two days apart from each other, therefore, each individual underwent two repetitions a week, until reaching a maximum of 5 repetitions. Because of occasional deaths and loss of individual marking, we also considered in our analysis individuals that went through 3 and 4 repetitions. In total, 154 individuals of the 6 sub-colonies underwent the personality tests, but only 54 individuals reached the minimal criterion of 3 repetitions and were considered in the analysis, in which, out of these 54 individuals, 10 individuals reached maximum repetition of 3, 6 individuals reached the maximum repetition of 4, and 38 individuals reached the maximum repetition of 5.

Four personality tests were performed: the open field test, the shelter test, an aggression test and a social test. The tests were performed on all subjects for a minimum of 3 and a maximum of 5 repetitions each (see details above). Individuals performed one repetition of all tests on the same day, one after the other, with the order of the tests randomized for each repetition. The order of nests and individuals were also randomized for each repetition. All tests were recorded with a *Powershot Canon* Sx620hs mounted 40 cm above the arena and stabilised with a tripod, with two opposite sources of light to avoid eventual shadows.

Open field test: Ants were tested in a circular open field arena (Petri Dish: 9 cm diameter), with a central area marked in pencil (8 cm diameter, hence peripheral area distanced 1 cm from the rim of the Petri Dish), filled with a clean filter paper floor that was changed after each trial. The ant was introduced into an acclimation tube placed in the centre of the arena (2 cm diameter) for 60 s, then the tube was removed, allowing the ant to explore, and behaviours were recorded for 300 s.

Shelter test: In the same arena as the open field test (with the filter paper changed), the focal ant was placed inside a shelter in the centre of the arena. The shelter was built as a rectangular apparatus (1,5 cm x 2 cm x 0,5 cm), in which the four walls were made by clip combs and a hard plastic guillotine door, removed after the individual had been acclimated for 60 s, and behaviours were recorded for 300 s.

Aggression test: In the same circular arena as described above (with the filter paper changed), the focal ant was introduced into an acclimation tube for 60 s (2 cm diameter). While the focal ant was acclimatising , a dead heterospecific sympatric ant (*Solenopsis saevissima*) was used as stimulus and was placed diametrically opposed at the central arena, then the tube was removed, and the behaviours were recorded for 300 s. The stimulus chosen was a dead worker from *Solenopsis* sp., from a colony also collected at the University of São Paulo. Individuals were frozen right after field collection, and for the trials, individuals that would be presented as stimulus were defrosted 30 minutes before the test. Since in preliminary observations stimulus inspection could happen within a short distance from stimulus, we considered a 2 cm circumference around the stimulus as stimulus periphery area, in which the focal ant was considered to be in contact with the stimulus the variables in further analyses.

Social test: In the same circular arena (with the filter paper changed), the focal ant was placed in the same acclimation tube for 60 s, while in a tube placed diametrically opposed in the central area, another acclimatization tube was introduced with other five nestmates individuals selected at random at the sub-colonies. Then, both tubes were removed at the same time, and trials were recorded for 300 s.

3.2.3 Behavioural variables

Seven behavioural variables were measured for the personality tests. For the open field test, we measured distance covered (cm) and exploration with percentage of time in the centre of the arena (see details of test settings above). For the shelter test, we measured latency to exit the shelter (s) and frequency of shelter exits. For the aggression test, we measured aggression with both the latency to get close to the stimulus (s) and percentage of time in the stimulus periphery. For the social test, we measured the mean of the minimal distances to the nearest nestmate. To do this, every 30 s the video was paused, and a screenshot taken, generating 10 images per trial, that were then used to calculate the distance to each nestmate. Each repetition was composed of 50 measurements (10 imagens with 5 nestmates), and the minimal distance per repetition was calculated with the average of the 10 minimal distances (one measurement per image). All open field test variables were calculated using the *Ethovision* software (Noldus, Wageningen, Netherlands). All shelter test and aggression test variables were calculated using the BORIS software (*Behavioural observation research interactive software,* Friard & Gamba, 2016). Social test variable was measured with the software *Image J* (Schneider & Rasband, 2012).

3.2.4 Statistical Analyses

Statistical analyses were done with the program R, version 4.0.3 (R Core Team, 2020). First, we tested for individual-level and colony-level repeatabilities over time of the seven behavioural variables collected during the five repeats of personality test. This analysis, based on N = 54 individuals, was done by linear mixed model (LMM)-based intra-class correlations (R package *rptR*) (Stoffel et al., 2021) with individual and nest identity as a random factor. P-values were calculated by 10,000 Monte Carlo permutations.

After that, we assessed individual-based associations between these seven different behavioural variables by a multivariate LMM, using the R package MCMCglmm (Hadfield, 2010), including individual and nest identity as random factors. Based on the among-individual variance matrix provided by this multivariate model, we calculated pair-wise correlation coefficients between the seven behavioural variables. If 95% credibility intervals did not include zero, the pair-wise associations tested were considered statistically significant (Houslay and Wilson, 2017). To understand further how novelty effect could have influence results, we ran the same analysis with all individuals but without the first repetition.

Using the individual variance matrix provided by this multivariate model, and the following pair-wise correlations, we also calculated the behavioural variance on time, to test whether behaviour response differed across repetitions.

3.3. RESULTS

3.3.1 Temporal consistencies across time

We found significant individual-level repeatability for two out of seven behaviours from two out of the four different tests, the distance covered in the open field test and the latency to exit the shelter (Table 1). No significant colony-level repeatabilities were found in any test (Table 1).

When the first repetition was excluded from the analysis, an additional behavioural variable was found to be significantly repeatable, the percentage of time in the centre of the open field (see appendix of chapter, Table C), but still no significant colony-level repeatabilities were found.

Table 1. Individual-level and colony-level repeatabilities of different behavioural variables quantified during repeated open field, shelter, aggression, and social tests.

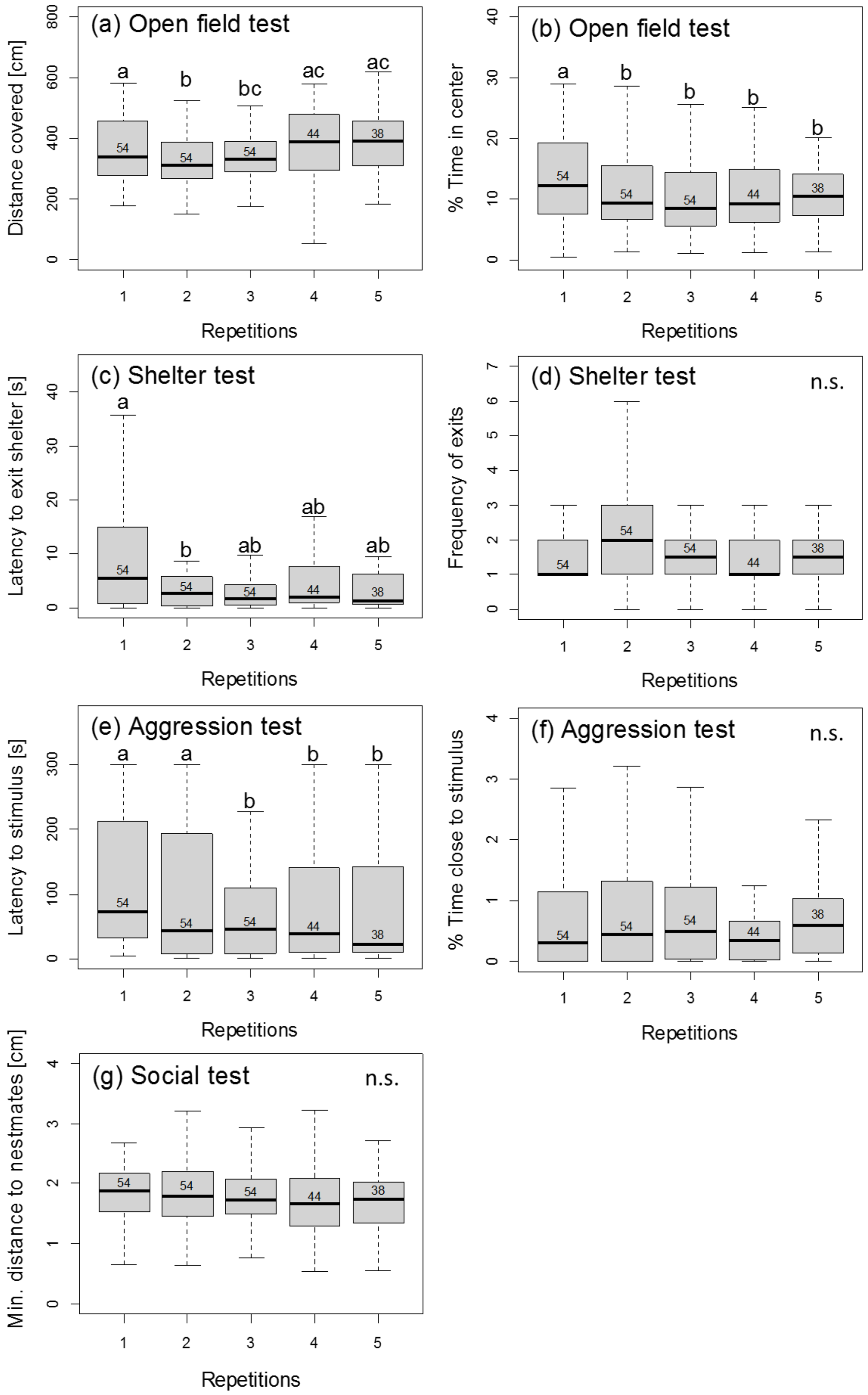
		Individual level			Colony level		
Tests	Behaviours	*R*	$CI_{95\%}$	*P*	*R*	$CI_{95\%}$	*P*
Open field	Distance covered	0.176	[0.037, 0.298]	< **0.001**	0	[0, 0.576]	0.999
	% Time in the centre	0	[0, 0.097]	0.999	0	[0, 0.038]	0.379
Shelter test	Latency to exit shelter	0.109	[0.001, 0.224]	**0.015**	0.013	[0, 0.086]	0.268
	Frequency of exits	0.02	[0, 0.124]	0.289	0.003	[0, 0.047]	0.299
Aggression test	Latency to the stimulus	0	[0, 0.101]	0.999	0.002	[0, 0.039]	0.324
	% Time close to stimulus	0	[0, 0.103]	0.999	0.02	[0, 0.086]	0.093
Social test	Min. distance to nestmates	0	[0, 0.095]	0.999	0.002	[0, 0.039]	0.336

Source: Jardim, V.A. (2022). Legend: Data from *N* = 54 adults *H. striatula* forager ants; each individual underwent these tests either 3, 4 or 5 times depending on its surviving time. Analysis by LMM-based intra-class correlations with 10,000 permutations, including individual identity and colony identity as random factors. Significant effects are given in bold.

3.3.2 Changes across time

Three out of the seven behaviours measured showed significant differences in their absolute levels across the five repetitions, the total distance covered (multivariate GLMM: $P < 0.05$) and the percentage time spent in the centre of the open field ($P < 0.05$), and the latency to get close to the stimulus in the aggression test ($P < 0.05$). Pairwise comparisons revealed that, for the variables latency to exit and % time in the centre, the first repetition was significantly higher than the following ones. A similar pattern was also found in the other two variables, but this time, the first two repeats were significantly higher than at least some of the following ones (post-hoc statistics in Figure 7). The other four behaviours, latency to exit shelter, frequency of exits, percentage time close to stimulus, minimal distance to nestmate, did not show significant changes across time (all *P* > 0.050).

Figure 7. Changes in behaviour across 5 repetitions for variables quantified during repeated open field, shelter, aggression, and social tests.



Source: Jardim, V.A. (2022). Legend: Variables quantified in the (a, b) open field test, (c, d) shelter test, (e, f) aggression test and (g) social test in *H. striatula* ants. The numbers of individuals are given inside the boxplots for each repetition. Pairwise comparisons by multivariate GLMM using MCMC methods (10,000 permutations). Different letters over the error bars show statistically significant differences between groups. Non-significant differences among groups (*P* > 0.05) are indicated by n.s.

3.3.3 Associations in behaviour across tests

There were no significant associations between the different behaviours either within or across tests. This was evident since confidence intervals of R values did include zero in all cases (Table 2). Also, no significant associations were found when taking out first repetitions from analysis (see Appendix of chapter, Table B)

Table 2. Associations between behavioural variables quantified in the open field, shelter, aggression, and social test.

Behaviours	%TC-OF	LE-SH	FE-SH	LS-AG	%TS-AG	MD-SOC
DC-OF	0.021 [-0.672, 0.730]	0.076 [-0.542, 0.649]	0.057 [-0.694, 0.712]	0.580 [-2.453, 2.742]	< 0.001 [-0.718, 0.702]	0.143 [-0.543, 0.827]
%TC-OF		0.128 [-0.589, 0.795]	0.085 [-0.633, 0.803]	0.071 [-0.712, 0.765]	0.068 [-0.701, 0.781]	0.028 [-0.750, 0.748]
LE-SH			-0.007 [-0.658, 0.764]	0.038 [-0.618, 0.775]	-0.005 -0.658, 0.727]	0.152 [-0.533, 0.874]
FE-SH				-0.031 [0.750, 0.719]	0.011 [-0.747, 0.754]	0.007 [-0.730, 0.717]
LS-AG					-0.053 [-0.806, 0.646]	-0.008 [-0.701, 0.824]
%TS-AG						-0.008 [-0.701, 0.824]

Source: Jardim, V.A. (2022). Legend: Data from N = 54 adults *H. striatula* forager ants; each individual underwent these tests either 3, 4 or 5 times depending on its surviving time. Analysis by multivariate GLMM using MCMC methods (10,000 permutations), including individual identity and colony identity as random factors. Repeatabilities including their 95% confidence intervals (in brackets) are given. None of the associations were statistically significant.

