Emergence et développement des différences comportementales individuelles chez les souris glaneuse, Mus spicilegus.

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“Look, if you had, one shot, or one opportunity

To seize everything you ever wanted. In one moment

Would you capture it, or just let it slip?”

Eminem

Content

1. General Introduction

1.1. Consistent individual differences in behaviour: the concept of personality

1.1.1. What is personality?

Behaviour is often assumed the most immediate and less costly way to respond to the environment, and thus suggested to be highly flexible (Pigliucci 2001). However, contrary to this assumption, animals from the same age, sex or habitat have been shown to constantly differ in their behaviour (Gosling & John 1999). Such consistent inter-individual differences in behaviour both across longer spans of time and across contexts, frequently referred to as 'animal personality', have been widely described in the animal kingdom (Gosling 2001; Sih *et al.* 2004a). Compared to non-human animals, "personality" in human has a long history of research, which mainly encompasses non-observable constant elements such as patterns in feeling, thinking and behaving (Pervin & John 1997). This definition has been adapted to animal personality research, as non-observable and psychological properties restrain the study of such behavioural variation in animals. Diverse and numerous study approaches have followed and consequently, this concept has received different terminology (e.g. temperament, coping style), and thus, it has suffered from a lack of rigorous definition leading to some confusion. **Animal personality** or **temperament** received convergent definition (Réale *et al.* 2007; but see: Mackay & Haskell 2015) and has been described as individual behavioural differences, which are consistent both across (periods of) time and contexts. "Consistent" has to be understood in the sense that differences between individuals are maintained, but this does not prevent individual trait values from changing across time or contexts (Réale *et al.* 2007; Stamps & Groothuis 2010). The term "**coping style**" defines how animals consistently cope with social or non-social challenging situations and two general behavioural profiles or coping styles are usually distinguished: "proactive" (or active) and "reactive" (or passive) (Benus *et al.* 1991; Koolhaas *et al.* 1999, 2007). Whatever the terminology used, animals are defined by specific personality traits assessed by repeatedly quantifying the behavioural responses of the animals in different contexts. At least five personality traits are usually reported in a wide range of animals: boldness, exploration tendency, aggressiveness, activity, and sociability (Gosling 2001; Réale *et al.* 2007). Except in strains artificially selected for certain personalities, these traits usually do not follow a bimodal distribution within a population of animals but typically vary along a continuum with two extremes (e.g. active-passive individuals). In addition, animals typically show suites of correlated behaviours, also called **behavioural syndrome** (Sih *et al.* 2004B; Sih & Bell 2008). In other words, different personality traits are frequently associated in a similar way. For example, bolder animals are frequently more active, more exploratory and more aggressive (e.g. Cote *et al.* 2010, Rödel *et al.* 2015). In this thesis, I will

use the term animal personality to refer to a single behavioural trait, which is consistent, both across a certain span of time and correlated across contexts.

1.1.2. Ultimate and proximate implications of the existence of personality

At first sight, consistent behavioural responses across time and context might not be easy to explain from an adaptive and evolutionary point of view, as this implies that an animal might potentially react in a way that does not allow it to be perfectly adapted to its (changing) environment (Réale *et al.* 2007; Bergmüller 2010). That is, in some conditions, the behaviour expressed may not be optimal to maximize its reproduction and survival while it might be more appropriate in another context. According to this, more flexible behavioural responses may provide a selective advantage. It has been argued that natural and sexual selection may favour a mean optimal phenotype, which should spread within the population and diminish variability (Krebs & Davies 1997). Thus, variation across the mean has been frequently considered as "noise" instead of an end-product of natural selection (Réale *et al.* 2007). To understand how personality is maintained within a population and across generations sheds light on the mechanisms how natural selection favours consistency rather than flexibility in behaviours. From a proximate point of view, animal personality suggests a common underlying architecture of behaviour based on common causal factors. Indeed, the different system that shape an organism (for instance, the neural, hormonal and immune system) does not act independently and any modifications in one of the system may induce modifications in the others, and consequently, potential high energetic demands (Lessells 2008). Switches between different behavioural phenotype and some associated physiological and neural changes might thus be highly costly and consistency in behaviour is thus more likely to occur (Duckworth 2010). To shed light on the underlying mechanisms associated with personality might thus provide information on intrinsic constraints diminishing behavioural flexibility (Duckworth 2010). Associations between hormones and behaviour have been extensively studied with regard to stress physiology and coping styles or personality (DeVries *et al.* 2003; Carere *et al.* 2010; Koolhaas *et al.* 2010). In addition to internal factors, the environment those animals are exposed to – and in particular the early developmental environment – can affect the individual (behavioural) phenotype as well (Lindström 1999; Stamps & Groothuis 2010). To investigate how underlying mechanisms and environmental cues are associated with personality might thus clarify how different personalities coexist within a population.

The existence of animal personalities suggests that an animal might be constrained by developmental processes, which might affect their capacities to respond freely to their environment. While empirical and theoretical models are frequently proposed and argued (e.g. Sih *et al.* 2004a; Réale *et al.* 2007; Sih & Bell 2007; Dingemanse *et al.* 2010; Dingemanse & Wolf 2010; Schuett *et al.* 2010; Stamps & Groothuis 2010; Wolf & Weissing 2010; Dall *et al.* 2012) and special issues and books

on this topic are regularly published (Bergmüller 2010; Carere & Maestripieri 2013; Trillmich *et al.* 2014), many questions concerning the causes and consequences of personality still remain unanswered. This thesis aims to add some more pieces of information to this field of research and will mainly focus on the emergence and development of personality. Additionally, a potential mechanism for the maintenance of personality will be discussed.

1.2. Proximate mechanisms: origins and development of behavioural traits

1.2.1. Emergence and development of behavioural responses during early life

In the following, I will first introduce the emergence of personality and describe how genes and environment shape the behaviour of an individual. I will then discuss the ontogeny of stable individual differences. Finally, I will emphasize on factors that can modulate the early environment, e.g. via parental and sibling effects.

1.2.1.1. Genes and environment shape individual differences in behaviour

A study reviewing the genetic basis of personality revealed that some traits have a higher heritability than others (e.g. 14% of heritability for aggression and 37% for activity; see Boomsma *et al.* 2002 for a review in humans; Van Oers & Sinn 2013). Apart from the genetic background, a multitude of pre- and post-natal experiential or environmental factors can affect the (behavioural) phenotype (reviews in: Stamps & Groothuis 2010; Houdelier *et al.* 2013).

Maternal or parental effects, the causal influence of the parental phenotype on the offspring phenotype (Mousseau & Fox 1998) are thought to be of major importance to affect the offspring phenotype, as they can modify the early environment experienced by the offspring both behaviourally and physiologically. During the prenatal phase, females transfer hormones, such as androgens and glucocorticoids, and nutrients to the young (e.g. through the yolk and albumen of the eggs in birds: Gil 2008 and placenta in mammals: Nowak *et al.* 2000). Differential quantity of hormones and nutrients can affect the foetal endocrine state and brain development (review in: Kaiser & Sachser 2005) and can exert long-lasting effects on behaviour (Groothuis & Carere 2005; Carere & Maestripieri 2013). For instance, socially stressed laboratory rat *Rattus norvegicus* mothers with higher concentrations of circulating glucocorticoids produced offspring, which showed comparatively lower levels of anxiety-related behaviours during adulthood and also lower increases in corticosteroid levels in response to stress (e.g. Götz & Stefanski 2007). Maternal or parental effects can also be transmitted during the postnatal phase. Because the presence and quality of care

provided by the mother or both parents to the dependent offspring are essential for their survival in most vertebrates (Nowak *et al.* 2000), the study of parental effects receives much attention (Stamps & Groothuis 2010; Houdelier *et al.* 2013). Maternal deprivation or separation during the early postnatal period can result for instance in an increase of anxiety-like behaviours (rodents: Ogawa *et al.* 1994; Lee *et al.* 2007; Veenema *et al.* 2007) and in a hyper-responsiveness of the hypothalamic pituitary axis HPA (zebra finches *Taeniopygia guttata*: Banerjee *et al.* 2011). Moreover, bi-parental deprivation alters the offspring's paternal behaviour and associated mRNA expression of different receptors in several regions of the brain (Yu *et al.* 2015). Apart from parental deprivation, naturally occurring variation in the quantity and quality of care behaviour (e.g. handling-licking or arched-back nursing) has consequences on the behavioural, neural and physiological development of the young (Liu *et al.* 1997; Francis *et al.* 1999a; Champagne *et al.* 2003; Braun & Champagne 2014). For example, higher amount of care displayed by older quail mothers *Coturnix coturnix japonica,* compared to younger ones, resulted in more fearful and more sensitive chicks to social separation (Pittet *et al.* 2013). In rodents, the amounts of licking-grooming and arched-back nursing during infancy have been related to the later offspring phenotype, and pups that received higher quantities of this care showed lower levels of fear behaviour in a novel environment (Caldji *et al.* 1998), higher levels of exploration (Francis *et al.* 1999b; Bales *et al.* 2007) and showed greater cognitive skills (Bredy *et al.* 2004).

The consequences of exclusive father-offspring interactions have received less attention. In house sparrows, *Passer domesticus*, transmission of sexual ornamentation is mediated by paternal care rather than by genetic influence, as revealed by cross-fostering experiments (Griffith *et al.* 1999). In mammals, potential effects of paternal care on developmental differences in the offspring have mostly been investigated by removing the father (Braun & Champagne 2014), and this removal has been shown to be associated with an increase in anxiety-related behaviours, with a lower sociality of the offspring (in mandarin voles *Microtus mandarinus*: Jia *et al.* 2009), and with a lower level of parental behaviour when adult (in mandarin voles: Jia *et al.* 2011; review in: Bales & Saltzman 2015; Yu *et al.* 2015). However, the effects of paternal care on offspring behavioural phenotype are mostly investigated through the removal of the father (Braun & Champagne 2014; Bales & Saltzman 2015), a method that does not allow to assess whether paternal effects are, in fact, transmit by direct effects of the quality of paternal care. Indeed, few studies actually report effects of variation in paternal care on the offspring development in units in which the father is present (Bales & Saltzman 2015), although variation among fathers in the quantity and quality of care behaviour might exist and affect offspring development. For example, notable differences in the quantity of paternal care have been reported in prairie voles *Microtus ochrogaster* (Perkeybile *et al.* 2013). Such differences can

indeed affect the offspring development, as shown in a study in marmosets *Callithrix geoffroyi*, in which a lower quality of paternal care led to a comparatively higher stress responsiveness in the young (Birnie *et al.* 2013).

1.2.1.2. Ontogeny of personality

One of the criteria included in the definition of animal personality is the consistency of individual behavioural responses over time. However, in young individuals, this assumption might be difficult to meet because in particular the early ontogeny entails strong developmental changes (West-Eberhard 2003) as exemplified by strong age-dependent differences in motor skills and behavioural performance (Muciño *et al.* 2009). To study the developmental process of personality, it is essential to understand whether and to what extent personality is stable over time, in particular during early life stages. Additionally, further research is needed to elucidate how personality develops, and what are the underlying physiological and neuronal mechanisms associated with personality variation (Stamps & Groothuis 2010; Groothius & Maestripieri 2013).

Behavioural responses of individuals are often suggested to be more flexible during the early development due to behavioural and physiological adaptations (Trillmich & Groothuis 2011). As described earlier, genetic and environmental components (including their interactions) shape the individual phenotype. If personality traits are predominantly governed by environmental factors, any important modification in their environment, triggered prenatally and postnatally via parental effects as mentioned above but also by sibling effects, may affect personality (e.g. Trillmich & Groothuis 2011; Dall *et al.* 2012; Favati *et al.* 2014).