DC-OF: Distance covered in the open field test
%TC-OF: Percentage of time in the centre in the open field test
LE-SH: Latency to exit shelter in the shelter test
FE-SH: Frequency exits in the shelter test
LS-AG: Latency to the stimulus in the aggression test

%TS-AG: Percentage of time close to stimulus in the aggression test
MD-SOC: Minimum distance to nestmates in the social test

3.4. DISCUSSION

3.4.1. Summary of key results

We found indications for the existence of personality in our study species, as two out of the seven behavioural variables showed significant consistency across time (Table 1), although only showing rather low repeatabilities (Bell et al., 2009). In disagreement with our hypothesis, we did not find significant associations between the different behavioural responses, either within or across our chosen behavioural tests (Table 2). Also, nestmates were not more similar to each other in their behavioural response than non-nestmates. Furthermore, three response variables showed significantly changes across time, as behavioural responses of the first one or two repeats were significantly different than at least some of the following ones (Figure 7), probably due to habituation and novelty effects.

3.4.2 Temporal consistency in behaviour

Finding no significant or only low repeatability of behavioural variables is not uncommon in personality studies (Carlson & Langkilde 2013, Liedtke et al., 2015, Parthasarathy et al., 2019, Wuerz, & Krüger, 2015). This is particularly true for invertebrates which, apart from courtship behaviours, tend to show a lower level of repeatability for behaviour traits then vertebrates (Bell et al., 2009). Behavioural variation occurs both at the intra- and inter-individual level (Roche et al., 2016), and thus repeatability calculation considers not only individual behavioural variation between trials but also the behavioural differences between individuals in a population. Consequently, a population who behaves very similarly interferes in how much a behaviour is considered consistent (Bell et al., 2009). This behavioural homogenization may have been one of the underlying causes for the lack of repeatability, especially in the aggression and social test, where individual scores showed a low variation (Figure 7).

The fact that we were using only one caste for our study could have contributed to homogenising inter-individual variation in behavioural responses of our study group. There is evidence of a strong relationship between caste and behavioural types, suggesting that social role is an important factor in behavioural differences (Chapman et al., 2001). It has been proposed that division of labour groups individuals with similar behavioural profiles inside the colony (due to similar thresholds, ontogeny, age, etc., see: Gartland et al., 2022, Pamminger et al., 2014, Wright et al., 2014). Although, there is some debate on whether personality predicts task allocation. Further factors other than personality can explain why individuals in a same caste behave similarly, such as differences in the environment and social interactions during their development and maturation, age polyethism and even plasticity (Gartland et al., 2022, Pamminger et al., 2014, Wright et al., 2014). We suggest that, regardless of the theory of why individuals of the same caste behave more similarly than with other nestmates, studying only one caste may be limiting when working with behavioural consistency.

We found significant, although low repeatability for two behaviour variables: the distance covered in the open field ($R = 0.176$), and in the shelter test for latency to exit shelter ($R = 0.109$). These behaviours variables may be related to personality axes such as, for the open field test, activity, exploration, and boldness for the shelter test (Beckmann & Biro, 2013, Carlson, 2013, Carter et al., 2013), Individuals that displayed higher values of distance covered can be considered to show more active and exploratory behavioural types, since higher values of this variable are directly related to time in activity and locomotion (Carlson & Langkilde, 2013, Russell & Williams, 1973), likely motivated by exploration (Verjat et al., 2021, Jardim et al., 2021). Although seemingly straight forward, the interpretation of the open field test is not as clear-cut as generally assumed (Carter et al., 2013, Walsh & Cummins, 1976). Many studies discuss the idea that the test is not only accessing exploratory and activity behaviours, but also emotionality, fear, boldness, gregariousness (Walsh & Cummins, 1976), and even neophobia (Carter et al., 2013). Although we believe that the open field test is capable of measuring many of these behavioural traits, the variables chosen for the test should be considered. In other studies with ants that used open field testing, variables such as distance covered have been interpretated as linked to exploration and activity (d'Ettorre et al., 2017, Maák et al., 2020, Udino et al., 2017), and that is the interpretation that we followed as well.

Moreover, a shorter latency to emerge from a shelter has been frequently assumed to reflect greater willingness to accept risks, thus representing a bolder behavioural type (, Beckmann & Biro, 2013, Burns, 2008). Other studies had suggested that latency to emerge in a novel environment should not be related to the boldness axis, but rather to exploration (see Koolhaas et al., 1999, Réale et al., 2007 for a broader definition of risk-taking behaviour).

However, in line with Beckmann and Biro (2013), we agree with the idea that a short latency to emerge from a dark refuge into an open novel environment may rather represent risk-taking (i.e., boldness), whereas in this context, exploration is reflected by space use following emergence (Brown et al., 2007, Cote et al., 2010). These traits are probably important for individuals of this caste, who spend a large part of their time in locomotor activity, as their function is to explore the surrounding landscape and forage for food (Hölldobler & Wilson, 1990). Forager can also be exposed to high risk of predation, as they also patrol outside the nest and can encounter aggressive predators or patrollers from other colonies (Chapman et al., 2011) requiring great risk-taking behaviour. Our experiments only worked with foragers and therefore did not investigate causality in the relationship between task allocation and individual behavioural phenotype, but it would be interesting for future studies to work with many castes so we can understand which trait underlies the other. It is important to point out that when discussing personality, we need to consider several axes in which personality is structured and/or more than one context in which it can be expressed (Koolhaas et al., 1999, Réale et al., 2007). In this study, we found repeatability of two behavioural traits in two different contexts, and although these behaviours were not correlated, we still can discuss them separately. These results suggest the existence personality for this species, which until now had not been proven.

We did not find higher levels of repeatability for our behavioural variables, but it is important to note that this does not imply that increasing the number of repetitions in time (numerous test repetitions, as we have done) decreases repeatability. Other studies have found different results regarding how increasing repetitions in time can influence behaviour repeatability (Araya-Ajoy et al., 2015, Carlson et al., 2020, David et al., 2012, Parthasarathy et al., 2021). Indeed, these studies suggested that an increasing number of observations per individual will decrease the error around the estimate, rather than the repeatability estimate itself (Bell et al., 2009). Moreover, some studies indicate that repeatability estimates decrease with an increasing time gap between trials rather than for how long or how many repeats are tested, as estimates are higher when repeated measures are taken closely in time (Bell et al., 2009, Boulton et al., 2014). It is therefore plausible that finding behavioural repeatability is mainly connected to factors other than the number of repeats itself, such as the methods applied (test and behaviours variables that were measured) or how repeats encompass more of the life history of the individual itself (observation time is relative when studying species with shorter life span, such as invertebrates) (Araya-Ajoy et al., 2015, Bell et al., 2009,).

. We predicted that aggressive behaviour would show greater repeatability, but our results revealed that interactions with the stimulus were short, and aggressive behaviour was rarely observed, with no significant repeatability. This fact could be related to the generally low levels of aggression in our study species (Giraud et al., 2001, Lommelen et al., 2010), but also possibly be due to the test design. It is possible that our setting using a dead (defrosted) heterospecific ant as stimulus did not provide a relevant simulation of a natural heterospecific encounter, thus influencing the motivation of the focal individual to interact and consequently, affecting the reliability of the variable as a measurement of intraspecific aggression.

We did not find colony-level repeatability, and this may be due to various methodological limitations, mostly revolving around colony closeness in many senses. Firstly, in a statistical sense, the low number of colonies collected may have led to a low power of the test. Thus, we cannot rule out the possibility of a false negative result. Although six sub-nests were set up for the experiment, it is important to remember that they came from two larger colonies. In addition to the analysis limitation of describing a nest profile from only two samples, there is the factor that most likely these nests also have similar behavioural profiles. The colonies were collected in near proximity to each other (less than 15 meters apart), and since *H. striatula* colonies are highly mobile (Giraud et al., 2000), genetic flow would be highly frequent, resulting in genetic closeness. On top of this, similarity in external conditions, such as temperature, humidity, food quality, availability, and heterogeneity, inter and intra specific competition etc., to which individuals were exposed to during the nest development could result in convergent strategies and similar colony profiles. All these factors may have synergistically contributed not only to the lack of a collective behaviour profile, but also for the behavioural homogenization of the group, that can possibly contribute to a lower repeatability.

3.4.3 Changes across time

It is well known that repeated exposures to a stimulus can modulate an individual’s behavioural response (Groves and Thompson 1970, Jardim et al., 2021, Mettke-Hoffmann et al., 2006, van Oers et al., 2005). For most of our behavioural variables we found significant differences in the ants’ behavioural response over time. For these variables, first trials generally showed a different response magnitude compared to the following one. This pattern is not uncommon when working with behavioural tests (Dingemanse et al., 2002, van Oers et al., 2003, Jardim et al., 2021) and can be linked to processes such as novelty effect, motivation and habituation to stimulus or test settings. Learning and habituation, here defined simply as decreased response to repeated stimulation (Groves & Thompson, 1970), may affect the novelty perception of a test replicated several times (Greenberg & Mettke-Hofmann, 2001, Wahlsten, 2001), affecting the individual motivation to explore, interact and respond to the stimulus and test settings (Jardim et al., 2021, van Oers et al., 2005). For the open field test variables, distance covered and percentage of time in the centre, a greater response in the first trial could be an indication of increased exploration and risk-taking behaviour (d'Ettorre et al., 2017), that decreased after first exposition, highlighting the novelty effect that the test can carry (Jardim et al., 2021). An opposite effect can also be observed, where individuals can show more fearful, caution and shy behaviour in face of the test novelty (Boissy, 1995), as we observed in the latency variable, where it took more time for individuals to interact with the stimulus in the first trial.

We have shown that removing the first trial had a small although significant effect on repeatability, as an additional behavioural variable shows low (*sensu* Bell et al., 2009), but repeatable behaviour: the percentage time in the centre during the open field test. For this variable, the mean response of the first repeat was significantly different from subsequent responses (Figure 7). Other studies have also discussed this issue, showing that behavioural responses can vary strongly with the individual's increasing familiarity with the test, and therefore variation in behavioural responses in repeated samplings must be interpreted with caution (Dingemanse et al., 2002, Jardim et al., 2021, Mettke-Hofmann et al., 2004, Russell & Williams, 1973,). In their work, Rudeck et al., (2019) showed that, in laboratory mice (*Mus musculus*), behavioural responses of activity and exploration in the open field test present stable patters after three days of habituation They also discuss the idea that a simple strategy can be established to introduce habituation as a preliminary step in behavioural experiments which requires a known environment, improving behaviour repeatability in personality studies.

3.4.4 Associations in behaviour across tests

We could not describe behavioural syndromes as we could not find any associations across behaviours in different contexts. This may have been due to the possibly most of the tests apparently have simply failed (at least under your experimental conditions) to detect personality differences. All too often in the field of animal personality, studies that corroborate the existence of behavioural syndromes are seen as more well-rounded than studies that do not find these associations. Discussing the idea of independently functioning behavioural axes should also be considered an equally valid result, since they are both valid premises. Although not much discussed in animal personality studies, the lack of association between multi context behaviours does not contradict the current theory on personality. For example, in their model, Réale et al., (2007) describe the existence of five distinct personality axes, that are not necessarily associated. The extent to which behaviour traits are independent or form part of a behavioural syndrome is a controversial and important issue discussed extensively (Koolhaas et al., 1999, Réale et al., 2007, Sih et al., 2004,).

It is possible that we did not find indications of a behaviour syndrome in our study species, not because we failed to access it, but possibly because it simply does not exist in our population, at least not for the behavioural contexts considered here. As described previously, the concept of behavioural syndromes is more complex and involves the notion of context-independent behavioural patterns and requires associations between distinct personality axes. These correlations are discussed in many approaches, and are proven through physiological, ethological, and evolutionary evidence (Gatland et al., 2022, Réale et al., 2007). But it is important to highlight that they are not a necessary requirement when working with personality, and as often as these correlations can exist, they cannot. Behavioural variables that do not correlate with others also highlight the idea that behavioural traits result from distinct evolutionary and physiological processes (Gatland et al., 2022, Lacasse & Aubin-Horth, 2014, Sih et al., 2004), that do not necessarily need to be correlated, and that individuals may show distinct behavioural type compositions.

3.4.5 Conclusions and recommendations

The findings of our study suggest that behavioural variables linked to activity and exploration can be useful tools when working with personality, especially in invertebrates, showing higher behavioural repeatability (for other invertebrate studies working with these behavioural parameters see Carere et al., 2018, d’Ettorre et al., 2017, Udino et al., 2017). We also highlight the importance that studies that adapt tests and behavioural variables from other groups or contexts, make sure that these parameters are still relevant and make sense for the studied species and behaviours. The lack of across context correlations for our behavioural variables suggests that behavioural axes can function independently. It would be interesting that future studies could also evaluate how different castes respond to the same multi personality essays tests, since the colony is composed of individuals with different experiences and development history executing different functions in different castes. Also, we highlighted the importance of considering repeated exposure effects such as habituation and novelty effect in behaviour responses, especially in first trials, as they could be, even if slightly, biasing results and interfering in the interpretations of the behaviours in question. Future studies could habituate individuals to the test setting lower novelty effects for the following trials.

Declaration of Competing Interest

The authors declare no competing interests. The authors have no conflicts of interest to declare that are relevant to the content of this article.

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Authors contributions

Veridiana Jardim: Conceptualization, Project administration, Funding acquisition, Methodology, Investigation, Data Curation, Formal analysis, Visualization, Writing - Original Draft. **Ronara Ferreira- Châline:** Conceptualization, Project administration, Supervision, Formal analysis, Visualization, Writing - Review and Editing. **Heiko G. Rödel:** Supervision, Formal analysis, Writing - Review and Editing. **Nicolas Châline:** Conceptualization, Project administration, Supervision, Formal analysis, Visualization, Writing - Review and Editing.

6. Appendix: Supplementary Information

Supplements to Results

Table C. Individual-level and colony-level repeatabilities of different behavioural variables quantified during repeated open field, shelter, aggression, and social tests.

		Individual level			Colony level		
Tests	Behaviours	*R*	$CI_{95\%}$	*P*	*R*	$CI_{95\%}$	*P*
Open field	Distance covered	0.187	[0.025, 0.327]	**0.003**	0	[0,0.076]	0.999
	% Time in the centre	0.153	[0.006, 0.298]	**0.01**	0	[0, 0.072]	0.999
Shelter test	Latency to exit shelter	0.136	[0.001, 0.273]	**0.028**	0.015	[0, 0.098]	0.272
	Frequency of exits	0	[0, 0.113]	0.999	0	[0, 0.047]	0.999
Aggression test	Latency to the stimulus	0.082	[0, 0.225]	0.109	0.015	[0, 0.101]	0.224
	% Time close to stimulus	<0.001	[<0.001, 0.002]	0.762	0.014	[0, 0.072]	0.192
Social test	Min. distance to nestmates	0	[0, 0.124]	0.999	0	[0, 0.055]	0.999

Source: Jardim, V. A. (2022). Legend: Data from *N* = 54 adults *H. striatula* forager ants; without the first repetitions. Analysis by LMM-based intra-class correlations with 10,000 permutations, including individual identity and colony identity as random factors. Significant effects are given in bold.