Such higher plasticity in young animals has been frequently related to their physiological changes during ontogeny. If personality is linked to physiological factors, any changes in their physiology such as hormonal profile might affect personality. Some brain structures, as well as the hypothalamic pituitary axis (HPA) still develop after birth (Giedd *et al.* 1999; Gage 2002). Such underlying structures have been shown to be associated with behavioural differences and to shape personality (Koolhaas *et al.* 2007; Capitanio 2010). For instance, behavioural inhibition is associated with the prefrontal cortex (Koolhaas *et al.* 2007). Because prefrontal cortex develops relatively late during early life, around adolescence, personality differences might consequently appear later in life (Trillmich & Groothuis 2011).

Personality has thus been proposed to be a function of age-dependent requirements and constraints (Gracceva *et al.* 2011; Rödel & Meyer 2011; Herde & Eccard 2013; Petelle *et al.* 2013). Studies actually assessing consistencies of behavioural traits over ontogeny present contradictory

results. Behavioural responses have been shown to be stable through different life stages in firebugs *Pyrrhocoris apterus* (Gyrius *et al.* 2012) and zebra finches (Wuerz & Krüger 2015), whereas they differ from fledging to sub-adult life stages in common ravens *Corvus corax* and carrion crows *Corvus corone corone* (Miller *et al.* 2016). In addition, consistencies across short versus long-term periods depend on the behavioural traits studied (Herde & Eccard 2013; Wuerz & Krüger 2015). Overall, such temporal consistencies in behavioural traits have been usually described to decrease over longer time intervals (Allen 1998; Bell *et al.* 2009; Herde & Eccard 2013). However, personality traits in young animals are frequently assessed by using specific tests originally developed for adult individuals. For example in laboratory rodents, anxiety-related behaviour and exploration are often assessed by open field or the elevated plus maze tests (Carola *et al.* 2002) and such tests are not always adapted to the ontogeny of the animal (Rödel & Meyer 2011); this could also explain the inconsistencies found in behaviour.

1.2.2. Personality tests over ontogeny

Studying the development of personality in young mammals is difficult for at least two reasons: first, due to the problem of testing dependent young without disturbing the often close mother-young relationship. Second, and as mentioned above, due to the limited but rapidly developing and thus changing behavioural repertoire, particularly in altricial species. All this makes it difficult to identify and test biologically meaningful behavioural variables that can be followed over a longer developmental time course (Réale *et al.* 2007; Stamps & Groothuis 2010). Lack of consistency in individual behavioural responses between two trials of a same test would thus not necessarily mean that personality is flexible but rather than the test does not have the same significance for the animal. In other words, consistent individual differences at least with respect to certain behavioural traits might be even present (very) early in life, but might be easily overseen. In this line, it remains often unsolved how personality in immature animals should be properly assessed and to this end, which tests might be adequate. The integration of tests in a social context might be especially relevant in young age classes, as many species of mammals and birds spend most of their time in the presence of parents and (same- or different-aged) siblings. Indeed, sociability is a trait generally used in animal personality research. For example, the amount of general motor activity and the display of directed, seemingly goal-oriented sibling interactions within the litter huddle have been used to assess early individual differences in domestic rabbits *Oryctolagus cuniculus* and in laboratory rats *Rattus norvegicus* (Rödel *et al.* 2008; Bautista *et al.* 2010, 2013; Reyes-Meza *et al.* 2011).

A particularly meaningful social context for young mammals can be the individual's responses to social separation from the mother and littermates. In mammals, the early developmental

environment includes numerous interactions with caregivers and littermates. In response to isolation, young of various species of altricial mammals often perform distress calls, which generally lead to the arrival of the caregiver (Briefer 2012). Vocal responses are closely related to the emotional state of an individual (Jürgens 2009; Briefer 2012), and separation calls have been shown to be consistent over time (sheep *Ovis aries*: De Passillé *et al.* 1995; cattle *Bos taurus*: Müller & Schrader 2005) and to be associated with sociability (Ligout *et al.* 2011). Separation calls might become a meaningful trait for the study of long-term consistencies of individuality across different life history stages (Stamps & Groothuis 2010). In addition, brief social separation tests might not demand high levels of motor abilities or in the pups' cognitive development as other standard tests do (e.g. measures of the locomotor activity in an open field).

1.2.3. Variation among siblings in a litter

While influences of the early life environment have been mainly investigated at the individual level, irrespectively of differences between siblings of the same litter or clutch, variation among siblings within litters has received much less attention (Hudson *et al.* 2011). However, heavier offspring within a litter have been shown to obtain more milk and consequently greater postnatal growths, more rapid motor development and higher level of testosterone than lighter offspring, with implications for their individual growth, development and survival (Drummond *et al.* 2000; Bautista *et al.* 2008; Rödel *et al.* 2008; Hudson *et al.* 2011). These heavier offspring have been shown to occupy more central positions in the litter huddle, with thermal advantages compared to lighter pups situated in more intermediate or peripheral positions (Bautista *et al.* 2008; Rödel *et al.* 2008; Hudson *et al.* 2011). Central offspring thus invest less in the maintenance of their body temperature with subsequent differences in their growth and development compared to their lightest littermates (Hudson *et al.* 2011; Bautista *et al.* 2013; García-Torres *et al.* 2015). Furthermore, the position in the litter huddle has been shown to be associated with their general motor activity and with proactive behaviours (Rödel *et al.* 2008; Bautista *et al.* 2010; Reyes-Meza *et al.* 2011).

In addition, individual differences in mother's interactions with their offspring within a litter might occur. As a consequence, it could lead to different developmental trajectories of the offspring within a litter (Cavigelli *et al.* 2010; van Hasselt *et al.* 2012a,b). Individual differences between human siblings and twins have always found lots of attention (e.g. Lytton 1977; Lobato & Kao 2002), as for example differences in parental behaviour have been shown to be associated with differences in the temperament of human siblings (Eley *et al.* 2004; Shanahan *et al.* 2007). The amount of maternal licking-grooming behaviour displayed by rat mothers toward the different individual pups of a litter can differ considerably, and such within-litter variation was even found to be as high as variation

among litters (Cavigelli *et al.* 2010; Ragan *et al.* 2012). High-licked rat pups within a litter were slower to approach novel objects as adults (Ragan *et al.* 2012) and showed less anxiety-related behaviour (Pan *et al.* 2014) compared to low-licked pups. As not all siblings experienced the same early-life environment, it could be useful to include such variance in parental care behaviour towards the different offspring of a litter into the study of the ontogeny of personality as well (Hudson *et al.* 2011; Zepeda *et al.* 2013).

1.3. Ultimate explanations: fitness consequences and the evolution of personality

The maintenance of personality types within a population has been explained by different processes of balancing selection, such as (among others) via heterogeneous environments heterogeneous environment and frequency-dependent selection (Dingemanse *et al.* 2010; Schuett *et al.* 2010).

1.3.1. Balancing-selection through heterogeneous environment and frequency-dependent selection

The association between fitness-related traits (e.g. reproductive performance or offspring condition) and certain personality types might greatly depend on the environmental conditions experienced. As environmental conditions can vary in space and time, they might trigger different selection pressures within and across generations. Therefore, fluctuations in environmental conditions could favour the coexistence of different personality types due to differential fitness advantages (Réale & Festa-Bianchet 2003; Dingemanse *et al.* 2004). For instance, in Siberian chipmunks, *Tamias sibiricus*, bolder individuals, those that were more trappable, had higher reproductive success in years with low food availability, whereas they had lower reproductive success in years with high food availability (Le Cœur *et al.* 2015).

A second mechanism that has been proposed is the frequency-dependent selection. Under some conditions, rare phenotypes might have a selective advantage, and evolutionary processes might lead to stable frequencies of different personality types (Maynard Smith 1982). A classic example of the coexistence of two behavioural types is illustrated in the 'producer-scrounger' situation (Barnard & Sibly 1981), where animals that actively search for hidden resources are the "producers", while animals that exploit food resources are the "scroungers". The more scroungers are present in a population, the less beneficial this strategy becomes, because these animals compete for fewer resources. In this case, the benefits of having one behavioural type depend

negatively on the frequency of that type in the population. Selection acts to increase the frequency of rare phenotypes through negative frequency-dependent selection. The rareness advantage could be extended in case selection favours more than two phenotypes, and might thus explain the coexistence of two or more behavioural types in stable frequencies (Wolf *et al.* 2007). Frequency-dependent selection has been used to explain adaptive state differences (e.g. benefits of particular physiological architecture or physical and social environment) among individuals or the existence of responsive and unresponsive individuals (Wolf *et al.* 2007, 2008).

1.3.2. Assortative phenotypic combination

Another hypothesis for the maintenance of personality variation comes from the combination of similar behavioural phenotypes within breeding pairs (Schuett *et al.* 2010). The correlation (positive or negative) between male and female phenotypes or genotypes between mated pairs has been frequently shown to have fitness effects. Most studies have explored these associations of personality combinations with parameters related to offspring condition (Budaev et al. 1999; Dingemanse *et al.* 2004; Both *et al.* 2005; Schuett *et al.* 2011a) and reproductive parameters such as clutch sizes, parturition success (Ariyomo & Watts 2013; Sinn *et al.* 2006). Assortative phenotypic combinations have been described in several taxa such as in fish, birds and squids (e.g. Both *et al.* 2005; Sinn *et al.* 2006; Ariyomo & Watts 2013), but rarely in mammals (Schuett *et al.* 2010). There are some studies suggesting that in mammals assortative mating could also lead to fitness advantages as well. For instance, in humans, similarity between partners in their psychological states is associated to marital satisfaction (Antill 1983; Richard *et al.* 1990; Gaunt 2006) and personal subjective well-being (Arrindell & Luteijn 2000). However, no studies have been carried out yet to investigate the association between similarity in personality traits between pairs and -traits correlated to reproductive fitness in a mammal. Fitness advantages might also arise in this taxa and might be especially probably in monogamous species with bi-parental care, as the coordination between partners is often considered an important mechanism underlying such advantages of assortative personality. Therefore, we decided to carry such a study in the monogamous mound building mouse *Mus spicilegus*.

1.4. Motivations and objectives of my thesis

The study of animal personality helps understanding why animals behave the way they do from a holistic point of view. The main goal of my thesis is to contribute to this field of research by a study on the emergence and the development of differences in personality in a rodent of wild origin, the mound building mouse. Such studies in animals of wild origin might be particularly useful as, in contrast to laboratory animals selected for particular behavioural traits, wild animals express higher

variation in personality traits (Koolhaas 2008). In addition, one of my studies generally deals with a topic related to the evolution of this phenomenon. This manuscript attempts thus to answer the following questions:

(1) How do environmental (non-genetic) factors during early life, specifically parental effects, affect personality later in life?

I attempted to answer to this question in manuscript I. Because personality' development are thought to be mainly influenced by the parents and mainly studied with regards to maternal effect. My aim is to assess how parental effects in a biparental species contribute to shape the personality of the young (and especially father care / presence) (page 10)

(2) How does personality develop across ontogeny?

Because studies in the ontogeny of personality show differential consistency across different life stages, one of the aim of these thesis is to assess the consistency of the behavioural responses of young mammals from the pre-weaning period until around maturity. This question is studied in manuscript II and III. Through a comparative approach using two altricial mammal species (domestic *cat Felis silvestris catus* and mound building mouse) and in collaboration with Dr. Robyn Hudson from the Instituto de Investigaciones Biomédicas (Universidad Nacional Autónoma de México), manuscript II attempts to develop an ethologically meaningful test for the study of personality in very young mammals, Making use of their responses to social separation. The main questions we wanted to answer were whether

(i) the frequencies of acoustic emissions and locomotor activity are consistent across time, and whether
(ii) kittens and young wild-type mice express similar patterns in their behavioural responses.

Manuscript III investigates the existence of a behavioural syndrome between several personality traits, including the social interactions displayed in undisturbed sibling groups. The specific goals of these manuscript are to investigate whether

(i) the social interactions displayed toward siblings are consistent across time,
(ii) behavioural responses during social interactions and in social separation test are associated with non-social standard test (across context), and whether

(iii) long-term consistencies within and across the different personality tests are detectable.

(3) What are the hormonal and immune correlates of personality variation in response to socially induced stress?

Physiological variations are associated with differences in personality. This is realised in manuscript IV, where the purpose of this project is to investigate whether and how animal personality traits modulate immunosuppressive effects of stress in a mammal of wild origin. This study is carried out in collaboration with Natacha Bessis and Sara Khalegparast Athari from the Laboratoire d'Immunologie et Immunopathologie (LI2P, Paris 13, Sorbonne Paris Cité). We attempted to assess whether

(i) personality types are associated with differences in corticosteroid levels, and whether
(ii) personality types are associated with differences in immune parameters in response to repeated social confrontations.

(4) Could similar personalities within breeding pairs affect the likelihood of reproduction?

This question is investigated in manuscript V. Personality combination is hypothesized an important balancing selection process for the maintenance of personality variation in many taxa. However, it remains to be studied in mammals. I explored potential benefits of assortative combinations in personalities within breeding pairs with respect to different parameters of reproduction. I investigated whether and how

(i) assortative combination in personality types influence the onset and the probability of reproduction and the litter size,
(ii) assortative combinations in personality types influence offspring body mass.
(iii) We also consider whether male or female personality *per se* and,
(iv) whether the body mass of the females might exert distinct effects on reproduction of the pair.

2. General Methods

2.1. My main study animal: the mound-building mouse

The study model used in this thesis is the mound-building mouse *Mus spicilegus* (Sokolov *et al.* 1998). Information on this taxonomic group is indicated in box 1.

Kingdom:	Animalia
Phylum:	Chordata
Class:	Mammalia
Order:	Rodentia
Family:	Muridae
Genus:	Mus
Species:	*M. spicilegus*

Box 1: Taxonomic group of *Mus spicilegus.*

2.1.1. Distribution

The mound-building mouse is distributed across the steppes of Eastern and Central Europe, but has recently been found in a discontinuous population in the southern Balkans (Krystufek & Macholán 1998) (Fig. 1.). Like most of the other non-commensal *Mus* species, the mound-building mouse lives in open habitats such as natural grasslands and agricultural fields (Coroiu *et al.* 2008).

Photo by Patrick Gouat.

Fig. 1. Current *Mus spicilegus* distribution.

2.1.2. Life cycle

The particular life cycle of the mound-building mouse splits this species in two distinct life history trajectories. In autumn, from mid-August to the beginning of November, juvenile mice born in late summer construct large mounds made of soil and vegetation (Murariu 1981; Sokolov *et al.* 1998), and overwinter under these structures in groups of 1-23 related individuals, almost all of them juveniles with the exception of one occasional adult (Muntyanu 1990; Garza *et al.* 1997; Poteaux *et al.* 2008; Canady *et al.* 2009; Hölzl *et al.* 2009; Szenczi *et al.* 2011). These mice are the only species in the genus known to build mounds (Auffray & Britton-Davidian 2012). It is assumed that most adults die before the next spring as it has hardly been found adults in the mound (Muntyanu 1990; Canady

et al. 2009). *Mus spicilegus* leave the mound in spring, disperse and breed seasonally from March to mid-October (Sokolov *et al.* 1998). Each female can have 4-5 litters in a breeding season with litter sizes ranging from 4 to 11 (Sokolov *et al.* 1998). Mice born from April to early summer will mostly die and only the ones born in late summer will reproduce next year.

2.1.3. Mating system and parental care

The mound-building mouse is the first *Mus* described as monogamous (Patris & Baudoin 1998; Auffray & Britton-Davidian 2012). The first evidence of social monogamy comes from female choice, whereby both oestrus and postpartum oestrus females preferred to copulate with their familiar mate than with an unfamiliar individual. These mice form pairs, and males and females cooperate in offspring care (Patris & Baudoin 2000). Compared to *Mus musculus domesticus*, male mound-building mice are more efficient at retrieving straying pups, alternate pup protection with females and spend significantly more time covering their week-old pups (Patris & Baudoin 2000). In addition, the time spent by the male in the nest with his mate is correlated with shorter intervals between litters (Féron & Gouat 2007), potentially reducing the energetic costs of reproduction.

2.2. Personality tests used in this project

A careful choice of the tests used in personality research has to be done to avoid conceptual or methodological difficulties (Réale *et al.* 2007; Carter *et al.* 2013). Behavioural tests used in this study were selected due to their validity to measure the targeted trait in other close species.

2.2.1. Elevated plus maze test

The elevated plus maze test exploits the natural aversion of rodents to exposed fields and is considered a reliable measure of anxiety-related behaviour (Archer 1973; review in: Carola *et al.* 2002).

The elevated plus maze used in our study was elevated 70 cm above the floor, and consisted of 2 opposing enclosed arms (by 50 cm high walls) and 2 open arms, each of the arms 30 cm long and 5 cm wide and connected by a central platform (10 × 10 cm). We recorded the number of entries/exits into the closed as well as the open arms, defined as crossing the respective thresholds with more than 50% of the animal's body length. We also measured the time the animal spent inside each arm (with all four paws) (Fig. 2).

Fig. 2. Elevated plus maze used in our research project. Photo by Marylin Rangassamy.

2.2.2. Open field test

As well as the elevated plus maze, the open field exploits the natural aversion of rodents to exposed fields (Carola *et al.* 2002). Among other traits, this test has been frequently used to assess the locomotor activity of the animals (Carola *et al.* 2002; Pogorelov *et al.* 2005).

According to the age of the animals (before or after weaning), two different sizes of white circular open fields were used. The one used prior weaning had a diameter of 48 cm and the surrounding walls were 50 cm high. The one used after weaning had a diameter of 60 cm with surrounding walls of 69 cm. For analysis, we defined a (circular) central zone of the arena, which represented 1/3 of the total diameter of the open field. We quantified the total distance covered by the animal, the distance covered in the centre of the arena and the latency to enter the centre of the arena (Fig. 3).

Fig. 3. Mouse in the arena used for the open field test. Photo by Marylin Rangassamy.

2.2.3. Novel object test

The aim of this test is to assess the neophobia of the animals. Following the recommendation given by Heiser and Chemero (2012), we only used objects where animals could climb on, allowing thus a greater discrimination index. We also habituated the animals to the arena before the introduction of the objects in order to exclude other sources of novelty.

Three objects were used in our research project. A colour plastic teddy bear (length: 3.1 cm, height: 3.6-3.8 cm), a coloured artificial hamburger made of soft, plastic PVC (diameter: 8.5 cm, height: 4.5-5.0 cm) and a kidney-shaped metallic box with smooth, varnished surface and with a slightly convex top (length: 9.5 cm, height: 2.2-2.7 cm; Fig. 4).

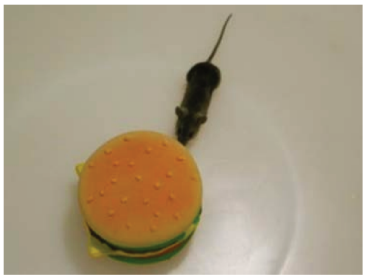

Fig. 4. Mouse approaching a novel object (plastic hamburger). Photo by Marylin Rangassamy.

2.2.4. Jump test

This test is usually performed with electric shock (“flinch-jump”) to assess pain sensitivity and pain tolerance in the animals (Kovacsics & Gould 2010; Li *et al.* 2013). However, without shock, it has successfully been used in rats to describe anxiety-related behaviours (Rödel & Meyer 2011). The high

motivation to jump in their cages of the model species makes this test very suitable (observation of our research group).

This test was carried out in the same arena as the one for the open-field test, and we added a thin layer of sawdust covering the floor. Animals were placed in the centre of a rectangular metallic box (length: 9.5 cm, height: 2.2-2.7 cm, width: 5-6 cm) and the latency until animals jumped from this platform was quantified (Fig. 5).

Fig. 5. Mouse in the metallic box used in the jump test. Photo by Marylin Rangassamy.

2.2.5. Social separation test

The aim of the social separation test is to assess consistent differences in the response of dependent young to social isolation, that is, separation from their mother, other care givers or littermates. Separation calls have been associated with negative states such as alarm, fear or pain (De Passillé *et al* 1995; Jürgens 2009; Briefer 2012) and could reflect the distress of the animals.

Pups were placed in a defined corner of a test arena, consisting of a rectangular plexiglas box (14.5 × 9.5 × 8.5 cm). We recorded pups' ultrasonic calls by the use of a bat detector (Batbox Baton, Batbox LTD, Steyning, UK) fixed 5 cm above the centre of the test arena. The frequency range of this detector was between 20 and 120 kHz. Recordings were automatically transformed by a conversion factor of 10 to make them detectable for the human ear (Fig. 6).

Fig. 6. Mouse in the arena (with the bat detector) during the separation test. Photo by Marylin Rangassamy.

2.2.6. Social interaction test

We developed a sociability test based on the observation of interactions in undisturbed sibling groups. Such approach could be easily done when animals were weaned but still not mature.

The home cage was covered with transparent plastic glass with vent holes in the periphery. Water and food were put directly inside the cage and mice were individually marked with a black permanent nontoxic hair dye (Nyanzol-D, Greenville Colorants, Jersey City, NJ, U.S.A.). These modifications allowed to identify individuals and to record their social interactions with a video camera. We recorded each sibling group for 10 minutes in three different occasions. For each of the 3 periods, the frequencies of nasal contacts (naso-nasal and naso-anogenital contact) and the number of approaches (defined as moving towards conspecifics to initiate a contact, with or without changing his initial direction) initiated by the focal individual was quantified (Fig. 7).

Fig. 7. A group of four siblings in their home cage. Photo by Marylin Rangassamy.