Table B. Associations between behavioural variables quantified in the open field, shelter, aggression, and social test.

Behaviours	%TC-OF	LE-SH	FE-SH	LS-AG	%TS-AG	MD-SOC
DC-OF	-0.171 [-0.774, 0.417]	-0.045 [-0.696, 0.564]	0.049 [-0.677, 0.775]	-0.423 [-1.691,1.954]	-0.081 [-0.781, 0.641]	0.229 [-0.475, 0.865]
%TC-OF		0.075 [-0.58, 0.756]	0.143 [-0.697, 0.801]	0.132 [-0.656, 0.756]	0.186 [-0.589, 0.844]	-0.16 [-0.859, 0.592]
LE-SH			-0.032 [-0.722, 0.688]	0.046 [-0.678, 0.701]	-0.056 [-0.729, 0.662]	0.073 [-0.66, 0.741]
FE-SH				-0.031 [-0.755, 0.725]	0.023 [-0.760, 0.81]	-0.02 [-0.742, 0.794]
LS-AG					-0.071 [-0.846, 0.658]	-0.094 [-0.818, 0.653]
%TS-AG						-0.094 [-0.818, 0.653]

Source: Jardim, V. A. (2022). Legend Data from *N* = 54 adults *G. striatula* forager ants without the first repetitions. Analysis by multivariate GLMM using MCMC methods (10,000 permutations), including individual identity and colony identity as random factors. Repeatabilities including their 95% confidence intervals (in brackets) are given.

None of the associations were statistically significant.
DC-OF: Distance covered in the open field test
%TC-OF: Percentage of time in the centre in the open field test
LE-SH: Latency to exit shelter in the shelter test
FE-SH: Frequency exits in the shelter test
LS-AG: Latency to the stimulus in the aggression test
%TS-AG: Percentage of time close to stimulus in the aggression test
MD-SOC: Minimum distance to nestmates in the social test

CHAPTER 4: GENERAL DISCUSSION

My thesis aimed to explore the plurality of the study of individual differences in behaviour in social animals, in an ant and in a mouse species. In the two main chapters (chapters 2 and 3) I undertook a more methodological approach, discussing how the observation of behaviour can be affected by several test aspects, ranging from the test apparatus, behavioural variables, to the number of repeats. Inherent of any young field of research, especially when working with invertebrates, the study of animal personality is still fraught with conceptual and methodological difficulties (Carter et al., 2013), especially about how personality traits are defined, measured, and interpreted.

4.1. METHODOLOGICAL LIMITATIONS AND INTERPRETATION OF BEHAVIOURAL RESULTS

As I have shown in my studies on ants, it is still needed to understand more about the way how invertebrate species perceive and interact with their environment, especially in complex social groups. This is especially important when studying the individual expression of behaviour, so that tests can be designed to have significant biological value and thus allow to choose proper behavioural variables to assess the behavioural traits of interest. It should be considered that many of the behavioural tests used in the field of animal personality were originally aimed at the specificities of vertebrate species, mainly mammals (Gosling, 2001; Kralj-Fišer & Schuett 2014). Many of the tests used in behaviour measurement can be applied almost universally across species, such as the open field test (Walsh & Cummins, 1976). However, more complex tests, such as tests measuring the latency to leave a shelter ("shelter tests"), or aggression tests, that relies on the identification of other stimulus or apparatus introduced in the arena, still need to consider what is biologically relevant for the studied species. More than the test itself, it is needed to evaluate the variables that were chosen in each test and what they are actually measuring (Carter et al., 2013). A frequently recurring critique in personality studies involves the validity of the tests that are used to measure the trait of interest (Duckworth & Kern, 2011). But even further than that, the interpretations of the variables obtained from the tests are also as fundamental as the choice of test and its execution.

As observed in the judgment bias experiments article with mound-building mice (chapter 2), the results obtained by repeated testing allowed us to evaluate the test bias and possible variables that could be affecting individual response. Just like we observed in the article on ants (chapter 3), repetition in time seems to be an essential factor to be considered in personality studies. In both articles it becomes obvious that a similar process in which the first exposition to the test generated a response pattern different from the following repeats. In our study on judgment bias in the mound-building mouse (chapter 2), the pattern was strongly related to personality, as some individuals with a specific personality type showed to be more affected by test novelty (Jardim et al., 2021). In turn, in the article on ants (chapter 3), we have shown that, excluding the first trial resulted in higher behaviour repeatability (higher R and one more significant repeatable variable), further highlighting the effect of the first repetition in behavioural tests. The discussions of the results in both articles (chapter 2 and 3), reiterate the need for caution in the interpretation of behavioural responses. As discussed, in the goal to deflect/lower the effects related to the test novelty, individual habituation to test settings should be part of the test protocol, especially for personality studies where behaviour repeatability is a requirement. This discussion also highlights the idea that the test and the choice of behavioural variables is only a small part of the study of animal personality. Without taking into account the interpretation of the result regarding how the behavioural variables are structured in time and across individuals, and without the understanding of which variables affect and are affected by the test design, the results may appear random and with no significance.

We also have shown that individuals with different personalities are not only affected by the test settings and novelty in different ways, as it became obvious in the paper presented in chapter 2, but also respond physiologically in different ways, as a result of conditions experienced during early life. In the article presented in the appendix, where we study oxidative parameters, we have shown that several early life parameters synergize and modulate various aspect of the adult physiology linked to the physiology of stress. In relation to personality, we have shown that oxidative DNA damage in skeletal muscle tissue was higher in animals with a relatively higher body mass as pups, but only in less exploratory individuals, a phenotype typically characterized by a higher adrenocortical activity. Interestingly, that shows that personality alone could not explain inter-individual variation in several oxidative status parameters in different tissues of adult individuals. However, the interaction between the animals' relative starting body mass within the litter, modulated by individual differences in exploration tendency was decisive. Furthermore, we have shown higher levels of oxidative damage to DNA in the liver in animals stemming from larger litters, suggesting long-lasting consequences of increased sibling competition during early life. These results reiterate the relevance of inter-individual differences in behaviour with respect to different aspects of an individual's life, not only punctually but also throughout the individual development. These results also highlight the importance of personality as a modulator of physiology, as it mediates the way individuals interact with their environment.

Difficulties and discussions regarding the methods applied in personality research are not new topics in the field of animal behaviour. Samuel Gosling in his review in 2001 proposed to analyse the personality studies that had been published at this time. He restricted his research only to vertebrates (which at the time was most studies published so far), and with more than 170 entries, more than 60 species considered deemed "impossible" to carry out comparative studies. He stated that the diversity of traits examined, species studied, methods used, and analyses performed made a quantitative comparison impossible to perform. More than 20 years had passed since this review has been published, and many aspects have changed in the personality field. Now, not only restricted to vertebrates, the field of personality also increasingly encompasses more diverse taxonomic groups, and the discovery of personality in new species increased with each publication. In the meantime, many theoretical articles have been published searching to standardize the techniques and terminologies in the field (Sih & Bell, 2004, Harding et al., 2004, Stamps & Groothuis, 2010), and many others that test and discuss such theoretical terms and methodologies, including the articles in this thesis. In this sense, studies that propose the discussion on methods, tests and variables are imperative for the future of the field of personality. Without the consensus of methods and terminologies there is no possibility for comparative studies, and without that, the future of the field will be limited, if not impaired. In this sense, I hope that the articles here presented can shed a light on many of the methodological limitations that difficult these important knowledge exchanges derived from comparative studies, and this time not only restricted to vertebrates.

4.2 BEHAVIOURAL SYNDROMES

Although the articles presented in chapters 2 and 3 have divergent conclusions about the corroboration of behavioural syndromes, this does not necessarily go against what has been proposed by Réale (2007) and the results from many other researchers (Sih et al., 2004, Bell, 2007, Sih & Bell, 2008, etc). As discussed in the article of chapter 3, significant correlations of behavioural traits are not an obligation when studying personality in a population. Many studies discuss the proximate and ultimate causes of behavioural syndromes, arguing that evolutionary, genetic, cognitive, and neuroendocrine processes can cause traits to have similar pathways, and thus leading to correlations among them (Koolhaas et al., 1999, Sih et al., 2004). It is important to highlight that the behavioural traits that correlate with each other (in studies that describe behaviour syndromes) are, many times, species-specific and relate to the evolutionary history of each species. In other words, many articles describe behavioural syndromes for many species where, for example, bolder individuals are also more active, more aggressive and less social (correlations between exploration, aggressiveness, boldness, speed and time in foraging behaviour, for example in common voles: *Microtus arvalis*, Herde & Eccard, 2013, European rabbits *Oryctolagus cuniculus*: Rödel & Monclús, 2011, fish: *Rivulus hartii*, Fraser et al., 2001, *Gasterosteus aculeatus*, Bell & Stamps, 2004, Bell, 2007, invertebrates: *Dolomedes triton*, Johnson & Sih, 2005). These correlations between behavioural traits are reinforced in a way that it makes it seem that, firstly, behavioural syndromes must exist for all study groups, and secondly, that the behavioural correlations follow necessarily these patterns. In the two articles presented in this thesis, we showed that, primarily, we do not necessarily need to find behavioural syndromes when discussing personality (chapter 3), but that in many cases these correlations across context exist (chapter 2, more exploratory individuals are faster). It is limiting to reinforce the idea that behavioural patterns exist in a certain conformality without considering that several factors (e.g., evolutionary trajectory, occupied niches, intra and interspecific competition, etc.) can modulate the way that species behave and how these behaviours correlate to each other to be adaptive for the individual.

Despite not finding any other study that describes behavioural syndromes for the studies genus, we did, however, find other studies on ants that described behavioural syndromes, at the individual and colony level. For example, in a study on *Myrmica* ants, Chapman et al. (2011) showed that the patroller caste exhibited a boldness–aggression syndrome (positive correlation) that was not present in brood carers caste, and moreover, at a colony level, sociability was positively correlated with boldness. In the ant species *Temnothorax rugatulus*, Bengston and Dornhaus (2014) found colony level syndromes suggesting that colonies consistently differ in coping style, as some were more risk-prone, whereas others were more risk-averse. These are a few examples that highlight that not only we can discuss behavioural syndromes in ants, but additionally we can discuss it in an individual and group level.

In complementary ways, the articles discuss the plurality of factors that interfere with individual behavioural expression. These interferences can either be due to methodological issues, limitations and biases, whether when performing the experiments, choosing tests and variables, or interpreting the behavioural results (Vazire et al., 2007; Carter et al., 2013; Bateson & Martin, 2021). Poor-quality measurements, as low sample sizes, can lead to low statistical power and unrepeatable findings (Bateson & Martin, 2021). Maximising the quality of measurements should therefore be a priority in the behavioural sciences

4.1 FINAL CONSIDERATIONS

In this doctoral thesis I discuss the pluralities of working with inter-individual differences in behaviour. I explored the proximate processes of differences in behaviours, either trying to understand cognition and learning, or how personality is structure and expressed and its interactions with early life parameters throughout the individual's development. As for the studied species, I investigated eusocial and social species from two distinct taxonomic groups (mammals and insects). I show the methods applied, such as test design or behavioural variables, must be chosen and executed with care, for they can impact greatly on observable behaviour and their interpretations.

I also have shown that, not only variables and tests can influence the observable behaviour, but also time. Repetitions are imperative for personality studies and have a significand impact in behaviour consistency. More than that, time is also a fundamental part in habituation, a process that have showed to be an important aspect on behaviour expression in personality tests. It is clear that the peculiarities of each species must be considered when planning and designing an experiment, but beyond that, this thesis has shown that we can design studies and discuss personality as unique process that permeate the divergences of taxonomic groups.

In this way, I hope that this thesis will contribute to the discussion on methods and theories regarding individual differences in animal behaviour. There is still much to be discovered in this growing field, even more so when we consider the range of species where we have not even begun to study such behavioural processes. I believe that each article in its peculiarities embarks on future study directions, but in general, we still have a lot to consider about the way we perform tests and the variables we choose to measure specific behavioural traits. On top of this, we must be careful when interpreting these results, seeking to understand the possible variables that may be influencing behaviour variation, whether between groups, individuals or over time. The effort to understand the real mechanisms behind measurable behaviour is a requirement in order to make pertinent conclusions and suggestions for these species, whether they are ants, mice or even us.

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CHAPTER 6: APPPENDIX

Early life parameters and personality affect oxidative status in an altricial rodent

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Abstract - It is increasingly recognized that alterations of the cellular oxidative status might be an important cost underlying challenging early life conditions. For example, an increased litter size can impose challenges as the offspring will face increased competition for maternal resources. Within a litter, individuals with relatively higher starting mass typically show higher growth rates, which can lead to increased oxidative damage. We investigated long-term consequences of these early life parameters on the oxidative status in mature mound-building mice (*Mus spicilegus*). Individual differences in the animals' exploration tendency were assessed by repeated open field and novel object tests. We predicted less exploratory phenotypes, which typically show a higher stress responsiveness, to be particularly susceptible to possible effects of these early life parameters on oxidative status. We quantified oxidative damage of DNA (8-hydroxy-2'-deoxyguanosine levels, 8-OHdG) and proteins (protein carbonyl content, PCC), and activities of the antioxidants catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) in liver and skeletal muscle tissue. 8-OHdG levels were positively associated with CAT and SOD in both tissues, indicating that increased oxidative DNA damage was associated with an upregulation of antioxidant production. Hepatic DNA damage after maturity was increased in animals from larger litters. In less exploratory animals, DNA damage and the activity of CAT and SOD in the muscle were increased, but only in individuals with higher relative starting mass (measured on postnatal day 9). This interaction may be explained by the higher adrenocortical activity in less exploratory phenotypes and the higher growth in relatively heavier pups, two factors known to increase oxidative stress. These findings contribute to enlightening the complex interplay between early life conditions, personality and oxidative status.