Manuscript I – in preparation for submission

Father absence during early life and long-term differences in offspring personality type in a monogamous rodent

Abstract Consistent individual differences in behaviour, also called personality, have been frequently shown to be modulated by the conditions experienced during an animal's early development. In mammals, maternal effects are a major source of variation in offspring phenotype, for example via differences in care behaviour provided to the young. In addition to maternal effect, the father has been shown to contribute to offspring phenotype. However, the mechanism by which the father exerts its influence remains unknown and father' effect on offspring personality has mainly focused through its removal. We investigated the contribution of the father in offspring behaviour in a rodent with a bi-parental care system, the mound-building mouse, *Mus spicilegus*. In particular, we studied whether the absence or presence of the father during the postnatal period modulates individual differences in behaviour of the offspring and whether differences in parental care due to the father's care contribution are the main pathway of such purported effect. To this end, fathers were removed shortly before birth in some litters, while the other ones remained with both parents. We assessed the time that mothers and fathers spent performing different care behaviours in the two groups over a period of 5 days after birth, such as handling-licking behaviour and their general presence in the nest. As expected, the summed-up care behaviours in litters with both parents were more frequent and longer compared to litters where mothers raised the pups alone. We then quantified the behavioural phenotypes of the offspring by repeated battery tests at different age classes. Behaviours reflecting anxiety, neophobia, activity and sociability were significantly repeatable across sub-adult and early adult life stages, thus indicating stable personality differences in the mound building mouse. The absence of the father altered the personality type of the offspring, at least with respect to their responses in some of the tests: pups raised in the presence of the father showed significantly shorter latencies to approach a novel object and to jump from the platform than the ones reared without the father, suggesting a higher responsiveness in the former group. Assuming that this comparatively higher responsiveness in pups raised in the presence

of the father would be due to the increased amount of care, we predicted that pups enjoying more parental or paternal care within the bi-parental groups would show a behavioural phenotype characterized by a higher responsiveness. This was, however, not supported by our data, as there were no such significant correlations within the bi-parental groups, and data even pointed towards associations in the opposite direction. We conclude that our study clearly shows consequences of father absence on the offspring's behavioural phenotype. However, we did not find support that gradual between-litter differences in paternal or parental care were the underlying mechanism for this association observed. We hypothesize that other differences in the early developmental environment arise from the removal of the father shortly before parturition. For example, maternal stress or differences in mothers' pregnancy status due the removal of the male might also play a role in priming the behavioural phenotype of the offspring.

Keywords: ontogeny, parental care, mound-building mouse, *Mus spicilegus*

Introduction

The early development of an individual is subject to diverse environmental influences. Especially, the quality and the composition of the early social environment have the potential to drive long-lasting consequences on the individual behavioural phenotype (Stamps & Groothuis 2010; Trillmich & Hudson 2011). In particular, maternal or parental effects, the causal influence of the parental phenotype on the offspring phenotype, are of major importance to affect the offspring's behavioural and physiological phenotype, as they can modify the early environment experienced by the offspring (Mousseau & Fox 1998; Houdelier *et al.* 2013).

The effects of maternal deprivation can dramatically alter the phenotype of the offspring (Harlow 1961) and result in an increase of anxiety-like and depression-like behaviour for instance (Lee *et al.* 2007; Veenema *et al.* 2007). In addition, the quantity and quality of maternal care have the potential to shape pups' behavioural, neural and physiological development (Champagne *et al.* 2003; Perkeybile *et al.* 2013; Braun & Champagne 2014). In rodents, the amount of care behaviours such as licking-grooming and arched-back nursing (Champagne *et al.* 2003) during infancy have been demonstrated to be key behaviours affecting or modulating the later phenotype. Pups that received high quantities of this care decreased fear behaviour in a novel environment (Caldji *et al.* 1998),

increased exploration in the elevated plus-maze (Bales *et al.* 2007) and in the open-field (Francis *et al.* 1999b), and showed greater cognitive development (Bredy *et al.* 2004).

Paternal effects have received less attention, although they are likely to occur through epigenetic transmission, paternal influence on mother-offspring interactions and / or through direct paternal care (Braun & Champagne 2014). The permanent absence of the father, frequently referred to as "paternal deprivation" can potentially affect the growth and the survival of the offspring (Wynne-Edwards 1987; Wynne-Edwards & Lisk 1989). In rodent species with bi-parental care, fathers can perform all care displayed by the females, except lactation (Kleiman & Malcolm 1981; Dewsbury 1985). Conditions of father absence lead to a general reduction in parental care behaviour towards the offspring (Helmeke *et al.* 2009; Pinkernelle *et al.* 2009; Bambico *et al.* 2013), and offspring raised under conditions of father absence usually show an increase in anxiety-like behaviour and an alteration of social interactions later in life (McGraw & Young 2010; Cao *et al.* 2014). For example, in the monogamous California mouse *Peromyscus californicus*, the presence of the father influences offspring social behaviour, by means of more contact between members of the litter (Vieira & Brown 2003). However, the effects of paternal care on offspring behavioural phenotype are mostly investigated through the removal of the father and the mechanism by which the absence of the father affects offspring personality remains therefore largely unknown (Braun & Champagne 2014; Bales & Saltzman 2015). Indeed, few studies investigate whether the quality of paternal care directly influences the offspring phenotype (Bales & Saltzman 2015). One of the rare studies that report effects of variation in paternal care on the offspring development in groups in which the father is present have been for instance carried out in marmoset monkeys *Callithrix geoffroyi*, showed that fathers differed in the quantity of care devoted to the offspring, and low quality of paternal care resulted in an increase in cortisol levels in the young (Birnie *et al.* 2013). There is a thus need for further studies to investigate whether and how paternal care might affect the offspring phenotype.

We conducted a study in a rodent of wild origin with bi-parental care (Patris & Baudoin 2000), the mound-building mouse, *Mus spicilegus*, to examine whether the care provided by father and mother (or their combination) results in long-term personality differences in the offspring. Males of this species are reported to alternate pup protection with the female, to spend significantly more time covering their pups during the first week and to be more efficient at retrieving straying pups than for example *Mus musculus domesticus* fathers (Patris & Baudoin 2000). For our study, offspring individuals were behaviourally phenotyped by repeated standard tests to determine individual variation in several personality traits quantified in non-social and social contexts. Our previous studies have shown that mound-building mice show notable individual differences in certain personality traits, making this species an extraordinarily suitable model for this kind of studies

(Hudson *et al.* 2015; Rangassamy *et al.* 2015). We followed a two-way approach. We removed the father in an assigned part of the litters to examine whether the rearing conditions in the absence of the father affected the behavioural phenotype of the offspring. If differences in care behaviour were due to the presence of the father, we expected an association between the quantity or quality of paternal and / or maternal care and the offspring behavioural phenotype between the two groups. We thus quantified the different care behaviours provided by the father and the mother separately in litters, in which both parents raised the pups. We expected that offspring raised in absence of the father would be more anxious, neophobic and less exploratory and, they might initiate fewer positive social interactions than offspring raised with both parents. In addition, we predicted that the amount of handling and licking received during early life would likely be the most relevant care to explain the behavioural variation between groups, as previously described (Champagne 2008).

Materiel & methods

Animals and Housing Conditions

Animals used in these experiments derived from individuals caught at different sites in Hungary in 1999 and bred in our animal facilities for 16 generations and was carried out over 2 years. To maintain genetic variation, additional individuals were captured at the same Hungarian collection sites every 2-4 years. Mice were kept in standard polycarbonate cages (26 × 14 × 16 cm, Iffa Credo, Lyon, France), containing wood shavings as bedding, with free access to rodents standard diet (Special Diet Services type M20, Witham, Essex, U.K) and water and underwent a 14:10 h light / dark cycle (lights off at 12:00 am). Cotton ball (diameter: approx. 5 cm) was always provided, which the animals (including juveniles) used for nest building in a corner of their cage. Temperature was maintained at 21 ± 0.5°C, and relative humidity at approximately 50%.

Formation of Pairs and Survey of Reproduction

We used thirty-four adult virgin male-female pairs of wild-type mound building mice for breeding. Each partner stemmed from different kin lines for at least two generations. The male and the female were isolated singly for one week before pairing to stimulate sexual receptivity (Féron & Gheusi 2003). Pairs usually start to reproduce at an age of 90 days, and this species usually matures sexually around the age of 70 days (Busquet *et al.* 2009). Males and females were weighed to the nearest 0.01 g once per week until pregnancy was detected by a steep increase in body mass in females. After pregnancy was confirmed, cages were checked every morning for the presence of litters, which

we considered as postnatal day 1. Cages were cleaned every 2 weeks except when pregnancy was detected, to avoid any disturbance.

Quantification of Parental Behaviour

Litters were randomly assigned to two different groups. In the first group, the male always remained in the cage with the mother (n = 22 litters) while in the second group, the male was removed from the cage shortly before parturition (preferably one day before) and the mother raised the pups alone (n = 12). Due to technical constraints, twenty-seven litters (both parents in the home cage: n =18; mother alone: n = 9) were following later on for quantification of care behaviour on the first litter produced by each pair (102 focal animals, $N_{females}$ = 41, N_{males} = 61). At birth, litter mass and litter size were noted. Parental behaviour was video recorded, starting at the beginning of the activity period of the animals (red light) for five full consecutive days. Later on, videos of days 1, 3 and 5 were fully analysed leading to 3 repeated measurements of the different care behaviour displayed in the nest by the father (when present in the cage) and the mother separately. We distinguished and quantified the duration of different behavioural categories:

Handling-licking: we quantified the time spent by the parents in nursing, licking, grooming or manipulating the offspring. These behaviours were pooled and reflected the time spent by the parents in "handling-licking" behaviour.
Contact with pups: we quantified the time spent lying in the nest and crouching over the pups with the four paws and in ventral contact with the pups. These behaviours were considered separately to the "handling-licking" category.
Non-directed behaviours: we quantified the behaviours displayed in the nest but not directed to the pups. These behaviours encompassed the mother-father interaction and the self-grooming.
Time spent in the nest: we measured the total time spent by the female and the total time spent by the male in the nest, which reflect the total time spent by at least one of the parents in the nest with the pups.

Sample Sizes and Behavioural Tests with Offspring

Sample Size

Four pups per litter were chosen to perform the behavioural tests later on. In total, we obtained 132 offspring from 34 litters in this study. These animals came from three experimental sessions performed at different period of time. At postnatal day 14, these animals were weighed to the

nearest 0.01 g and marked with different symbols on their back using a black, permanent non-toxic hair dye (Nyanzol-D, Greenville Colorants, New Jersey, USA) and were re-marked around 20 days later to allow individual recognition within the sibling groups. At 28 days, they were weaned, sexed by external genital inspection and kept in cages of 4 siblings of different sexes ($N_{females}$ = 52, N_{males} = 80) until postnatal day 55. From this day on, before mound building mice reach maturity (at around an age of 70 days, Busquet *et al.* 2009), all individuals were transferred to new cages and were housed singly.

Behavioural Tests

Five behavioural tests were performed with the offspring at two periods of time, at around day 40 - 44 after weaning when they were kept in cages of four, and at day 70 to 74 when individuals were isolated singly in a home cage. Between these two batteries of standard tests, the social interactions among siblings within the different groups were recorded on postnatal day 50. Experiments were always conducted in the dark phase (red light), during the animals' activity period. Immediately after each behavioural test, individuals were weighed to the nearest 0.01 g before they were returned to their home cages – except for the recording of sibling interactions, which were carried out in the animals' home cages. The different test apparatuses were always cleaned with water and soap (Cleansinald, Johnson Diversey, Fontenay-sous-Bois, France) between the different trials. Note that we obtained different samples sizes in the different tests, as some tests failed (e.g. due to individuals jumping from the elevated plus maze) or video recordings were not successful. Details on sample sizes can be found in Fig .1.

Open field test: This test was carried out on postnatal day 40 (post-weaning) and on day 70, around the time when animals reached maturity. The apparatus consisted of a white polyethylene circle arena with a diameter of 120 cm surrounded by walls of 69 cm. Each individual was placed singly on a defined position at the edge of the arena, and its behaviour was recorded for 5 min using a digital video camera mounted 120 cm above the centre of the arena. During later analysis, the distance covered by the animals were automatically quantified with the aid of Ethovision XT7 (Noldus Information Technology, Wageningen, The Netherlands). A total of 132 individuals underwent this repeated test.