Keywords: DNA damage, exploration tendency, litter size, mound-building mouse, *Mus spicilegus*, oxidative stress

6.2.1 INTRODUCTION

Challenging conditions experienced during early development can have profound, and even life-long consequences on an individual's health, reproduction and survival (Costantini & Marasco, 2022; Lindström, 1999; Lummaa & Clutton-Brock, 2002; Metcalfe & Monaghan, 2001; Mousseau & Fox, 1998). It has been suggested that one of the key mechanisms involved in the mediation of such long-term effects might be oxidative stress, i.e. an increased production of reactive oxygen species (ROS) relative to the body's antioxidant defense (Costantini, 2008; Costantini, 2019; Romero-Haro & Alonso-Alvarez, 2020; Selman et al., 2012; Smith et al., 2016; Yang et al., 2020). Such an imbalance of the redox system, resulting in oxidative damage in different cells and tissues can accelerate aging and limit reproductive success, and can contribute to the development of various chronic diseases (de Araújo et al., 2016; Liguori et al., 2018; Romano et al., 2010; Yousefzadeh et al., 2021).

A first essential step to better understanding the possible mediating role of oxidative stress in life-history trade-offs and phenotypic programming is to explore long(er)-term consequences of early life conditions on parameters of oxidative status (Song et al., 2009). In this context, litter or brood size have been highlighted as a prominent factor shaping the early developmental environment in mammals and birds. Competition among offspring for restricted maternal/parental food resources is usually higher in larger litters or broods (Mock & Parker, 1997). In altricial mammals, this is evident by the typically lower growth rate in offspring from larger litters at least until weaning, as the share of milk obtained by an individual pup typically decreases with increasing litter size (Koskela, 1998; Machin & Page, 1973; Mendl, 1988; Rödel et al., 2008b).

The stress-inducing character of increased sibling competition in enlarged broods or larger litters is further supported by studies reporting higher glucocorticoid levels prior to weaning or fledging in offspring developing under such conditions (birds: Gil et al., 2019; Hardt et al., 2017; Vitousek et al., 2017; mammals*:* Cohas et al., 2021; Fey & Trillmich, 2008, but see Kozlowski & Ricklefs, 2011; Rödel et al., 2010). Accordingly, as chronic stress is known to deteriorate an animal's oxidative status (Costantini et al., 2011), several studies have found indications of higher oxidative damage and/or lower antioxidant activity in offspring from larger or enlarged broods or litters (Bourgeon et al., 2011; Costantini et al., 2006; Gibson et al., 2015; Gil et al., 2019, but see López-Arrabé et al., 2016; Losdat et al., 2010).

The relative differences in starting mass among litter siblings (i.e. within a litter) can be another relevant feature of the early development potentially affecting an individual's oxidative status. Such within-litter differences can lead to a cascade of effects reinforcing differences in growth between siblings (Hudson et al., 2011). In general, the increased metabolic activity during periods of increased growth comes at some expenses, as it can lead to increased ROS production, thus causing oxidative damage to cells and tissue (Alonso-Alvarez et al., 2007; Smith et al., 2016). Studies in some species of altricial small mammals have shown that pups with a relatively higher body mass at birth get access to a higher share of milk, and also consistently occupy central positions in the litter huddle, while relatively smaller ones are more often displaced to the periphery (European rabbit *Oryctolagus cuniculus*, Bautista et al., 2015; laboratory rat *Rattus norvegicus*, Bautista et al., 2010; house mouse *M. musculus*, Zepeda et al., 2018). As a consequence, relatively heavier offspring profit more frequently from the warmer and energetically more favorable center of the huddle, resulting in a higher conversion of mother's milk into biomass, thus further contributing to their faster early growth (Rödel et al., 2008a; Zepeda et al., 2019).

Such early differences in body mass among siblings can even persist into later life stages, possibly due to different growth trajectories between heavier and lighter siblings (e.g., European rabbits, Rödel et al., 2020). However, there are examples that individuals experiencing growth-restricting conditions early in life might accelerate their growth later on, to at least partly catch up in body mass and size (Finkielstain et al., 2013; Metcalfe & Monaghan, 2001). Thus, when studying the potential consequences of increased early growth rates on parameters of oxidative status, it may be useful to consider potential periods of accelerated catch-up growth during postweaning life.

The individual behavioural phenotype is a further important aspect which may alter or modulate an animal's physiological response to a challenge. Studies in mammals and birds have shown a generally higher or more chronic activation of the hypothalamic-pituitary-adrenal (HPA) axis in more reactive, less aggressive and less exploratory phenotypes, as opposed to more proactive (Koolhaas et al., 1999), aggressive (Veenema et al., 2003) and exploratory ones (Baugh et al., 2017; Carere et al., 2003; Lavergne et al., 2019; Lendvai et al., 2011; Montiglio et al., 2012; Rossi et al., 2018; Stöwe et al., 2010). An increased HPA axis activity, releasing higher levels of circulating glucocorticoids, is well known for its potential to induce cellular oxidative stress

(Costantini et al., 2011; Spiers et al., 2015). The typically higher resting metabolic rate of more reactive and less exploratory animals (Hürlimann et al., 2019; Réale et al., 2010) may further contribute to a higher ROS production in such individuals (Frisard & Ravussin, 2006). This might be one of the main mechanisms leading to associations between certain personality traits and oxidative profiles, as it has been shown in several studies on vertebrates, including humans (Matsuzawa et al., 2005; Vida et al., 2018). For example, studies in non-human vertebrates indicate a tendency that more reactive, less aggressive and less exploratory individuals have a lower antioxidant capacity compared to more proactive, aggressive and exploratory ones (Costantini et al., 2012; Herborn et al., 2011; Isaksson et al., 2011, but see Costantini et al., 2008).

Thus, considering aspects of an individual's behavioural phenotype could significantly add to our understanding of possible downstream effects of early life challenges on its oxidative status during later life. We carried out such a multifactorial approach for the first time, investigating long-term effects of litter size and of within-litter differences in starting body mass as proxies of the conditions experienced during early life, and of exploratory tendency as a key behavioural ('personality') trait commonly used to phenotype mammals and birds (Carere & Maestripieri, 2013; Réale et al., 2007; Rödel et al., 2015). We used the mound-building mouse (*Mus spicilegus*) as study species, an altricial and polytocous small rodent showing clear and consistent individual differences in its tendency to explore novel environments and objects (Duparcq et al., 2019; Jardim et al., 2021). Mound-building mice give birth to litters of variable sizes (4-11 pups, Sokolov et al., 1998), and in our study we made use of the natural variation in this parameter; thus, we did not manipulate litter size. For evaluating the animals' oxidative status, we quantified (a) from liver and skeletal muscle tissues two parameters of oxidative damage, 8-hydroxy-2'-deoxyguanosine [8-OHdG] mutagenic DNA damage (Takeuchi et al., 1996) and protein carbonyl content [PCC] as a marker of protein damage (Dalle-Donne et al., 2003). Damage caused by ROS has important implications in various tissues, although DNA and proteins in the liver have been reported to be among the cellular structures primarily affected by oxidative damage (Cichoż-Lach & Michalak, 2014). Furthermore, we chose to sample skeleton muscle tissue due to the high functional importance to maintain the body's muscle integrity (Marasco et al., 2021). In these tissues, we also measured (b) three markers of enzymatic antioxidant activity reflecting the organism's performance to remove harmful ROS (Ighodaro &

Akinloye, 2018), superoxide dismutase [SOD], catalase [CAT], and glutathione peroxidase [GPx].

Our main goal was to investigate whether individual traits as well as conditions experienced during early life exert long-term consequences on the oxidative status, thus evident during adulthood. Accordingly, we (*i*) expected indications of higher oxidative stress, i.e. higher oxidative damage and/or lower antioxidant activities, in animals from larger litters. We (*ii*) explored the effects of within-litter differences in starting mass (measured on postnatal day 9) on parameters of oxidative status. Since an accelerated growth can entail oxidative costs (Smith et al., 2016), we expected faster growing individuals to show higher levels of oxidative damage and/or lower activities of antioxidants. On the one hand, higher growth rates until around weaning (Rödel et al., 2008b; Zepeda et al., 2019) and possibly beyond can be expected in individuals with a relatively higher starting body mass, with potential negative consequences on their oxidative status. On the other hand, animals with lower starting body mass may show increased catch-up growth after weaning, possibly leading to increased oxidative costs. Furthermore, we (*iii*) predicted that negative consequences of early life conditions may be more pronounced in behavioural phenotypes well known for their comparatively higher HPA axis activity, i.e. in less exploratory individuals compared to more exploratory ones (Baugh et al., 2017; Lavergne et al., 2019; Montiglio et al., 2012). To this end, animals were behaviourally phenotyped for individual differences in exploration tendency by repeated open field and novel object tests (Duparcq et al., 2019; Jardim et al., 2021).

6.2.2 METHODS

6.2.2.1 *Study animals*

We studied mound-building mice of wild origin, descendants from animals caught in Hungary in 1999 and bred at the animal facilities of the Laboratoire d'Ethologie Expérimentale et Comparée at the Université Sorbonne Paris Nord, France. Every 4-5 years since then, additional animals have been captured in the same region in Hungary and integrated into the breeding stock to maintain genetic variation (details in Duparcq et al., 2019).

Animals were kept in polycarbonate cages (32.5 × 16.2.5 cm and 14.2 cm high, Plexx, Elst, The Netherlands) with a layer of wood shavings, in a room with a temperature of around 20

± 1 °C and a 14/10 light/dark cycle (red light on at 12:30 pm). Cages were enriched with two cardboard rolls (length: 10 cm, diameter: 6 cm) and three cotton balls per individual as material for building the nest. Food (rodent standard diet; Special Diet Services type M20, Witham, Essex, UK) and water were provided ad libitum.

We used n = 35 females originating from 15 litters (from 15 different mothers), with litter sizes between 4 and 9 individuals and 1–5 females per litter (more details in the *Ethics note*). On postnatal day 9, animals were individually marked with different symbols drawn on their backs, using a black permanent non-toxic hair dye (Prodye, Weaver Leather Livestock, Ohio, USA; see (Rangassamy et al., 2015) for details on this procedure). These symbols were checked every few days and were redrawn if necessary to keep individual identities. Furthermore, animals were weighed individually on that day (postnatal day 9), as well as on postnatal days 32 (first behavioural test session), 43 (second behavioural test session) and when they were killed off (around postnatal day 145). On postnatal day 28, animals were weaned, and their sex was determined by external genital inspection. After that, they were kept in mixed-sex sibling groups until day 55, shortly before this species reaches maturity (Busquet et al., 2009). Afterward, females were kept in same-sex groups of 2 to 3 individuals (usually litter sisters) per cage (n = 17 cages). Two litters of more than 4 sisters were split up into different cages, and 4 single females were merged together into two cages. Thus, for statistical analysis, we used the cage identity (next to litter identity) as a random intercept factor to consider possible differences among the different cages (see below).

6.2.2.2 *Behavioural phenotyping*

Individual differences in exploratory activity were assessed by repeated behavioural tests, by the combination of an open field test and directly after by a novel object test. Both tests were carried out two times, on postnatal day 32 (T_1) and on day 43 (T_2). The apparatus was made of white polyethylene and was used for both tests. It consisted of a circular open field arena with a diameter of 60 cm surrounded by walls with a height of 65 cm. All behavioural tests were carried out during the dark (red light) phase, i.e. during the activity period of this species. More details on procedures are given in (Jardim et al., 2021). The behavioural tests were carried out with N = 35 focal females from which oxidative status parameters were taken.

6.2.2.2.1 *Open field test*

During each of the two test repeats, individuals were placed singly in a defined peripheral position at the edge of the arena and were video recorded for 5 min while exploring the arena. From the video footage, we quantified the total distance covered using the software Ethovision, version XT10 (Noldus Information Technology, Wageningen, The Netherlands).

6.2.2.2.2 *Novel object test*

After 5 min of open field test, the individual was caught and kept in an opaque plastic box inside the arena for around 20 s, while a novel object was introduced into the center of the test arena. The individual was then released from the box into the arena. After 5 min of testing, animals were caught and returned to their home cage. On postnatal day 32 the mice were confronted with a small transparent and round glass pot, and on postnatal day 43 with a rounded kidney-shaped metallic box (details in Jardim et al., 2021). Video footage was analyzed using the software Boris, version 7.9.8 (Friard & Gamba, 2016). We recorded (a) the latency to touch the novel object and (b) the time subjects spent exploring the object by climbing and moving while being on top of it.

6.2.2.3 *Measurements of parameters of oxidative status*

Around postnatal day 145 (± 2 days), the animals were killed off by decapitation. Immediately after, animals were dissected and the muscles of both hindlegs and the liver were taken. These tissue samples were stored in 2 ml labelled plastic tubes and were immediately frozen and stored at –80 °C.

Three to four months later, samples were defrosted. We homogenized liver and muscle samples, separately, in Dulbecco’s Phosphate Buffered Saline (Sigma-Aldrich, France) supplemented with 1 mM of phenylmethylsulfonyl fluoride (Sigma-Aldrich, France) as an inhibitor of proteases using a TissueLyser II (Qiagen) at 30 Hz for 1 min. Afterwards, we centrifuged tubes for 10 min at 4 °C to obtain clean supernatants to be used for the assays. We measured (*i*) the concentration of 8-hydroxy-2'-deoxyguanosine (8-OHdG; marker of oxidative DNA damage with mutagenic properties) using the 8-hydroxy-2'-deoxyguanosine ELISA Kit

(Abcam, France), (*ii*) the concentration of protein carbonyls (PCC) using the Protein Carbonyl Content Assay Kit (Abcam, France), (*iii*) the activity of the antioxidant enzyme catalase (CAT) using the Catalase Activity Assay Kit (Abcam, France), (*iv*) the activity of the antioxidant enzyme glutathione peroxidase (GPx) using the Ransel assay (Randox Laboratories, France), and (*v*) the activity of the antioxidant enzyme superoxide dismutase (SOD) using the Ransod assay (Randox Laboratories, France). We standardized values of markers by the concentration of proteins as quantified using the Bradford protein assay with albumin as reference standard (Sigma-Aldrich, France). All assays were run according to manufacturer's instructions. Concentrations of markers were expressed as: ng/mg proteins for 8-OHdG, nmol/mg proteins for PCC, nmol H_2O_2/min/mg proteins for CAT, and units/mg proteins for GPx and SOD.