Jump test: This test was carried out at postnatal days 40 and 70 immediately after the 5 minutes of the open field test in the same arena. Animals were taken out from the open field and a rectangular metallic box (length: 9.5 cm, height: 2.2-2.7 cm, width: 5-6 cm) was introduced in the centre of the arena with a thin layer of sawdust covering the floor. Animals were then placed centrally on top of this metallic box and the latency until animals jumped from this platform was quantified. After 300

seconds (5 min), the experiment was stopped and for those animals that did not jump, we noted down the maximum time (300 s) as "latency" to jump for later analysis. In total, 5 animals did not jump during the post-weaning period and 7 animals during the early-adult period. A total of 132 individuals underwent these tests.

Novel object test: This test was carried out in the same apparatus than the open field test; that is, even during the first time of testing, the arena was not novel to the animals. As for the open field test, the animals were placed in a defined position in the edge of the arena. After 10 minutes of habituation, a novel object was introduced in the centre of the arena. Tests were done on postnatal days 42 and 72 using different novel objects, a coloured, artificial hamburger made of soft, plasticized PVC (diameter: 8.5 cm, height: 4.5-5.0 cm) on day 42 and a 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) on day 72. Immediately after the introduction of the novel object, behaviours were recorded with a digital video camera mounted 120 cm above the centre of the arena for 5 minutes. We quantified the latency until the animals sniffed the novel object for the first time, and the summed-up time the animals spent exploring the object by means of sniffing, and touching with the front paws. A total of 132 individuals underwent these tests.

Elevated plus maze test: This test was carried out on postnatal days 44 and 74. The apparatus was mounted 70 cm above the floor by a stable wooden and metal construction and consisted of a white, rigid PVC divided in four arms of 5 cm wide and 30 cm long, arranged at 90° angles. Two opposite closed arms by 30 cm high walls and two open arms without walls were connected by a 10 × 10 cm central platform. To prevent jumps at the beginning of the trial, a closed box with holes (5 cm in diameter) on each side, made of clear Plexiglas, was added on the central platform. Each animal was placed on this central platform and its behaviour was recorded for 5 min by use of a video camera mounted 120 cm above the centre of the maze. We quantified the time an animal spent in the closed arms, defined as crossing the respective thresholds with more than 50% of its body length. Due to failures of video recordings, a total of 128 individuals underwent these tests.

Behaviour in sibling groups: Only groups of 4 litter siblings underwent this test in order to control for the number of potential interaction partners (n = 100 individuals stemming from 20 litters). Social interactions were recorded on day 50, when focal individuals were kept in groups of four (see details above). A few hours prior to the start of the recording, a part of their nest (made of cotton balls) was removed and the grid cover of the cage was replaced by a flat, transparent plastic glass cover with vent holes at the periphery for air ventilation. These modifications allowed us to identify individuals by their colour marks on their backs and to record their social interactions by a video camera

mounted 30 cm above the centre of the cage. Water was available in the corner of the cage and a small amount of pellets was dropped on the floor of the cages during the recording.

Three periods of 10 min length were video recorded for each sibling group. Recordings were started at 02:00 pm for 10 minutes and were then automatically re-started for two times during the next two full hours. For each of the 3 periods; we quantified the frequencies of nasal contacts (naso-nasal and naso-anogenital contact) and the number of approaches initiated by the initiator towards one of its siblings – defined as a movement, including translocation of the whole body, of the initiator towards the receiver, whereat the initiator did or did not change its initial direction. This behaviour was considered only when the focal individual approached the receiver at a distance equivalent to 5 cm between the initiator and the receiver. Nasal contacts were considered only when the initiator previously approached the receiver and not when both partners were already engaged in huddling together. Differences in the frequency of approaches and nasal contacts have been frequently used as measures of sociability, for example in young laboratory mice *Mus musculus domesticus* (Brodkin, 2007; Brodkin *et al.* 2004; Fairless *et al.* 2008). Agonistic interactions by means of events of chasing and displacement did not occur during the time of recording.

Experiments and observations were recorded with video cameras in night vision mode (Sony HDR-XR 200) for later analysis. Behavioural recordings were done by the aid of EthoLog, version 2.2.5 (Ottoni 2000) and Ethovision XT7 (Noldus Information Technology, Wageningen, The Netherlands).

Ethics Note

Animals were kept and treated according to the ethical and animal care guidelines of France, and experimental procedures were approved by the local authority for laboratory animal care and use (Comité d'Ethique en Expérimentation Animale Charles Darwin; Ce5/2011/068; Ce5/2012/212; 00809.02). After the experiments, breeding pairs were killed off in accordance to French animal law. This procedure was carried out in two distinct steps; subjects were first anesthetized by putting them into a closed transparent plastic box filled with isoflurane gas of a concentration of 3% (IsoFlo, Axience, France), administered by an automatic system (Univentor 400 Anaesthesia Group, Uno Roestvststaal BV, The Netherlands). Immediately after, subjects were euthanized with a high dose of CO2 gas, delivered by a compressed gas cylinder. We always observed the animals until all muscle activity and other signs of life had been absent for at least 30 s. Finally, we carried out cervical dislocation in order to ensure death. All procedures were conducted by a qualified and experienced person. The offspring of the breeding pairs were used for further behavioural experiments (not included in this paper).

Statistical Analyses

Consistencies of Behaviour over Time

Statistical analyses were done with R, version 3.2.3 (R Core Team 2014). We checked for repeatabilities across time in parental care and in the subjects' behavioural responses by intra-class correlation, calculated as the proportion of phenotypic variation that can be attributed to between-subject variation (Lessels & Boag 1987). This was done by LMM-based repeatability using R package *rptR* (Nakagawa & Schielzeth 2010). We assessed 95 % confidence intervals by 1000 bootstrap steps. We previously scaled the data for the analysis of the consistencies in subjects' behavioural responses in personality assays and the latency to sniff the objects and the latency to reach the centre were square-root transformed prior to analysis in order to adjust these variables to a normal distribution.

Parental Behaviour and Individuals' Behavioural Responses

For the following analyses, each of these behaviours displayed during red light and white light were pooled together, thus the percentage time spent in each behaviour were calculated over a full day of 24 hour. We analysed the total amount of parental behaviour provided by fathers and mothers toward the pups for each behavioural variable recorded. This was done by linear mixed-effects models based on restricted maximum likelihood estimates using the package *lme4* (Bates *et al.* 2014). Litter identity and the identity of the pup was included as random factors (random intercept) to adjust for the same origin of litter siblings tested. *P*-values were extracted by Wald Chi-square tests (type III). We assure that the model residuals were well adjusted to a normal distribution by visually checking normal probability plots. The latency to sniff the objects and the latency to reach the centre were square root transformed prior to analysis in order to adjust them to a normal distribution. Furthermore, we verified homogeneity of variances by plotting residuals versus fitted values (Faraway 2006).

Parental Care and Associations with Individuals' Behavioural Responses

We first tested by linear mixed-effects models based on restricted maximum likelihood estimates using the package *lme4* (Bates *et al.* 2014) for differences between father-absent and father-present groups and subjects' responses in the personality assays. Session was included as random factors (random intercept) to adjust for the different number of animals per room and the different experimental rooms used between the sessions. For all linear mixed-effects models, we tested for interactions among the following predictor variables: littersize, individual mass at weaning, sex and session to test if these predictor variables did not primary explained the differences between groups.

Non-significant interaction terms or predictor variables were sequentially removed from the models before these were re-calculated. To test for associations between the parental behaviours displayed by the mothers and the fathers and the responses in the personality assays, we also used linear mixed-effects models based on restricted maximum likelihood estimates using the package *lme4* (Bates *et al.* 2014). Session and litter identity were included as random factors (random intercept) to adjust respectively for the different number of animals per room and the different experimental rooms used between the sessions and for the same origin of litter siblings tested. We assure that the model residuals were well adjusted to a normal distribution by visually checking normal probability plots. Furthermore, we verified homogeneity of variances by plotting residuals versus fitted values (Faraway 2006).

Results

Parental Behaviour

Consistency in Parental Care Behaviour

Maternal and paternal care in groups with both parents: On average, the *time spent in nest* by the mother was 72.07% ± 6.71% SD and the *time spent in nest* by the father was 68.08% ± 16.60% SD of the total time. The parents showed different behaviours such as *handling-licking* (mother: 48.41% ± 10.96% SD; father: 15.99% ± 9.82% SD), *contact with pups* (mother: 19.46% ± 9.04% SD; father: 47.99% ± 15.87% SD) and *non-directed behaviours* (mother: 3.45% ± 7.33% SD; father: 2.35% ± 1.65% SD).

Sum-up maternal and paternal care in groups with both parents: The *time spent in nest* by at least one of the parents was, on average, 83.80% ± 7.01% SD of the total time. Parents spent in *handling-licking* 64.40% ± 17.32% SE; in *contact with pups* 67.45% ± 23.12% SD and in n*on-directed behaviours* 5.80% ± 7.57% SD (see above).

Maternal care in father-absent groups: The *time spent in nest* by the mother alone was on average 72.36% ± 8.01% SD and, during this time, they allocated in *handling-licking* 54.29% ± 6.38% SD, in c*ontact with pups* 14.02% ± 6.70% SD and in *non-directed behaviours* 0.70% ± 0.62% SD.

During the days 1, 3 and 5 after parturition, all the parental behaviour measured were repeatable for mother alone, mother with father and father (all $P < 0.05$; all $R > 0.43$). Due to these findings, we later on used the average of the three days for the following analyses.

Comparison of the Parental Behaviour expressed between Father-Absent Groups and Groups with Both Parents

Comparison between maternal care in both groups: There were no significant differences between the *time spent in nest* by the mothers alone and the *time spent in nest* by the mothers in groups where the fathers were present (χ_1^2 = 1.90, β = -1823 ± 1323 SD, *P* = 0.17). However, the behaviours expressed by mothers alone or by mothers in the group where the fathers were present were significantly different. Mothers alone displayed significantly more *handling-licking* than mothers in groups where the father was present (χ_1^2 = 17.24, β = -0.78 ± SD 0.19, *P* < 0.001), and these mothers spent significantly less time in *contact with pups* (χ_1^2 = 8.40, β = 0.53 ± SD 0.18, *P* = 0.003).

Comparison of the total amount of total care between father-absent groups and groups with both parents: Compared to the group in which only the mother raised the pups, the summed-up *time spent in nest* of both parents was significantly higher (χ_1^2 = 37.61, β = 1.02 ± 0.17 SD, *P* < 0.001). With respect to their summed up *time spent in nest* (mother + father behaviour) they also spend comparatively more time in *handling-licking* (χ_1^2 = 7.42, β = 0.56 ± 0.20 SD, *P* = 0.007) and in *contact with pups* (χ_1^2 = 213.2, β = 1.76 ± 0.12 SD, *P* < 0.001).

Association between Maternal and Paternal Care

In the groups where both parents raised the pups, there were significant positive associations between the following behaviours expressed by the mother and by the father: the *time spent in nest* (χ_1^2 = 4.59, β = 3517 ± 1641 SE, *P* = 0.032), *handling-licking* (χ_1^2 = 5.29, β = 2045 ± 1063 SD, *P* = 0.021) and *contact with pups* (χ_1^2 = 43.99, β = 8447 ± 1274 SD, *P* < 0.001).

Offspring Behaviour in Repeated Tests

Consistency in offspring behaviour across time: Except for the latency to reach the centre of the open field, the percentage of time spent in closed arms in the elevated plus maze; the distance covered in the open field, the latency to jump from the platform, the latency to sniff and approach the novel object as well as the time spent to explore it were significantly repeatable between the post-weaning period and the time around which the animals reached maturity (Table 1). With respect to positive social interactions displayed in sibling groups, the frequencies of the initiation of nasal contacts as well as the initiation of approaches were significantly repeatable across the three period of time considered (Table 1). To investigate association with care behaviours, we averaged the behaviours between the post-weaning period and the time around which the animals reached maturity in the

elevated plus maze, the open field, the novel object and the jump test and we averaged all three period of times for all positive social behaviours quantified during the three consecutive observations of sibling groups.