6.2.2.4 Ethics note

Animals were kept and treated according to accepted international standards (Vitale et al., 2018) and to the ethics and animal care guidelines of France, where the experiments were carried out. Experimental procedures were approved by the French Ethics Committee for Animal Experimentation 'Charles Darwin' (APAFIS#17922-2018112916198301 v8) and by the ethics committee of our institution (SBEA-LEEC-USPN). Thirty-five females were bred especially for this study and the remaining male siblings were used for another research project (Jardim et al., 2021). At around postnatal day 145, these females were killed off by decapitation to obtain tissue samples for the analysis of parameters of oxidative status.

6.2.2.5 Statistical analysis and sample sizes

Statistical analyses were carried out with the program R, version 4.0.3 (R Core Team, 2021). All statistical tests reported in this study are two-tailed. The statistical units were the values obtained from the different study animals, details on sample sizes below.

We tested for litter size effects on (a) pup body mass on postnatal day 1 (*n* = 87 litters with a total of 662 pups, from 51 breeding pairs) and (b) the increase in body mass from postnatal day 1 to day 20 (*n* = 34 litters with a total of 263 pups, from 28 breeding pairs), using a larger data set from our mound-building mouse breeding facility data base. For these analyses, averaged values over all pups per litter were used. We fitted linear mixed-effects models (LMM) using the R package *lme4* (Bates et al., 2015) including the identity of the parental pair as a random intercept factor. Response variables were right skewed, resulting in non-normally distributed model residuals. Thus, data were log[x] transformed, leading to the adjustment of model residuals to a normal distribution, which we verified by normal probability plots.

For each of our focal females (n = 35 individuals from 15 litters), we calculated, within their litter of origin, the body mass ranking relative to their (male and female) siblings, using the body masses determined on postnatal day 9 (i.e., at the youngest age when it was possible to individually mark the animals with black hair dye, i.e. in a low invasive way). This proportional ranking ranged between 0 (lightest pup) and 1 (heaviest pup) and was independent of differences in litter size, thus could be used as predictors within the same multifactorial model (see also below). First, by a multifactorial LMM (Bates et al., 2015), with litter identity and cage identity as random intercept factors, we tested for associations between the ranked body mass (first predictor), litter size (second predictor) and the increase in body mass from postnatal day 9 to 43.

We ran principal component analyses (PCA) based on the (scaled) three different behavioural variables recorded during open field and novel object tests (see above). This was done separately for the variables recorded on postnatal days 32 (T_1) and 43 (T_2). The aim of this analysis was to reduce these three different variables to a single score, expressing individual differences in exploration tendency. The three behavioural parameters considered, the distance covered in the open field arena, the latency to touch the novel object, and the time spent climbing and exploring, have been shown to be significantly repeatable over time in previous studies on the mound-building mouse (Duparcq et al., 2019; Jardim et al., 2021) (see details on the repeatability of these parameters in Table C of Suppl. Materials). The two latter variables were log[x+1] transformed prior to analysis, as they showed a strong right-skewed distribution. Consequently, the resulting PCA scores were well adjusted to a normal distribution, facilitating the use of parametric statistics for further analysis (see below; cf. Jardim et al., 2021). The first axes of both PCAs (at T_1, T_2), which showed highly similar loadings in terms of the direction of how the input variables were associated with it, were averaged for later analyses, hereafter referred to as 'exploration tendency'. Before averaging, we verified that this exploration score was significantly repeatable over time (T_1, T_2), by using an LMM-based intra-class correlation with individual identity as a random factor (R package *rptR* Stoffel et al., 2017).

We assessed individual-based associations between the ten different parameters of oxidative status by a multivariate LMM using the R package *MCMCglmm* (Hadfield, 2010), including litter identity and cage identity as random intercept factors. Pair-wise correlation coefficients between the different parameters of oxidative status were calculated based on the among-individual variance matrix provided by this model (Houslay & Wilson, 2017).

Coming back to the main questions of the study, we tested in a first step the effects of the females' original litter size (covariate), their proportional within-litter body mass ranking on postnatal day 9 (earliest time of individual marking; covariate), and their exploration tendency (PCA score; covariate) on the different parameters of oxidative status (8-OHdG, PCC, CAT, GPx and SOD in liver and skeletal muscle tissue, respectively) by LMMs (Bates et al., 2015). Litter identity and cage identity were used as random intercept factors. Some of the dependent variables (8-OHdG, GPx and SOD) were log[*x*] transformed to adjust model residuals to a normal distribution. The success of these transformations was verified by checking the distribution of model residuals by normal probability plots. We considered all possible 2-way interactions between the 3 covariates (predictors). There was a statistically significant and negative, although weak correlation between two of these predictors, the ranked within-litter body mass and exploration tendency (*marginal* $R^2 = 0.185$, $\beta = -0.444 \pm 0.141$ SE, $P = 0.004$). This is in line with previous studies on animal personality, revealing a certain contribution of early life conditions to the emergence of personality differences (e.g., Rödel & Meyer, 2011), in addition to its genetic basis (van Oers & Mueller, 2010). However, the analysis of variance inflation factors (VIF) for all covariates and 2-way interactions between them (see Table 4) revealed that VIFs were always lower than 3.3, thus showing no indications of interfering (multi)collinearities in any of our models (Faraway, 2006). VIFs were even lower than 1.6 when considering the final models, after step-wise exclusion of statistically non-significant interaction terms (Engqvist, 2005).

In a second step, we substituted the two predictor variables litter size and the proportional within-litter ranking on postnatal day 9 by the growth in body mass between postnatal days 9 and 43 (i.e. until around 2 weeks after weaning). This was done as we found, that early growth was negatively associated with litter size and positively with the within-litter ranking in early body mass relative to littermates (see results for details), thus confirming the findings in various other small mammals (Hudson et al., 2011; Mendl, 1988; Rödel et al., 2008b). As described above, analyses were done by multifactorial LMMs (with litter and cage identity as random factors), separately for all parameters of oxidative status quantified in the liver and in skeletal muscle tissue. We also tested for the interaction of growth with exploration tendency; the latter was included as the second covariate in these models (see Table D in Suppl. Materials). Again, we did not find any indications of interfering collinearities, as VIFs were always lower than 1.6.2. We obtained the same results when using the growth from postnatal days 32 to 43 (instead of from postnatal days 9 to 43) as covariate for these analyses (see alternative statistics in Table E in Suppl. Materials).

LMMs were checked for normal distribution of model residuals by normal probability plots, and we verified that variances were homogeneous by plotting residuals versus fitted values (Faraway, 2006). P-values were calculated by corrected F-tests with Satterthwaite approximation (Bolker et al., 2009). Statistically non-significant interaction terms were eliminated from the models before these were re-calculated (Engqvist, 2005). For all statistically significant LMM, we calculated the *marginal* R^2 (Nakagawa et al., 2017), which can be interpreted as the proportional variance explained by the fixed effects (R package *partR2* Stoffel et al., 2021). We controlled for the potential occurrence of false positives in our set of multiple models (n = 10 different models, see Table 4) by applying an α-level correction (Benjamini & Hochberg, 1995). All initially statistically significant P-values reported in the table remained below the corrected α level (same for Tables A, B in Suppl. Materials). In general, in our results section, statistically significant results ($P < 0.05$) and statistically non-significant results ($P > 0.05$) are hereafter referred to as "significant" and "non-significant".

6.2.3. RESULTS

6.2.3.1 *Effects of litter size and within-litter body mass ranking on growth*

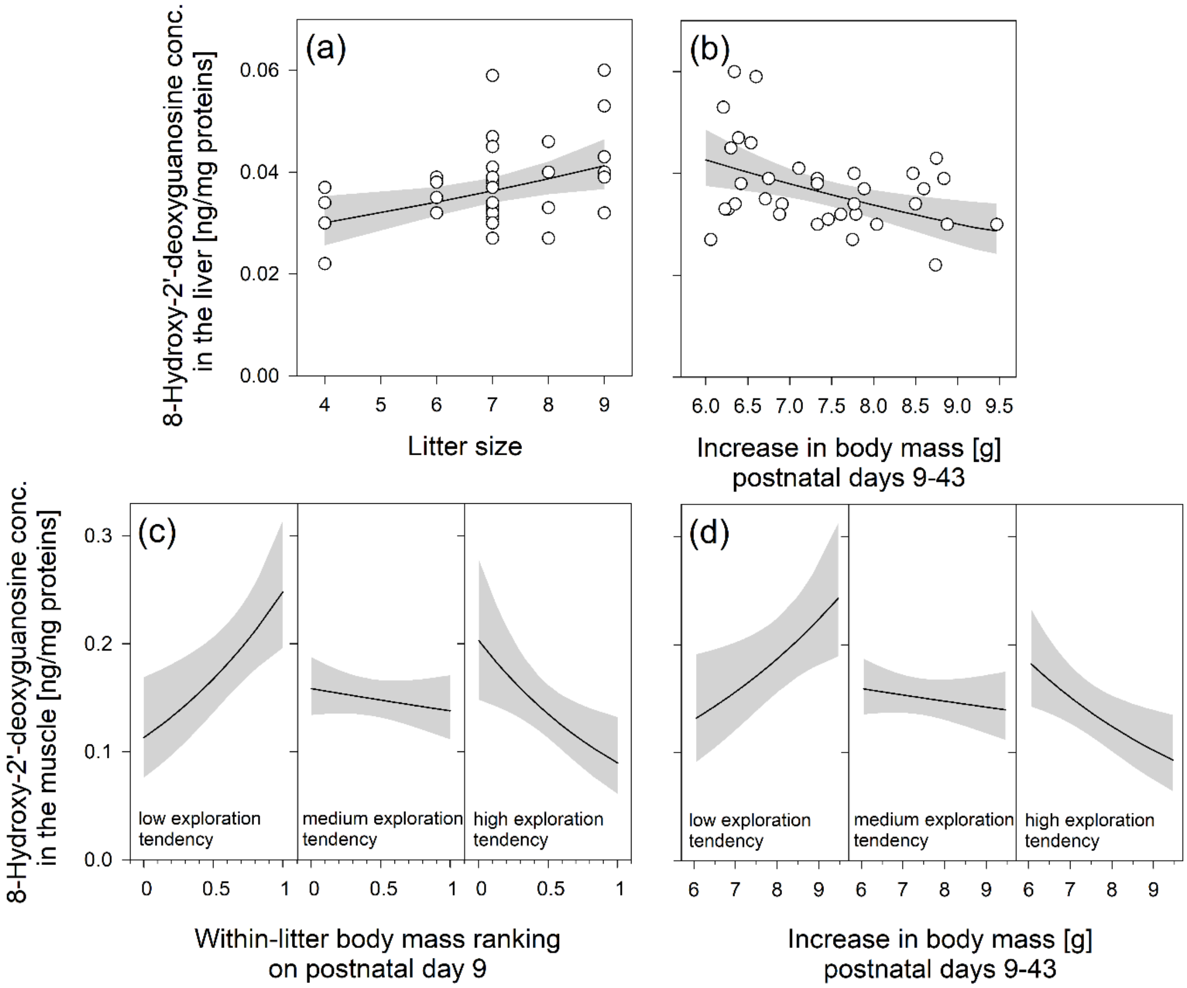
Litter size effects – The analysis of a larger data set from our breeding stock (n = 87 and 34 litters, respectively) revealed that the mean body mass on postnatal day 1 (LMM: $F_{1,74} = 13.55$, *marginal* $R^2 = 0.141$, $\beta = -0.350 \pm 0.095$ SE, $P < 0.001$) as well as the pre-weaning growth from postnatal day 1 to day 20 ($F_{1,31} = 7.91$, *marginal* $R^2 = 0.189$, $\beta = -0.440 \pm 0.156$ SE, $P = 0.008$) were significantly and negatively associated with litter size. That is, mound building-mouse pups from smaller litters were on average heavier shortly after birth and showed a higher pre-weaning growth.

When only considering growth from postnatal days 32 to 43 (weaning was at day 28), litter size also showed a significant and negative effect indicating a lower growth in juveniles from larger litters during the first few weeks after weaning ($F_{1,32} = 4.374$, *marginal* $R^2 = 0.083$, $\beta = –0.293 \pm 0.140$ SE, $P = 0.044$).

Finally, we also considered in our analysis the increase in body mass between postnatal day 44 until the time of sampling of oxidative status parameters at day 145 (sexual maturity in the mound building mouse is at around day 70; Busquet et al., 2009). However, during this period we did not find a significant association between litter size and growth ($F_{1,14} = 0.517$, *marginal* $R^2 = 0.014$, $\beta = 0.133 \pm 0.185$ SE, $P = 0.484$).

Effects of within-litter body mass ranking – Based on the data from our 35 focal females, the growth from postnatal days 9 to 43 was significantly and positively associated with the relative, within-litter body mass ranking on postnatal day 9 ($F_{1,30} = 13.795$, *marginal* $R^2 = 0.258$, $\beta = 0.398 \pm 0.139$ SE, $P < 0.001$). That is, females with a higher-ranked body mass relative to their littermates showed a higher growth at least until two weeks after weaning. Such a significant and positive association was also apparent when only considering early postweaning growth from day 32 to day 43 ($F_{1,32} = 14.056$, *marginal* $R^2 = 0.259$, $\beta = 0.525 \pm 0.140$ SE, $P < 0.001$). However, we did not find a significant effect of within-litter ranking in starting mass (measured on postnatal day 9) on later growth between postnatal days 44 and 145 ($F_{1,31} = 0.513$, *marginal* $R^2 = 0.014$, $\beta = –0.123 \pm 0.172$ SE, $P = 0.479$).

Figure 8. Association between the original litter size of female mound building mice ($n = 35$ females from 15 litters) and their 8-hydroxy-2'-deoxyguanosine concentration (as a marker of oxidative DNA damage) in the liver at adult age (4.8 months).



Source: Rödel et al. 2022. Legend: Association between the original litter size of female mound building mice (n = 35 females from 15 litters) and their 8-hydroxy-2'-deoxyguanosine concentration (as a marker of oxidative DNA damage) in the liver at adult age (4.8 months). The three conditions plotted for each significant interaction are exemplary categorizations (low: 10% percentile, medium: 50% percentile, high: 90% percentile) of the females' within-litter body mass ranking prior to weaning (continuous variable, see Table 4). The regression line with 95% confidence intervals (gray shading) is based on parameter estimates of a linear mixed-effects model given in Table 4a.