Table 1. Intra-class correlations across time of different behavioural responses of young mound building mice in repeated open field tests, elevated plus maze tests and novel object tests. Repeatabilities (*R*) between the post-weaning period (tests during postnatal days 40-44) and the time around maturity (tests during postnatal days 70-74) are presented. The nasal contacts and the approaches toward sibling initiated by the focal animal were tested on postnatal day 50 for three periods of ten-minute test. There were no sex differences. Significant results are given in bold.

Time	Repeats	Test	Behaviour	*N*	*R*	$CI_{95\%}$	*P*
Post-weaning / around maturity	2	Novel object	Latency to sniff[1]	132	0.182	0 / 0.352	**0.022**
Post-weaning / around maturity	2	Novel object	Time spent to explore the object	132	0.186	0.02 / 0.34	**0.010**
Post-weaning / around maturity	2	Elevated plus maze	% Time spent in closed arms	128	0.354	0.211 / 0.498	**0.001**
Post-weaning / around maturity	2	Open-field	Latency to central area[1]	132	0.043	0 / 0.214	0.33
Post-weaning / around maturity	2	Open-field	Total distance	132	0.297	0.139 / 0.456	**0.002**
Post-weaning / around maturity	2	Jump	Latency to jump[2]	132	0.388	0.227 / 0.522	**< 0.001**
Across 3 periods of 10-min test	3	Sibling behaviour	Initiation of nasal contacts[2]	100	0.41	0.263 / 0.521	**0.002**
Across 3 periods of 10-min test	3	Sibling behaviour	Initiation of approaches[2]	100	0.382	0.249 / 0.499	**0.002**

[1] $[x]^{0.5}$ transformed [2] log transformed

Father-Absent Rearing Conditions in Early Life and Differences in the Increase in Body Mass

The increase in body mass measured between day 1 to day 14 (3.84 g ± 0.65 SE) as well as day 1 to day 28 (7.03 g ± 1.11 SE) did not differ significantly between pups from groups where the father was present and groups where the father was absent ($P > 0.10$).

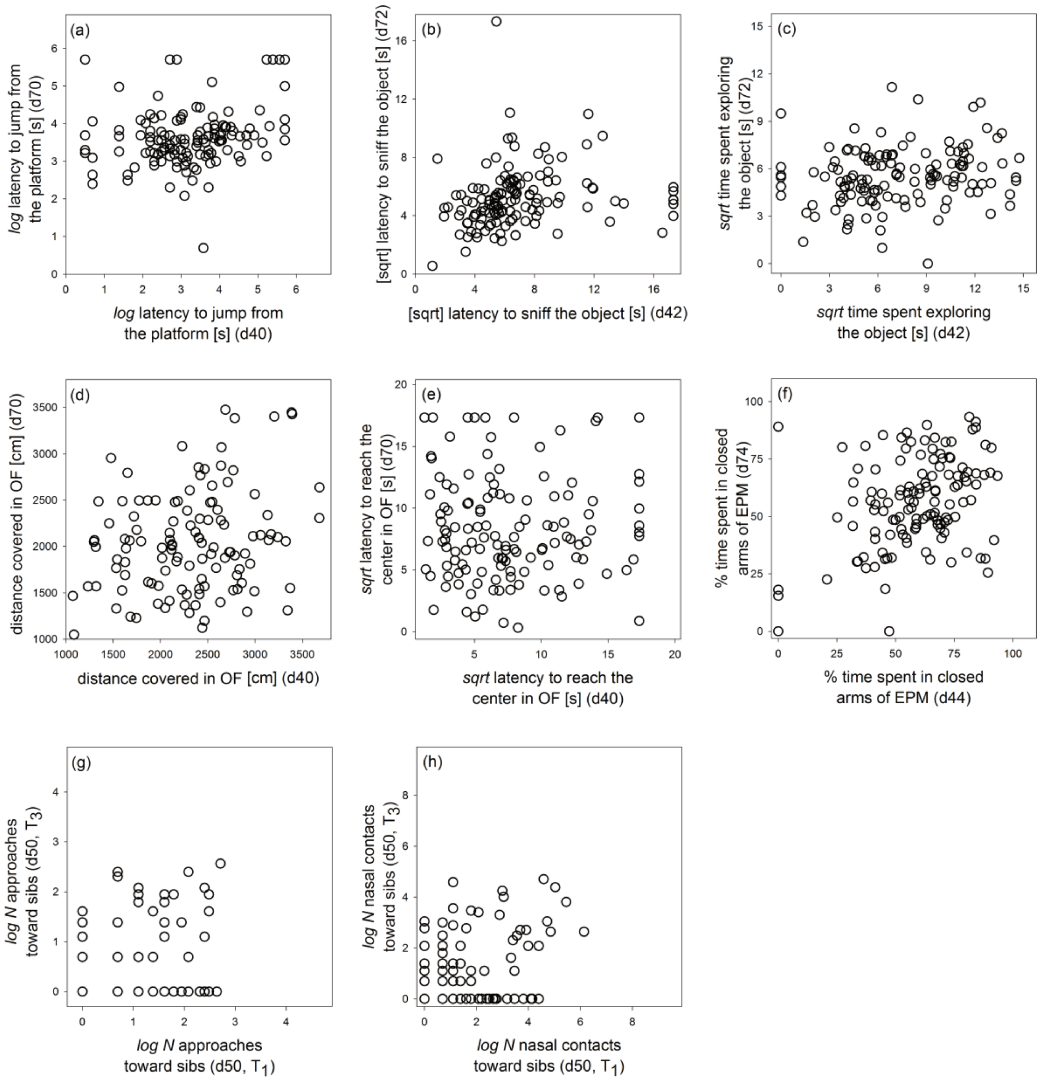

Fig. 1. Consistencies across time in individual behavioural responses of mound building mice during different tests. (a) The latency to jump from the platform in the jump test, (b) the latency to sniff the novel objects and (c) to explore it in a novel object test (NO), (d) the distance covered and (e) the latency to reach the centre in the open field (OF), (f) the percentage time spent in the closed arms of the elevated plus maze (EPM), and (g-h) the frequency of approaches and nasal contacts with litter siblings (groups of 4), initiated by the focal animal in consecutive recordings with 1 hour time lag. The days of testing are always given in the figure legends, (d: Day; T: Time). All associations shown are significant (tested by intra-class correlations), see Table 1 for statistics and sample sizes.

Paternal Absence in Early Life and Differences in Behaviour

There was a significant association between the absence of the father and the individuals' behavioural responses in the jump test, as pups raised without father exhibited a significantly longer latency to jump from the platform (Table 2; Fig. 2). Furthermore, pups raised without father showed a longer latency to approach and to sniff the objects in the novel object test (Table 2; Fig. 2). There were no significant differences between the other behavioural responses and paternal absence ($P > 0.10$; Table 2).

Table 2. Multiple linear mixed-effects models of the effects of sets of independent predictor variables in the responses of different behavioural responses of young mound building mice displayed in the personality assays. Significant results are given in bold.

Response variable	Predictor	χ^2	*P*
Time spent in closed arms (EPM)	Sex	0.05	0.82
Time spent in closed arms (EPM)	Litter size	7.86	**0.005**
Time spent in closed arms (EPM)	Mass at weaning	0.47	0.49
Time spent in closed arms (EPM)	Father presence	0.027	0.96
Latency to center (OF) [1]	Sex	1.80	0.18
Latency to center (OF) [1]	Litter size	0.75	0.38
Latency to center (OF) [1]	Mass at weaning	0.90	0.34
Latency to center (OF) [1]	Father presence	0.038	0.85
Total distance covered (OF)	Sex	0.61	0.43
Total distance covered (OF)	Litter size	0.92	0.33
Total distance covered (OF)	Mass at weaning	0.45	0.56
Total distance covered (OF)	Father presence	1.16	0.28
Latency to jump (JT) [2]	Sex	0.08	0.78
Latency to jump (JT) [2]	Litter size	0.03	0.86
Latency to jump (JT) [2]	Mass at weaning	0.03	0.86
Latency to jump (JT) [2]	Father presence	9.12	**0.003**
Latency to sniff (NO) [1]	Sex	4.29	**0.038**
Latency to sniff (NO) [1]	Litter size	3.42	0.064
Latency to sniff (NO) [1]	Mass at weaning	0.15	0.70
Latency to sniff (NO) [1]	Father presence	13.12	**<0.001**
Exploration (NO)	Sex	0.89	0.35
Exploration (NO)	Litter size	1.00	0.32
Exploration (NO)	Mass at weaning	0.008	0.93
Exploration (NO)	Father presence	0.50	0.48
Nasal contacts (SB) [2]	Sex	1.00	0.31
Nasal contacts (SB) [2]	Litter size	0.39	0.53
Nasal contacts (SB) [2]	Mass at weaning	0.08	0.77
Nasal contacts (SB) [2]	Father presence	0.11	0.75
Approaches (SB) [2]	Sex	1.74	0.19
Approaches (SB) [2]	Litter size	0.0003	0.99
Approaches (SB) [2]	Mass at weaning	1.04	0.31
Approaches (SB) [2]	Father presence	0.38	0.54

[1] $[x]^{0.5}$ transformed [2] log transformed

The latency to approach the objects and latency to jump from the platform were not correlated (χ^2_1 = 0.02, β = 3.06 ± 0.32 SE, *P* = 0.46), we thus considered this 2 variables separately to test for association with the different care quantified.

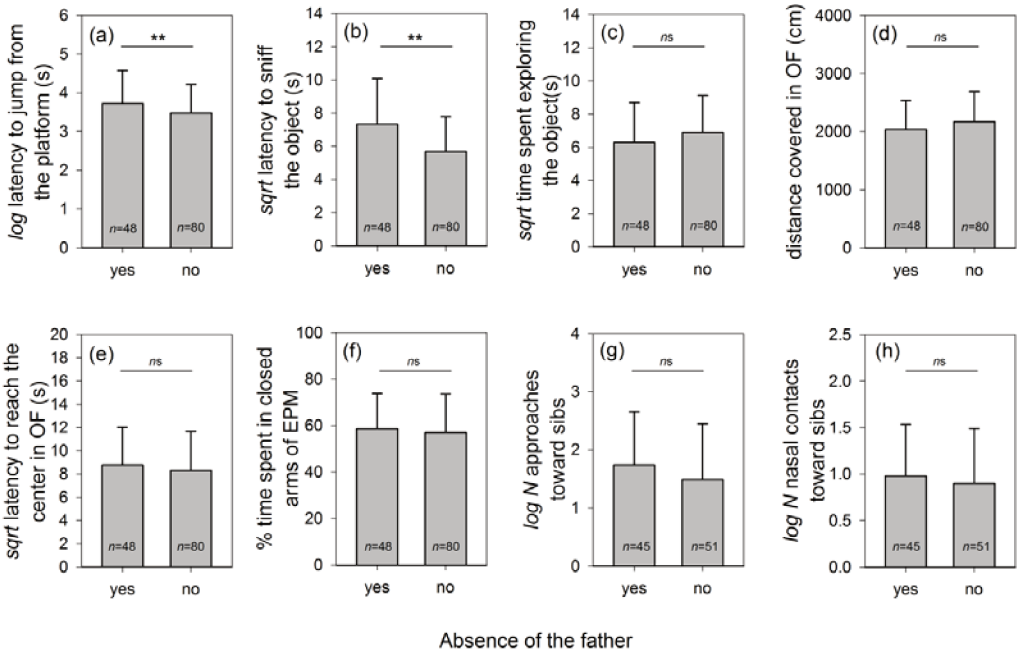

Fig. 2. Father absence affected behavioural responsiveness of mound-building mice but only for some behavioural variables. Father-absent mice had a significant longer latency to (a) jump from the platform in the jump test and (b) a higher latency to approach and to sniff the novel objects. (c) The time spent exploring the objects in the novel object test, (d) the distance covered and (e) the latency to reach the centre in the open-field test, (f) the number of approaches and (g) the number of nasal contacts did not differ significantly between paternal deprived offspring and offspring raised with both parents. All the statistics are shown in the Table 2.

Between-litter Differences among Parental Care Behaviour and Differences in Offspring Personality

We investigated whether variation in parental care behaviour was directly associated to the emergence of individual differences in offspring behaviour. To this aim, we first only considered the variables that differed between father-absent offspring and offspring in groups where both parents raised the pups. Absent-father offspring showed a higher latency to approach and to sniff an object and a higher latency to jump from the platform. In case when care behaviours drive differences in the behavioural responses, we thus expected that higher care displayed by the father, the mother or their combination results in lower latencies for these behavioural variables in the offspring.

However, we did not find significant association between the latency of the offspring to approach and to sniff the novel object and the time spent *handling-licking* displayed by the mother or the father separately, the *time spent in nest* by the father or the mother and the time that father or mother were in *contact with pups* ($P > 0.10$). We also did not find any significant associations between the latency to jump from the platform and the different care quantified ($P > 0.10$). Furthermore, there was no negative, but a positive association between the latency to approach and to sniff the novel objects and the time spent *handling-licking* by the parents (χ_1^2 = 4.49, β = 0.68 ± 0.32 SE, $P = 0.034$).

We assessed each care behaviours displayed by the females, the males or their combination with respect to the other behaviours displayed by the offspring in the different tests (not being significantly different between the father presence / absence). Also here, there were no significant associations (all $P > 0.10$).

Discussion

Our results demonstrate that individuals raised in the absence of their father showed differences in their behavioural phenotypes during later life, when compared to individuals raised by both parents. These differences in father-absent offspring consisted of a comparatively longer latency to approach the novel objects and to jump from a platform when tested during post-weaning and adult life stages, suggesting a higher responsiveness in such individuals. Overall, pups raised in the presence of the father enjoyed higher levels of parental behaviour during the first postnatal days compared to father-absent pups. However, in the group where both parents raised the offspring, we did not find that higher levels of parental care behaviour were associated with shorter latencies in offspring behaviour with respect to these two tests. These findings contradict our expectation that the observed differences in paternal care behaviour between bi-parental and uniparental groups were the underlying mechanism causing effects on offspring behavioural phenotype.

Through repeated social and non-social behavioural tests quantified across pre-weaning period and around maturity, we showed that offspring expressed consistent individual differences in the latencies to approach and to sniff the novel objects, the exploration of the objects, the distance covered in the open field and the time spent in closed arms in the elevated plus maze. Such findings are in accordance with previous work on this species and are usually considered to reflect the levels of neophobia, activity, and anxiety of the animals (Rangassamy *et al.* 2015). This study showed also that animals were consistent across time in their latency to jump from the platform. In addition, although measured in a short term, animals showed consistencies across the three periods in their positive social contacts in sibling groups, likely reflecting the sociability of the individuals. In none of the cases, approaches and the initiation of nasal contacts with litter siblings were followed by obvious signs of aggression. Consistent individual differences in patterns of positive social interactions have been frequently used to determine sociability in a wide range of species (e.g. in rhesus macaques *Macaca mulatta*: Capitanio 1999; laboratory mouse: Moy *et al.* 2004; sheep *Ovis aries*: Ligout *et al.* 2011, or European rabbits *Oryctolagus cuniculus*: Rödel *et al.* 2015). It is likely that the mound-building mouse also expresses consistencies in their sociability over longer time spans as

measured in these other species, although future studies might be done to confirm the existence of such long-term consistencies.

Our study clearly demonstrated long-lasting consequences of paternal absence on offspring personality type. Indeed, animals reared in absence of the father showed significantly higher latencies in two different behavioural tests, that is, the latencies to approach and sniff the novel objects and the latency to jump from the platform. Differences in the latencies quantified in these two tests could reflect a differential responsiveness in the animals. Generally, differences in an animal's responsiveness are likely be associated with differences in the sensitivity of the animals to environmental stimuli (Verbeek *et al.* 1994; Koolhaas *et al.* 1999; Groothuis & Carere 2005). Indeed, while some animals might be less affected by a change in their environment and show a more rigid, routine-like behaviour (also called unresponsive animals), other might be especially affected by any environmental stimuli in their current environment and quickly respond accordingly. The absence of the father in early life is likely to occur frequently under natural conditions due to predation or diseases, and an adjustment of the pup's behavioural phenotype might be adaptive to better cope with a challenging environment during adulthood (Sachser *et al.* 2011). To be raised without father might represent a critical challenge early in life, and offspring might thus be especially vigilant towards any changes in their current environment. Therefore, these animals might be more cautious, reflected by a longer latency to approach and sniff the novel objects or to jump from the platform. In great tits *Parus major* for instance, slow (responsive) birds differed in their exploration in a novel environment compared to fast (unresponsive) birds (Drent & Marchetti 1999).

Furthermore and contrary to our expectations, offspring raised without the father and those raised with both parents did not differ in any other behavioural variables. Such results are in contradiction with previous studies in prairie voles and mandarin voles, showing that offspring raised without fathers generally showed higher levels of anxiety-related behaviour, a lower exploration tendency and lower levels of positive social behaviour (Jia *et al.* 2009; McGraw & Young 2010; Cao *et al.* 2014). However, in the monogamous California mouse with bi-parental care, a species more closely related to the mound-building mouse, father-absent offspring did not differ either from offspring raised with both parents with respect to anxiety-like behaviour, locomotor activity and active social avoidance (Bambico *et al.* 2013). Particular life histories can drive differential effects of father presence / absence on differences in personality types (Oliveras & Novak 1986; Réale *et al.* 2007; Carter *et al.* 2013). An alternative, non-exclusive explanation could come from the fact that the father might have the potential to affect some behaviours in the offspring, while other behaviours could be shaped by the mother or by both parents. Indeed, it has been demonstrated that even if mother and father display similar care, the way that father provide the care could differ from the

mother with subsequent consequences on the offspring (review in: Braun & Champagne 2014). For instance, although both parents may participate in social play, offspring interactions with the father might exert greater influences on offspring phenotype than interactions with the mother (Guerra *et al.* 1999; Becker *et al.* 2005).

We quantified the levels of maternal and paternal care in groups where both parents raised the pups to investigate the mechanism which might explain the observed behavioural differences between offspring raised with or without fathers. We hypothesized that the lower responsiveness (by means of a longer latency to approach and sniff the novel objects and to jump from a platform) of the offspring in groups where both parents raised the offspring might be based on higher levels of care received. Mothers who raised the young alone devoted fewer time in care behaviours than the sum-up of father and mother care in groups, where both parents raised the pups. Higher levels of handling-licking behaviour have been regularly associated with a reduction of anxiety-related behaviours and fear in a novel environment (Caldji *et al.* 1998; Francis *et al.* 1999b; Menard *et al.* 2004). Based on our prediction, high level of care might thus results in a lower responsiveness of the offspring. Contrary to our expectations, however, our data did not support that a higher level of parental care behaviour was associated with a lower responsiveness in the offspring during later life. This was most probably not due to a false negative, as we even found a correlation in the opposite direction.

However, the removal of the father shortly before the birth of the offspring not only results in differences in the total amount of offspring care behaviour but can also exert potential consequences for the mother (Gubernick & Alberts 1987; Gubernick & Alberts 1989). Thus, it could be that only considering the care displays *per se* is not sufficient to disentangle the underlying mechanism in shaping differences in offspring personality types, and other indirect mechanisms might play a role. The removal of the father one day before birth provokes that mothers of this pair were not pregnant during lactation. On the contrary, most of the mothers where the fathers were present in the home cages might have been probably pregnant again shortly after giving birth due to post-partum oestrus in this species (Patris & Baudoin 1998). Mothers in father-absent groups and mother in groups where the fathers were present might thus differ in pregnancy status and consequently, in their hormonal status (Lambert *et al.* 2011; Siegeler *et al.* 2011). It has been reported that pregnancy and the associated hormonal changes may alter hippocampal neurons that regulate some non-pup-directed components of maternal behaviour and consequently the care that mothers provide to the pup (Kinsley *et al.* 2006; Pawluski *et al.* 2009). For instance, pregnant lactating rats have been shown to spend more time outside the nest and less time nursing than non-pregnant mothers, which resulted in a more anxious state in the former mothers' offspring (Uriarte

et al. 2008). In addition, in monogamous species, animals that are isolated from their partner show behavioural and neuroendocrine alterations (Grippo *et al.* 2007a, b; Bosch *et al.* 2008). The monogamous mound-building mouse develops a permanent bond with one partner (Patris & Baudoin 1998; Auffray & Britton-Davidian 2012) and our couples were kept in pairs for some weeks prior to the experiment, thus allowing the creation of such a bond. Thus, the absence of the father, by its removal shortly before the birth of the pups, might constitute a stressor with potential consequences on the behavioural and neuroendocrine state of the mother (Nowak *et al.* 2000; Götz & Stefanski 2007). Therefore, offspring raised in the absence of the father were likely to be confronted to a different hormonal intra-uterine environment than offspring raised in the presence of the father. Thus, independently of differences in care behaviour, different conditions of the mother such as the pregnancy status or the level of stress due to unstable social conditions and its associate hormonal changes can potentially act in priming the differences in offspring personality type (Uriarte *et al.* 2008; Siegeler *et al.* 2011; Sullivan *et al.* 2011; Kaiser *et al.* 2015).

In conclusion, we showed in the mound building mouse that the absence of the father shortly before birth results in an alteration of the behavioural phenotype of the offspring. Offspring from groups where both parents raised the pups overall received more care behaviour than offspring from the father-absent group. However, an increased level of parental care in the group where both parents raised the pups only affected one behavioural variable in the offspring and in a counter-intuitive direction. Indeed, more handling-licking behaviour by the parents resulted in a higher latency to sniff and to approach the novel objects. Such findings suggest that the causal association between parental effects and the behavioural phenotype of the offspring remain unclear. We hypothesise that a change in the maternal condition following the removal of the father might play a role on the differences in offspring behaviour. More studies are thus needed to disentangle which effects might finally drive the differences in the behavioural phenotype between offspring raised without fathers and offspring raised with both parents.