6.2.3.2 *Consistent individual differences in exploration tendency*

We quantified individual differences in exploration tendency of the 35 focal females for which we measured parameters of oxidative status later on. PCAs based on the three behavioural parameters recorded during open field and novel object tests, and separately calculated for T_1 (tests on postnatal day 32) and for T_2 (postnatal day 43), revealed a first axis explaining 48.4% (T_1) and 51.7% (T_2) of the variation of the data (eigenvalues T_1: 1.45; T_2: 1.55), respectively. The eigenvalues of all further axes were < 1, and thus these axes were not considered in our analysis.

The distance covered in the open field arena (loadings, T_1: +0.594, T_2: +0.276) and the time spent climbing and exploring the novel object (T_1: +0.446, T_2: +0.704) were positively associated, and the latency to touch the novel object (T_1: –0.669, T_1: –0.654) was negatively associated with the score of the first axis. This score, hereafter refer to as 'exploration tendency', was significantly repeaTable Ccross the two test sessions (LMM-based intraclass correlation: $R_{\mathrm{ICC}} = 0.567$, $P < 0.001$).

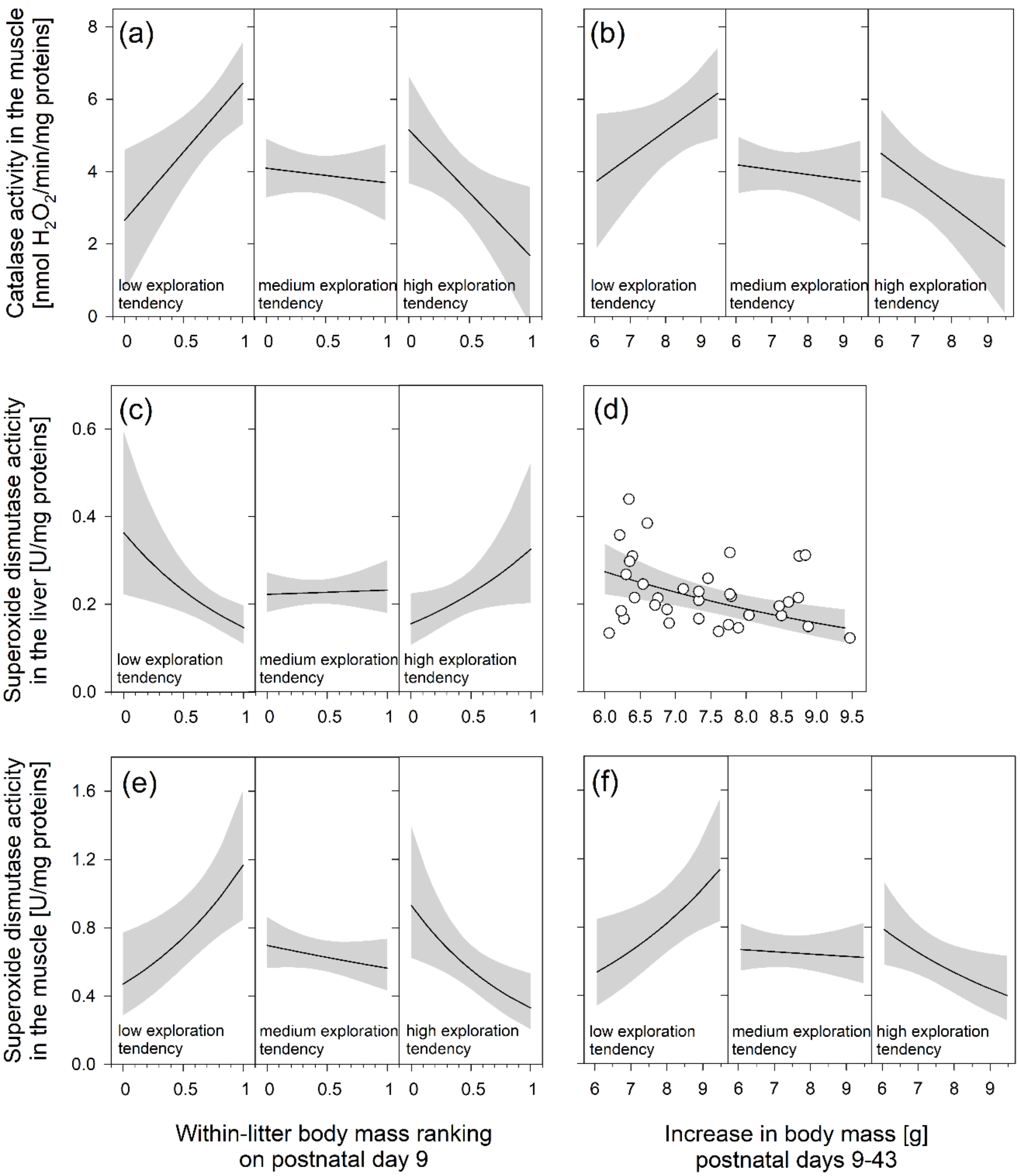
Table 3. Effects of exploration tendency (PCA score), litter size and the ranked pup body mass relative to litter siblings on postnatal day 9 on parameters of oxidative damage and on the activity of antioxidants

Dependent variables	Predictors	*F* (*df*)	$\beta \pm$ SE	*P*
(a) 8-OHdG concentration in the liver	Exploration tendency E	0.054 (1,31)	–0.043 ± 0.185	0.817
	Litter size L	6.478 (1,31)	0.417 ± 0.164	**0.016**
	Pup body mass rank R	0.258 (1,31)	0.093 ± 0.184	0.615
	$E \times R$	2.985 (1,30)	0.269 ± 0.156	0.094
	$E \times L$	3.609 (1,29)	0.265 ± 0.139	0.067
	$L \times R$	0.479 (1,28)	–0.187 ± 0.270	0.494
(b) 8-OHdG concentration in the muscle	Exploration tendency E	0.939 (1,30)	–0.168 ± 0.174	0.340
	Litter size L	0.029 (1,17)	0.034 ± 0.197	0.867
	Pup body mass rank R	0.259 (1,28)	–0.077 ± 0.152	0.615
	$E \times R$	10.544 (1,30)	–0.470 ± 0.145	**0.003**
	$E \times L$	0.005 (1,26)	0.013 ± 0.188	0.946
	$L \times R$	0.084 (1,22)	–0.044 ± 0.152	0.774
(c) PCC in the liver	Exploration tendency E	0.192 (1,28)	–0.090 ± 0.205	0.665
	Litter size L	0.024 (1,14)	0.028 ± 0.183	0.879
	Pup body mass rank R	0.212 (1,31)	–0.093 ± 0.203	0.649
	$E \times R$	0.014 (1,27)	–0.022 ± 0.183	0.905
	$E \times L$	1.102 (1,28)	–0.167 ± 0.159	0.303
	$L \times R$	0.754 (1,28)	–0.279 ± 0.321	0.393
(d) PCC in the muscle	Exploration tendency E	0.324 (1,29)	–0.116 ± 0.204	0.573
	Litter size L	0.185 (1,15)	0.080 ± 0.186	0.674
	Pup body mass rank R	0.875 (1,30)	0.031 ± 0.199	0.875
	$E \times R$	2.304 (1,30)	–0.258 ± 0.172	0.143
	$E \times L$	0.016 (1,28)	–0.029 ± 0.234	0.901
	$L \times R$	0.234 (1,29)	0.105 ± 0.217	0.632
(e) CAT activity in the liver	Exploration tendency E	0.088 (1,31)	–0.059 ± 0.198	0.769
	Litter size L	0.221 (1,15)	0.097 ± 0.207	0.645
	Pup body mass rank R	0.079 (1,27)	0.051 ± 0.181	0.781
	$E \times R$	1.975 (1,30)	0.239 ± 0.170	0.170
	$E \times L$	0.057 (1,27)	–0.054 ± 0.224	0.812
	$L \times R$	0.225 (1,20)	–0.088 ± 0.186	0.640
(f) CAT activity in the muscle	Exploration tendency E	1.350 (1,30)	–0.205 ± 0.177	0.254
	Litter size L	0.025 (1,17)	–0.030 ± 0.192	0.876

	Pup body mass rank *R*	0.012 (1,28)	–0.018 ± 0.162	0.193
	E × *R*	9.326 (1,30)	–0.439 ± 0.144	**0.005**
	E × *L*	1.281 (1,25)	0.134 ± 0.118	0.269
	L × *R*	0.001 (1,22)	–0.001 ± 0.241	0.996
(g) GPx activity in the liver	Exploration tendency *E*	0.077 (1,31)	–0.053 ± 0.190	0.784
	Litter size *L*	1.418 (1,16)	0.238 ± 0.200	0.252
	Pup body mass rank *R*	1.998 (1,27)	–0.244 ± 0.173	0.169
	E × *R*	2.147 (1,30)	–0.239 ± 0.163	0.153
	E × *L*	0.001 (1,28)	0.004 ± 0.211	0.984
	L × *R*	1.457 (1,23)	0.216 ± 0.179	0.240
(h) GPx activity in the muscle	Exploration tendency *E*	0.042 (1,30)	–0.042 ± 0.204	0.838
	Litter size *L*	0.228 (1,20)	–0.090 ± 0.188	0.638
	Pup body mass rank *R*	0.568 (1,30)	0.149 ± 0.197	0.457
	E × *R*	3.538 (1,29)	0.320 ± 0.170	0.070
	E × *L*	0.186 (1,28)	0.098 ± 0.229	0.669
	L × *R*	0.209 (1,26)	0.094 ± 0.205	0.651
(i) SOD activity in the liver	Exploration tendency *E*	0.461 (1,31)	–0.121 ± 0.179	0.502
	Litter size *L*	1.444 (1,15)	0.219 ± 0.182	0.248
	Pup body mass rank *R*	0.024 (1,29)	–0.026 ± 0.166	0.878
	E × *R*	10.076 (1,30)	0.463 ± 0.146	**0.003**
	E × *L*	1.560 (1,26)	0.157 ± 0.125	0.223
	L × *R*	0.516 (1,22)	0.177 ± 0.246	0.408
(j) SOD activity in the muscle	Exploration tendency *E*	1.017 (1,30)	–0.171 ± 0.219	0.321
	Litter size *L*	0.002 (1,16)	–0.008 ± 0.169	0.969
	Pup body mass rank *R*	0.712 (1,26)	–0.124 ± 0.198	0.407
	E × *R*	10.615 (1,30)	–0.460 ± 0.141	**0.003**
	E × *L*	0.063 (1,26)	–0.046 ± 0.182	0.803
	L × *R*	0.067 (1,22)	–0.038 ± 0.147	0.798

Source: Rödel et al. 2022. Legend: Postnatal day 9 proportional within-litter score between 0 and 1. Oxidative damage (a-d) and activity of antioxidants (e-j) in the liver and in skeletal muscle tissue of adult female mound-building mice. Data stem from 35 subjects from 15 litters. Sampling of oxidative parameters around postnatal day 145; see short-cut definition in Table 3. Analysis by multifactorial LMMs including cage identity and litter identity as random factors, with Satterthwaite's approximate F-tests. P-values given in bold are still significant after controlling for false discovery rate due to multiple testing (Benjamini & Hochberg, 1995).

Figure 9. Interactive effects of exploratory activity (PCA score) and the ranked within-litter pup on different parameters of oxidative status measured in adult female mound building mice



Source: Rödel et al. 2022. Legend: Body mass 0 = lightest, 1 = heaviest pup per litter. *n* = 35 animals from 15 litters; 4.8 months old. The three conditions plotted for each significant interaction are exemplary categorizations (low: 10% percentile, medium: 50% percentile, high: 90% percentile) of the females' within-litter body mass ranking prior to weaning (continuous variable, see Table 4). The regression lines with 95% confidence intervals (gray shading) are based on parameter estimates of linear mixed-effects models given in Table 4.

6.2.3.3 *Factors affecting parameters of oxidative status during adulthood*

6.2.3.3.1 *Oxidative DNA damage*

Liver tissue – The 8-OHdG (8-hydroxy-2'-deoxyguanosine) concentration in the liver was significantly and positively associated with litter size (*marginal* R^2 = 0.156, Table 4a), indicating that females born to larger litters had a higher degree of DNA damage in this tissue (Figure 8a).

When substituting litter size by the growth in body mass from postnatal days 9 to 43 (*marginal* R^2 = 0.202, p = 0.007, details in Table D in Suppl. Materials) or by postweaning growth from days 32 to 43 (*marginal* R^2 = 0.157, P = 0.017, Table E in Suppl. Materials), these variables also showed significant associations with 8-OHdG levels in the liver, indicating higher DNA damage in this tissue in individuals with a lower growth (Figure 8b).

Skeletal muscle tissue – The concentration of 8-OHdG in the muscle was significantly explained by the interaction between the females' within-litter body mass rank and their exploratory tendency (*marginal* R^2 = 0.301, Table 4b). This interaction indicates that in females with low exploration tendency, oxidative DNA damage in the muscle was notably higher in individuals with relatively heavy pup body mass compared to lighter littermates, whereas in individuals with high exploration tendency, this positive association tended to inverse (Figure 8c).

Such significant interactions were also apparent when substituting the within-litter body mass ranking and litter size by the growth in body mass from postnatal days 9-43 (*marginal* R^2 = 0.253, P = 0.004, Table D in Suppl. Materials), or by the postweaning growth from days 32-43 (*marginal* R^2 = 0.293, P < 0.001, Table E in Suppl. Materials). That is, in females with low exploration tendency, oxidative DNA damage in the muscle was notably increased with increasing growth, whereas in females with higher exploration tendency, such a positive association was absent and tended to inverse (Figure 8d).

6.3.3.2 *Oxidative protein damage*

There were no significant effects by any of the predictors considered on PCC (protein carbonyl content) concentration, a marker of oxidative protein damage, either in the liver or in skeletal muscle tissue (all P > 0.10; Table 4c, d, and Tables C, D in Suppl. Materials).

6.3.3.3 *Catalase activity*

Liver tissue – There were no significant effects of any of the predictors considered on CAT activity in this tissue (all $P > 0.10$; Table 4e, and Tables C, D in Suppl. Materials).

Skeletal muscle tissue – CAT activity in the muscle was significantly explained by the interaction between the animals' within-litter body mass rank and their exploratory tendency (*marginal* $R^2 = 0.279$, Table 4f). In low-exploratory females, CAT was notably increased along with increasing within-litter body mass ranking as a pup. In females with higher exploratory tendency, this positive association between body mass ranking and CAT disappeared and tended to inverse (Figure 9a).

When substituting within-litter body mass ranking and litter size by the growth from postnatal days 9-43 (*marginal* $R^2 = 0.224$, $P = 0.021$; Table D in Suppl. Materials) or in postweaning growth from days 32 to 43 (*marginal* $R^2 = 0.296$, $P = 0.001$; Table E in Suppl. Materials), there were also significant interactions between growth and exploration tendency. In low-exploratory females, muscle CAT activity increased with increasing growth. However, in females with higher exploratory tendency, CAT activity generally remained at a lower level, and the positive association with growth turned around (Figure 9b).

6.3.3.4 *Glutathione peroxidase activity*

We did not find significant effects by any of the predictors considered, neither on GPx activity in the liver nor in the muscle (all $P > 0.05$, Table 4g, h and Tables C, D in Suppl. Materials).

6.3.3.5 *Superoxide dismutase activity*

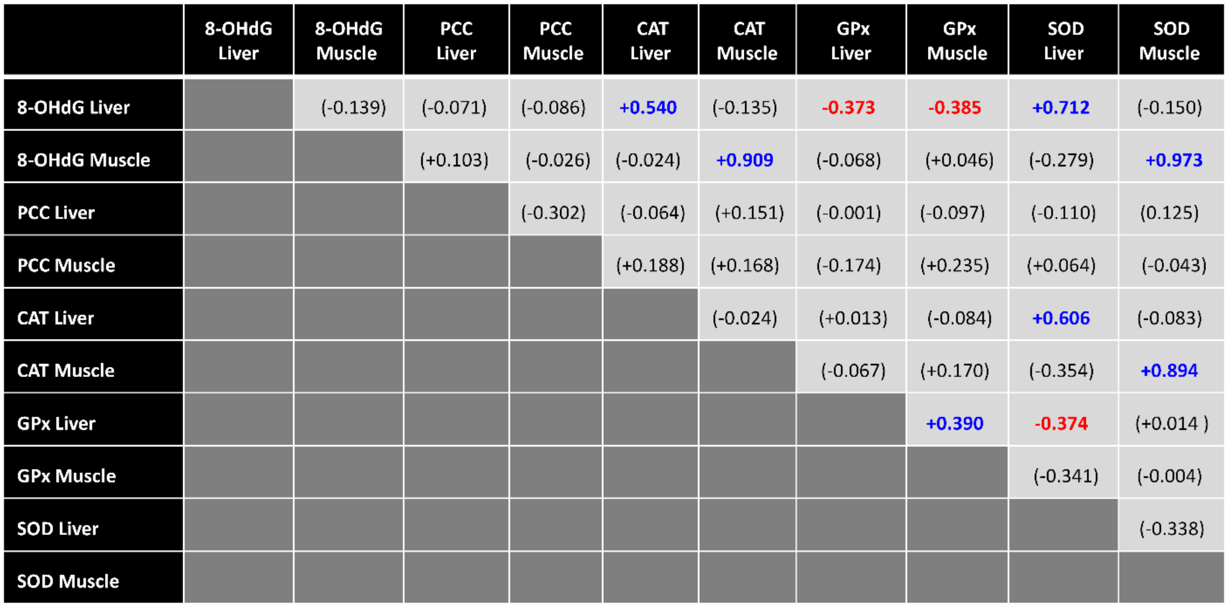
Liver tissue – The significant interaction (Table 4i) revealed that SOD activity in the liver was negatively associated with their within-litter body mass ranking in less exploratory individuals, but was positively associated with this variable in more exploratory ones (*marginal* $R^2 = 0.159$, Figure 9c).

When substituting the within-litter body mass ranking and litter size by the growth from postnatal day 9 to day 43 (*marginal* $R^2 = 0.217$, $p = 0.006$; Table D in Suppl. Materials) or by postweaning growth from days 32 to 43 (*marginal* $R^2 = 0.162$, $P = 0.008$; Table E in Suppl. Materials), the latter variables showed significant and negative associations with hepatic SOD activity. That is, the higher the growth was, the lower was the activity of SOD in this tissue (Figure 9d).

Skeletal muscle tissue – In the muscle, the significant interaction between the within-litter body mass ranking on postnatal day 9 and the animals' exploration tendency predicted a contrasting pattern to that of the liver (Table 4j). In low exploratory individuals, the activity of SOD in the muscle increased with the relative pup body mass of the females compared to their littermates, whereas in more exploratory individuals the direction of this association tended to be inversed (*marginal* $R^2 = 0.261$, Figure 9e).

When rerunning the model by substituting body mass ranking and litter size by the growth from postnatal days 9 to 43 (*marginal* $R^2 = 0.210$, $P = 0.008$; Table D in Suppl. Materials) or by postweaning growth from days 32 to 43 (*marginal* $R^2 = 0.249$, $P = 0.001$; Table E in Suppl. Materials), we again found significant interactions between these variables and exploration tendency. These interactions revealed that in low-exploratory individuals, SOD activity in the muscle increased with increasing growth, whereas this positive association disappeared in individuals with higher exploration tendency (Figure 9f).

Table 4. Associations between the different parameters of oxidative stress



	8-OHdG Liver	8-OHdG Muscle	PCC Liver	PCC Muscle	CAT Liver	CAT Muscle	GPx Liver	GPx Muscle	SOD Liver	SOD Muscle
8-OHdG Liver		(-0.139)	(-0.071)	(-0.086)	**+0.540**	(-0.135)	**-0.373**	**-0.385**	**+0.712**	(-0.150)
8-OHdG Muscle			(+0.103)	(-0.026)	(-0.024)	**+0.909**	(-0.068)	(+0.046)	(-0.279)	**+0.973**
PCC Liver				(-0.302)	(-0.064)	(+0.151)	(-0.001)	(-0.097)	(-0.110)	(0.125)
PCC Muscle					(+0.188)	(+0.168)	(-0.174)	(+0.235)	(+0.064)	(-0.043)
CAT Liver						(-0.024)	(+0.013)	(-0.084)	**+0.606**	(-0.083)
CAT Muscle							(-0.067)	(+0.170)	(-0.354)	**+0.894**
GPx Liver								**+0.390**	**-0.374**	(+0.014)
GPx Muscle									(-0.341)	(-0.004)
SOD Liver										(-0.338)
SOD Muscle										

Source: Rödel et al. 2022. Legend: Measurements taken from 35 adult females stemming from 15 litters. Analysis by a multivariate LMM including litter identity and cage identity as random factors. Correlation coefficients are given; statistically significant ($P < 0.05$) negative effects are indicated in red and significant positive effects are indicated in blue. 8-OHdG = 8-hydroxy-2'-deoxyguanosine (marker of oxidative DNA damage), PCC = Protein carbonyl content (marker of oxidative protein damage), CAT = Catalase activity (antioxidant), GPx = Glutathione peroxidase activity (antioxidant), SOD = Superoxide dismutase activity (antioxidant)

6.2.4. DISCUSSION

6.2.4.1 *Summary of key results*

In relation to the main goal of our study, our findings on female mound-building mice demonstrate long-term effects of parameters experienced during early life on the oxidative status during adult stage. This was evident by the higher levels of oxidative damage to DNA (i.e. higher 8-OHdG concentrations) in the liver of animals stemming from larger litters, suggesting long-lasting consequences of increased sibling competition during early life.

Furthermore, several oxidative status parameters were associated with the animals' relative starting body mass differences within the litter, and these associations were modulated by individual differences in exploration tendency (as evident by the significant interactions between relative starting mass and exploration tendency). First, oxidative DNA damage in skeletal muscle tissue was higher in animals with a relatively higher body mass as pups, but only in less exploratory individuals, i.e. in a phenotype typically characterized by a higher adrenocortical activity (Baugh et al., 2017; Carere et al., 2003; Lavergne et al., 2019; Lendvai et al., 2011; Montiglio et al., 2012; Rossi et al., 2018; Stöwe et al., 2010). Furthermore, activity levels of the antioxidants CAT and SOD in skeletal muscle tissue were also increased in such animals.

6.2.4.2 *Effects of litter size and within-litter body mass ranking on growth*

Our study confirms a negative association between litter size and early growth, as it has been shown in a wide range of other small mammals (Mendl, 1988; Rödel et al., 2008b). Furthermore, individuals with a lighter relative starting mass compared to littermates showed a lower early growth. The latter finding is also in accordance with studies in other small altricial mammals, and might be explained by the fact that heavier pups are typically occupying more central, energetically more advantageous positions in the litter huddle, leading to feedback loops positively affecting their growth (Bautista et al., 2010; Rödel et al., 2008a).

The lower growth in individuals from larger litters and in such with a relatively lower starting mass was still apparent during the early post-weaning period with ad libitum access to food. Thus, given the consistent effects of litter size and relative starting mass before and at least for some time after weaning, we conclude that there were no indications of postweaning compensatory growth (cf. Sikes, 1998) of lighter individuals in the mound-building mouse.

6.2.4.3 *Litter size effects on parameters of oxidative status*

Indications for an increase in oxidative stress in offspring from broods or litters experimentally enlarged in size have already been found in studies on birds (Bourgeon et al., 2011; Gil et al., 2019) and in one on mammals (Gibson et al., 2015). Furthermore, in a study on wild Eurasian kestrels (*Falco tinnunculus*), nestlings from naturally larger brood sizes had the highest levels of oxidative stress (Costantini et al., 2006). Extending these findings, our study shows for the first time that the natural variation in litter size has the potential to exert such effects in the long-term, as evident by the increased hepatic 8-OHdG levels in mature individuals stemming from larger litters. Our study did not reveal compensatory post-weaning growth in offspring born to larger litters. Thus, there is no support for the hypothesis that the observed long-term effects, i.e. the higher liver DNA damage in individuals from larger litters were driven by a higher ROS production related to enhanced, compensatory growth in such individuals (cf. Smith et al., 2016).

One of the possible pathways through which early life conditions, such as growing up in different-sized litters, may disrupt the oxidative status homeostasis is via increased levels of circulating glucocorticoids, as a consequence of chronic stress (Costantini et al., 2011; Spiers et al., 2015). A higher behavioural activity including increased competition among offspring from larger compared to smaller litters can be observed in some polytocous mammals characterized by scramble competition (e.g., laboratory rat *Rattus norvegicus*, Bautista et al., 2010) as well as by direct contest for mother's milk (e.g., domestic pig *Sus scrofa*, Kobek-Kjeldager et al., 2020). The stress-inducing character of increased sibling competition in larger litters is further supported by some studies showing increased concentrations of circulating glucocorticoids (guinea pigs *Cavia aperea*, Fey & Trillmich, 2008) or a higher adrenocortical capacity in mounting a stress response (Alpine marmots *Marmota marmota*, Cohas et al., 2021) in such offspring before or around weaning. At first sight, it appears unexpected that challenging conditions experienced early in life translate into changes in oxidative status during adulthood, given that oxidative (DNA) damage can be repaired enzymatically, at least to a certain extent (Cooke et al., 2003). Base and nucleotide excision repair mechanisms cleave 8-OHdG from DNA (Halliwell & Gutteridge, 2015); thus, our results might suggest an increased generation of DNA damage, but also increased repair activity. Whatever the exact molecular mechanism, our findings are in line with some other studies, highlighting that early life stress can lead to long-term alterations of an animal's oxidative phenotype. For example, laboratory mice (*M. musculus*) from experimentally enlarged litters had consistently decreased aconitase activities around weaning and when reaching maturity (Gibson et al., 2015), indicative of increased oxidative damage in such individuals (Yan et al., 1997). In Japanese quails (*Coturnix japonica*), enduringly stressful conditions experienced during pre- and post-natal development led to long-term shifts in different antioxidant defenses in the blood and in post-mitotic neuronal tissue (Marasco et al., 2013).

Interestingly, in the current study, animals with (litter size-dependent) increased early growth rates also showed an increased activity of the antioxidant SOD in the liver (see Figure 8d), which is contrary to findings obtained in some studies in birds reporting lower antioxidant activities in enlarged broods (Bourgeon et al., 2011; Gil et al., 2019). Furthermore, in our study, higher 8-OHdG levels were associated with higher activities of the antioxidants SOD and CAT, both in the liver as well as in muscle tissue (see Table 3). In contrast, several studies highlight negative correlations, i.e. higher levels of oxidative damage in individuals characterized by a lower antioxidant capacity, in particular in energetically challenged or sick individuals (Kapusta et al., 2018; Tabur et al., 2015). However, as discussed above, challenging early life conditions, such as a more competitive environment in larger litters, may not only induce increased oxidative damage during early postnatal life but also cause longer-lasting, possibly priming effects (Gibson et al., 2015; Marasco et al., 2013). Thus, we hypothesize that the increased antioxidant enzyme activity observed during adult stage may represent the body's protective response to such long-term effects on cellular ROS production.

Nevertheless, this explanation does not exclude the possibility that antioxidant enzymatic activities were decreased when the animals were facing challenging conditions early in life, but may have increased adaptively over lifetime, thus leading to such a positive association during adulthood. Unfortunately, our study based on single endpoint measures only allows restricted insights. Further follow-up studies using repeated measurements of parameters of oxidative status across different life stages will be useful to explore mechanisms leading to the here observed strong and positive, tissue-specific associations between parameters of oxidative damage and antioxidant activity (see Table 3).

6.2.4.4 *Effects of relative starting body mass and exploration tendency on oxidative status*

Our findings support a link between exploratory tendency and oxidative DNA damage in skeletal muscle tissue. Most other studies exploring personality-dependent differences in oxidative status report associations with the animals' antioxidant capacity (Costantini et al., 2008; Costantini et al., 2012; Herborn et al., 2011; Isaksson et al., 2011; Matsuzawa et al., 2005), and only few have found associations with parameters of oxidative damage. A study on green finches (*Carduelis chloris*), in general accordance with our findings, supports a negative relationship between exploration tendency and oxidative damage, showing that less exploratory individuals had higher concentrations of malondialdehyde, an indicator of cellular lipid peroxidation (Herborn et al., 2011). In the same study, birds with extremely high or low neophobia had lower malondialdehyde concentrations than intermediate responders (Herborn et al., 2011). Furthermore, a study in humans found indications of higher oxidative damage in terms of increased concentrations of malondialdehyde in individuals with high scores of neuroticism (Vida et al., 2018).

However, our study draws a more complex picture, revealing that the increased oxidative DNA damage in muscle tissue of less exploratory phenotypes was only apparent in individuals with a relatively heavier early body mass compared to littermates. First, and as hypothesized upfront, the potentially higher HPA axis activity in low-exploratory individuals (Baugh et al., 2017; Carere et al., 2003; Lavergne et al., 2019; Lendvai et al., 2011; Montiglio et al., 2012; Rossi et al., 2018; Stöwe et al., 2010), and thus their higher levels of circulating corticosteroids in response to challenge may be a key mechanism explaining the increased oxidative damage in such animals (e.g., Costantini et al., 2012; reviews in Costantini et al., 2011; Spiers et al., 2015). Second, higher growth rates can carry significant oxidative costs in terms of the accumulation of ROS as a by-product of an increased metabolism, and a meta-analysis on this topic suggests that such negative consequences appear particularly visible in terms of increased oxidative damage (review in Smith et al., 2016). This combination of a higher HPA activity and growth rate might explain the increased DNA damage in the muscle tissue in faster-growing individuals with a relatively heavier starting body mass (see Figure 8c, d). Although, as predicted by the significant interaction in our statistical model, only low-exploratory individuals were affected by such negative consequences, most likely due to their higher sensitivity or predisposition to oxidative stress related to their higher adrenocortical activity (Costantini et al., 2011; Spiers et al., 2015).

Not only the oxidative DNA damage, but also the enzymatic activities of the two antioxidants CAT and SOD in muscle tissue were notably increased in low-exploratory individuals with a higher relative starting body mass and higher associated growth rate. As already discussed above, this pattern could reflect an adaptive, tissue-specific upregulation of the antioxidant machinery in animals with particularly high ROS production, thus leading to the positive associations between DNA damage and antioxidant activity levels (see Table 3). As already mentioned above, the single measurements of our study only provide a snap shot of the current condition, and follow-up studies using repeated measurements may help to better understand cause and consequence of the here observed phenomenon.

In contrast, in the liver, we found indications for a lower SOD activity in such low-exploratory individuals with a higher relative starting body mass (see Figure 8c). Although little is known about the energetic costs of antioxidant defenses, we speculate that the lower SOD activity in the liver may be the outcome of a trade-off with observed upregulation in antioxidant capacity in skeletal muscle tissue of such animals. Accordingly, as has been concluded in a study on the oxidative status in common quails (*C. coturnix*), our findings support the assumption that the body's maintenance of muscle integrity might be of higher priority than its protection of the liver during the growing period (Marasco et al., 2021).

Coming back to the effects of litter size on oxidative status; it may appear surprising that the higher early growth rates in offspring from smaller litters did not result in detectable higher oxidative damage in such individuals. One might expect such an effect, in accordance to the above-discussed negative consequences of higher growth on oxidative DNA damage in initially heavier siblings. We suggest that one of the reasons for the absence of such an association may be that the effect of litter size on post-weaning growth was relatively small ($\beta = -0.293 \pm 0.140$ SE), notably smaller compared to the growth effect driven by within-litter differences in starting mass ($\beta = 0.525 \pm 0.140$ SE), as evident by the comparison of the absolute values of the standardized regression slopes β. Thus, in our correlational study, we suggest that negative effects of increased sibling competition in larger litters may have simply masked the detectability or have outweighed the possible detrimental consequences of higher growth in offspring from smaller litters on their oxidative status.

6.2.4.5 CONCLUSIONS

Our findings provide some novel insights in the long-term consequences of early life conditions on oxidative damage and antioxidant activity during adulthood. We show that the naturally occurring variation in litter size is sufficient to exert such effects, in terms of an increased oxidative DNA damage in adult mice born to larger litters. Our findings also suggest synergistic effects of the typically higher stress sensitivity of low-exploratory phenotypes together with increased oxidative costs in faster growing littermates, possibly leading to higher oxidative DNA damage and to an upregulation of antioxidant defenses in such individuals. Although the character of our study is correlational, and although we can only speculate about the causes and consequences of concomitantly increased levels of oxidative damage and antioxidant levels in some animals, the patterns we observed may help to shed light on potential mechanisms explaining how early life conditions and aspects of animal personality are linked to cellular senescence and other processes of aging (Selman et al., 2012; Yousefzadeh et al., 2021).

AUTHOR CONTRIBUTIONS

H.G.R., C.F., R.M. and D.C. conceived and designed the study; H.G.R. supervised the project and statistically analyzed the data; H.G.R. and D.C. wrote the original manuscript; V.J., M.R., L.J and D.J. carried out experimentation and cared for the animals; V.J. analyzed videos, and V.J., L.J. and D.J. dissected the animals and took tissue samples; D.C. quantified oxidative parameters. All co-authors contributed to the manuscript by correcting and editing it.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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6.2.6 SUPPLEMENTARY MATERIAL

Supplements to Methods

Repeatability of behavioural variables

Analyses based on the 35 females of the present study revealed that 2 out of the 3 chosen behavioural parameters were significantly repeatable (Table C). The third parameter, the latency to approach the novel object, missed the level of significance when only testing 35 females, although was also significantly repeatable when increasing the sample size with data from other females from our data base to a total of *n* = 86. This underlines the usefulness of all three chosen behavioural parameters (as given in Table C) to contribute to the assessment of individual differences in exploration tendency in the mound building mouse.

Table C. Repeatability of different behavioural variables quantified during repeated open field and novel object tests carried out in female mound building mice.

	(a)		(b)	
Behavioural variables	R_{ICC}	*P*	R_{ICC}	*P*
Distance covered in Open field	0.563	**< 0.001**	0.425	**< 0.001**
Latency to touch Novel object	0.182	0.139	0.410	**< 0.001**
Time spent climbing on Novel object	0.576	**< 0.001**	0.484	**< 0.001**

Source: Rödel et al. 2022. Legend: Data from (a) 35 females stemming from 15 litters, and from (b) 86 females from 42 litters (including the 35 females analyzed separately for (a)). Analyses by linear mixed-model based intra-class correlations (1). Statistically significant results are given in bold.

Supplements to the Results

Analysis using early growth as a predictor

Growth from postnatal days 9 to 43

Table D Effects of exploration tendency (PCA score) and body mass growth **day 9 to 43** on parameters of oxidative damage and on the activity of antioxidants activity in the liver and in skeleton muscle tissue of adult female mound-building mice.

Dependent variables	Predictors	*F* (*df*)	*β* ± SE	*P*
(a) 8-OHdG concentration in the liver	Exploration tendency *E*	2.696 (1,30)	–0.295 ± 0.180	0.111
	Growth d9-d43 *G*	8.412 (1,30)	–0.552 ± 0.180	**0.007**
	E × *G*	0.172 (1,31)	0.061 ± 0.148	0.681
(b) 8-OHdG concentration in the muscle	Exploration tendency *E*	1.115 (1,31)	–0.187 ± 0.177	0.299
	Growth d9-d43 *G*	0.250 (1,29)	–0.082 ± 0.164	0.621
	E × *G*	10.127 (1,25)	–0.384 ± 0.121	**0.004**
(c) Protein carbonyl content in the liver	Exploration tendency *E*	0.193 (1,31)	–0.093 ± 0.211	0.663
	Growth d9-d43 *G*	0.936 (1,31)	–0.194 ± 0.201	0.341
	E × *G*	0.360 (1,31)	–0.098 ± 0.163	0.553
(d) Protein carbonyl content in the muscle	Exploration tendency *E*	0.001 (1,28)	–0.001 ± 0.208	0.998
	Growth d9-d43 *G*	1.165 (1,28)	–0.214 ± 0.198	0.290
	E × *G*	0.367 (1,31)	–0.089 ± 0.160	0.584
(e) Catalase activity in the liver	Exploration tendency *E*	1.102 (1,30)	–0.208 ± 0.198	0.165
	Growth d9-d43 *G*	0.596 (1,31)	–0.156 ± 0.202	0.170
	E × *G*	0.842 (1,28)	0.140 ± 0.153	0.437
(f) Catalase activity in the muscle	Exploration tendency *E*	1.975 (1,31)	–0.264 ± 0.188	0.170
	Growth d9-d43 *G*	0.190 (1,29)	–0.056 ± 0.171	0.744
	E × *G*	6.056 (1,25)	–0.315 ± 0.128	**0.021**
(g) Glutathione peroxidase act. in the liver	Exploration tendency *E*	0.136 (1,31)	0.077 ± 0.208	0.715
	Growth d9-d43 *G*	0.170 (1,31)	0.081 ± 0.196	0.683
	E × *G*	1.376 (1,27)	–0.172 ± 0.147	0.251
(h) Glutathione peroxidase act. in the muscle	Exploration tendency *E*	0.529 (1,29)	0.038 ± 0.052	0.472
	Growth d9-d43 *G*	1.070 (1,29)	0.051 ± 0.049	0.309
	E × *G*	3.573 (1,31)	–0.075 ± 0.040	0.068
(i) Superoxide dismutase act. in the liver	Exploration tendency *E*	1.095 (1,31)	–0.198 ± 0.189	0.030
	Growth d9-d43 *G*	8.877 (1,29)	–0.514 ± 0.173	**0.006**
	E × *G*	1.538 (1,24)	0.161 ± 0.130	0.227
(j) Superoxide dismutase act. in the muscle	Exploration tendency *E*	0.717 (1,30)	–0.149 ± 0.176	0.404
	Growth d9-d43 *G*	0.007 (1,28)	–0.013 ± 0.163	0.935
	E × *G*	8.470 (1,23)	–0.344 ± 0.118	**0.008**

Source: Rödel et al. 2022. Legend: Body mass growth from postnatal days 9 to 43. Oxidative damage (a-d) and activity of antioxidants (e-j) activity. Data stem from 35 subjects from 15 litters. Sampling of oxidative parameters around postnatal day 145; see short-cut definition in Table 3. Analyses by multifactorial LMMs including cage identity and litter identity as random factors, with Satterthwaite's approximate *F*-tests. *P*-values given in bold are still statistically significant when controlling for false discovery rate due to multiple testing (2).

Growth from postnatal days 32 to 43

Table E Effects of exploration tendency (PCA score) and body mass growth **days 32 to 43** on parameters of oxidative damage and on the activity of antioxidants activity in the liver and in skeleton muscle tissue of adult female mound-building mice.

Dependent variables	Predictors	*F* (*df*)	β ± SE	*P*
(a) 8-OHdG concentration in the liver	Exploration tendency *E*	1.087 (1,30)	–0.179 ± 0.172	0.306
	Growth d32-d43 *G*	6.386 (1,32)	–0.430 ± 0.170	**0.017**
	E × *G*	0.170 (1,31)	0.055 ± 0.135	0.680
(b) 8-OHdG concentration in the muscle	Exploration tendency *E*	0.245 (1,31)	–0.181 ± 0.164	0.624
	Growth d32-d43 *G*	0.028 (1,28)	–0.019 ± 0.138	0.893
	E × *G*	15.756 (1,27)	–0.414 ± 0.104	< **0.001**
(c) Protein carbonyl content in the liver	Exploration tendency *E*	0.001 (1,31)	–0.001 ± 0.193	0.999
	Growth d32-d43 *G*	0.816 (1,31)	–0.164 ± 0.181	0.373
	E × *G*	1.871 (1,31)	–0.195 ± 0.143	0.181
(d) Protein carbonyl content in the muscle	Exploration tendency *E*	0.335 (1,27)	–0.117 ± 0.202	0.568
	Growth d32-d43 *G*	0.094 (1,31)	0.057 ± 0.187	0.762
	E × *G*	0.003 (1,31)	–0.008 ± 0.146	0.954
(e) Catalase activity in the liver	Exploration tendency *E*	2.292 (1,30)	–0.292 ± 0.193	0.140
	Growth d32-d43 *G*	2.058 (1,30)	–0.241 ± 0.168	0.162
	E × *G*	1.419 (1,28)	0.151 ± 0.127	0.243
(f) Catalase activity in the muscle	Exploration tendency *E*	0.886 (1,31)	–0.157 ± 0.167	0.354
	Growth d32-d43 *G*	0.134 (1,29)	–0.016 ± 0.140	0.907
	E × *G*	12.704 (1,28)	–0.386 ± 0.108	**0.001**
(g) Glutathione peroxidase act. in the liver	Exploration tendency *E*	0.131 (1,30)	0.072 ± 0.201	0.720
	Growth d32-d43 *G*	0.519 (1,29)	0.126 ± 0.175	0.477
	E × *G*	0.830 (1,29)	–0.122 ± 0.134	0.370
(h) Glutathione peroxidase act. in the muscle	Exploration tendency *E*	0.030 (1,29)	–0.009 ± 0.052	0.864
	Growth d32-d43 *G*	0.012 (1,31)	0.005 ± 0.048	0.914
	E × *G*	0.609 (1,31)	–0.029 ± 0.038	0.441
(i) Superoxide dismutase act. in the liver	Exploration tendency *E*	0.001 (1,32)	–0.002 ± 0.170	0.990
	Growth d32-d43 *G*	8.232 (1,29)	–0.449 ± 0.157	**0.008**
	E × *G*	1.0748 (1,28)	0.127 ± 0.122	0.309
(j) Superoxide dismutase act. in the muscle	Exploration tendency *E*	0.154 (1,31)	–0.064 ± 0.164	0.697
	Growth d32-d43 *G*	0.005 (1,28)	–0.010 ± 0.137	0.942
	E × *G*	13.196 (1,27)	–0.376 ± 0.104	**0.001**

Source: Rödel et al. 2022. Legend: Body mass growth from postnatal days 32 to 43. Oxidative damage (a-d) and activity of antioxidants (e-j) activity. Data stem from 35 subjects from 15 litters. Sampling of oxidative parameters around postnatal day 145; see short-cut definition in Table 3. Analyses by multifactorial LMMs including cage identity and litter identity as random factors, with Satterthwaite's approximate *F*-tests. *P*-values given in bold are still statistically significant when controlling for false discovery rate due to multiple testing (2).

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