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Stable individual differences in separation calls during early development in cats and mice

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Abstract *Background*: The development of ethologically meaningful test paradigms in young animals is an essential step in the study of the ontogeny of animal personality. Here we explore the possibility to integrate offspring separation (distress) calls into the study of consistent individual differences in behaviour in two species of mammals, the domestic cat *Felis silvestris catus* and the mound-building mouse *Mus spicilegus*. Such vocal responses in young mammals are a potentially useful test option as they represent an important element of mother-offspring communication with strong implications for offspring survival. In addition, the neural control of vocalisation is closely associated with emotional state. *Results:* We found marked similarities in the pattern of individual responses of the young of both species to separation from their mother and littermates. In the domestic cat as well as in the mound-building mouse, individual differences in the frequency of calls and to a lesser extent in locomotor activity were repeatable across age, indicating the existence of personality types. Such consistencies across age were also apparent when only considering relative individual differences among litter siblings. In both species, however, individual patterns of vocalisation and locomotor activity were unrelated. This suggests that these two forms of behavioural responses to isolation represent different domains of personality, presumably based on different underlying neurophysiological mechanisms. *Conclusions:* Brief separation experiments in young mammals, and particularly the measurement of separation calls, provide a promising

approach to study the ontogeny of personality traits. Future long-term studies are needed to investigate the association of these traits with biologically meaningful and potentially repeatable elements of behaviour during later life.

Keywords: development, personality, siblings, open field test, vocalization, locomotion, *Felis silvestris catus, Mus spicilegus*

Introduction

Interest has been growing among behavioural biologists in the existence of individual differences in behavioural phenotypes of a kind now frequently referred to as animal personality (Wilson *et al.* 1994; Bell 2007; Briffa & Weiss 2010; Dingemanse & Wolf 2010; Carere & Maestripieri 2013). Once considered to be the exclusive domain of human psychologists, differences in animal personality are now considered to be the result of adaptive evolutionary processes (Dall *et al.* 2004; Wolf *et al.* 2007; Réale *et al.* 2010a), and to occur across a wide range of taxa (Gosling & John 1999; Stamps & Groothuis 2010; Kralj-Fišer & Schuett 2014; Müller & Müller 2015). Despite some uncertainty as to an exact definition of personality (Uher 2011; Zipser *et al.* 2013) and differences in theoretical applications of this construct, it is generally agreed that individual differences in behavioural traits need to be stable across an appreciable time span and / or different contexts to qualify (Sih *et al* 2004a; Réale *et al.* 2007; Briffa & Weiss 2010; Dingemanse & Wolf 2010; Stamps & Groothuis 2010).

More recently, attention has been drawn to the fact that there are still few studies of the ontogeny of such differences; when and how they emerge across development and how they relate to differences at later life stages (Stamps & Groothuis 2010; Trillmich & Groothuis 2011; Trillmich & Hudson 2011; Chang & Dingemanse 2015; Würz & Krüger 2015). This is particularly true for mammals, although an increasing number of recent studies have assessed and could successfully show the existence of personality traits in young animals around weaning by means of behavioural consistencies across time and context (Brommer & Class 2015). For example, personality assays such as open field, novel object or elevated plus maze tests have been used in young rodents before and / or shortly after weaning under laboratory conditions (laboratory rat *Rattus norvegicus:* Rödel & Meyer 2011, guinea pigs of wild origin *Cavia aperea:* Guenther *et al.* 2014) or in animals captured in the field (eastern chipmunks *Tamia striatus:* Bergeron *et al.* 2013, European rabbits *Oryctolagus cuniculus:* Rödel *et al.* 2015). Studies have also been made in very young mammals from birth until

weaning of differences in behaviour among littermates under undisturbed conditions in the litter huddle (domestic rabbits: Bautista *et al.* 2008, 2013; Reyes-Meza *et al.* 2011; domestic cats *Felis silvestris catus:* Raihani *et al.* 2014; review in Hudson *et al.* 2011). Furthermore, tests in wild animals such as flight initiation distances or responses to trapping (juvenile yellow bellied marmots *Marmota flaviventris*: Petelle *et al.* 2013) and handling responses of young during the nest period (European rabbits: Rödel *et al.* 2015) have been used to assess personality types. In a study of little brown bats *Myotis lucifugus*, exploration in animals caught from the wild has been repeatedly tested across development using modified hole board tests (Menzies *et al.* 2013).

Studying the development of personality in mammals is difficult for at least two reasons: the problem of testing dependent young without disturbing the often close mother-young relationship and affecting normal development; and the limited but rapidly developing and thus changing behavioural repertoire, particularly of altricial species. All this makes it difficult to identify and test biologically meaningful behavioural variables that can be followed over a longer developmental time course (Stamps & Groothuis 2010).

A notable example of behaviour largely free of such difficulties is the response of dependent young to social isolation, that is, to separation from their mother or other care givers, and in the case of polytocous species, also from littermates. Under such conditions the young of various mammals quickly start to emit persistent vocalizations (separation distress calls) of clear adaptive significance. This behaviour usually results in the arrival of the mother or other care giver(s) (e.g. humans: Christensson *et al.* 1995), often leading to the rapid retrieval of the young to the nest or burrow (e.g. various rodent species: Noirot 1972; Hahn & Lavooy 2005; Schneider & Fritzsche 2011; domestic cat *Felis silvestris catus*: Haskins 1977, own observations) or in mothers preparing to defend their young against potential predators or infanticidal conspecifics (Špinka *et al.* 2000; Lingle *et al.* 2005; Held *et al.* 2006; Rödel *et al.* 2013).

Vocalizations are a particularly useful behavioural measure. In mammals, distress or separation calls of dependent young typically consist of enduring trains of often high-pitched calls of variable frequency (Lingle *et al.* 2012). Not only can the frequency of emission and acoustic properties be readily measured and quantified (Schrader & Hamerschmidt 1997), but the neural control of vocalizations is closely integrated with and reflects the emotional state of an individual (e.g. Stoeger *et al.* 2011, 2012; Scheumann *et al.* 2012; reviews in: Jürgens 2009; Briefer 2012). This is particularly the case for vocalizations associated with negative states such as alarm, fear or pain, due to the close neural connections within the brain of vocal centres, with limbic structures such as the amygdala importantly involved in the regulation of negative affect (Jürgens 2009; Briefer 2012).

However, despite the clear functional significance of separation calling in young mammals and the close link to an individual's emotional state, it has scarcely been used in the study of personality in non-human mammals. One of the few published studies related to this is in cattle *Bos taurus*, where vocalisation was quantified in repeated open field tests (De Passillé *et al.* 1995). Furthermore, a study in lambs of domestic sheep *Ovis aries aries* showed that individual differences in the emission of high pitched bleats after separation were positively associated with lambs' sociability (Ligout *et al.* 2011).

Here, we present data from two altricial mammals with rather different life histories, the domestic cat *Felis silvestris catus* and the mound-building mouse *Mus spicilegus*. Domestic cats can be readily kept under semi-natural free-ranging conditions and mothers allow observation and handling of their new born young by familiar care takers without apparent protest or negative effects on the kittens' growth or survival (Hudson *et al.* 2009; Raihani *et al.* 2009, 2014). Although altricial, the kittens are mobile from birth (Prechtl 1952; Levine *et al.* 1978) and during the first postnatal month they emit persistent distress calls within seconds of being separated from their mother or nest (Brown *et al.* 1978; Scheumann *et al.* 2012). The mound-building mouse is a monogamous altricial rodent species (Baudoin *et al.* 2005), occurring in a variety of open habitats including steppe grassland, pastures and agricultural areas of central and south-eastern Europe (Sokolov *et al.* 1998). Mound-building mice can be successfully bred under laboratory conditions (Gouat *et al.* 2003a), and we have found that adolescent mice show consistent individual differences with respect to their behavioural responses in open field and elevated plus maze tests (Rangassamy *et al.* 2015). Both parents show retrieval behaviour when pups are displaced from the nest (Patris & Baudoin 2000). As has been reported for various other rodent species (Branchi *et al.* 2001), our preliminary studies have shown that mound-building mouse pups emit ultrasonic distress calls after separation from parents and littermates, well detectable at least until postnatal day 16.

It was therefore our aim in this study to investigate the existence of stable individual differences in behaviour during early development in these two species of altricial, taxonomically distant mammals. In addition, we tested whether such purported consistent individual differences were also present within litters, i.e. among siblings. We recorded individuals' responses in brief separation tests on repeated occasions before weaning using two behavioural measures, the number of separation calls and the amount of locomotor activity. In both species we expected stable individual differences in the performance of the two behaviours and a stable negative association between these; that is, we expected that more timid or fearful individuals would emit more vocalizations but show less locomotor activity ('freezing').

Results

Changes across Age in Vocalisation and Locomotor Activity

(a) Domestic cat

There were significant differences across age in the total number of calls emitted (GLMM for count data: χ^2_3 = 16.46, P < 0.001; Fig 1a). This showed a non-linear pattern with highest values during weeks 2 and 3 (post hoc analyses in Fig. 1a), and was significantly lower in males than in females (χ^2_1 = 16.46, P < 0.001). The interaction between the two independent variables (age × sex) was not significant (P > 0.10), indicating that differences between males and females consistently occurred across all age classes. There was no association between the number of calls emitted by individual animals and their body mass.

There were no significant changes in the animals' locomotor activity during the first 4 postnatal weeks (LMM: χ^2_3 = 6.12, P = 0.11). Furthermore, there was no relation between the animals' amount of locomotor activity and their body mass, and no difference on any measure of locomotor activity between males and females (all P > 0.10).

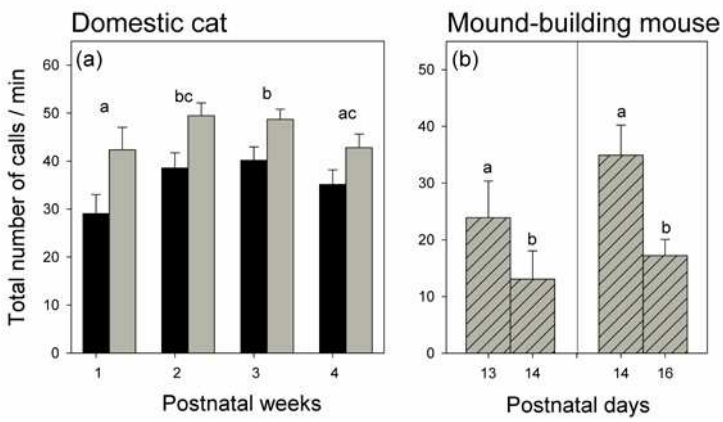

Fig.1. Developmental time course of separation calls emitted by domestic kittens and mound-building mice. (a) Kittens. Average (± SE) number of calls emitted in separation tests by males (n = 19; black bars) and females (n = 14; grey bars) during four experimental sessions across the first postnatal month. (b) Mound-building mice. Average (± SE) calls emitted in separation tests by young mice tested on postnatal days 13 and 14 (n = 18) and postnatal days 14 and 16 (n = 59). Note that data in (a) are repeated measurements, whereas 2 sets of different individuals are presented in (b). In (a), the number of calls emitted by males was significantly lower than in females. Call frequencies differed across weeks; significant post hoc comparisons are indicated by different letters. In (b), differences are significant in both subsamples; see text for statistics.

(b) Mound-building mouse

The number of vocalizations decreased significantly between postnatal days 13 and 14 as well as between postnatal days 14 and 16 (GLMM: both P < 0.05; Fig. 1b). This decline is consistent with published results in laboratory mice *M. musculus*, where vocalizations of pups also decreased sharply and even disappeared after about postnatal day 14 (Elwood & Keeling 1982; Branchi *et al.* 2001).

However, such a decrease was already apparent in our study in the independent data set between days 13 and 14, indicating that this decline might have been rather the consequence of habituation to the separation.

Locomotor activity increased significantly from days 13 to 14 (LMM: χ_1^2 = 31.74, P < 0.001) and from days 14 to 16 (χ_1^2 = 10.95, P < 0.001).

Neither the number of vocalizations nor the amount of locomotor activity were associated with pup body mass, and there were no significant differences between males and females with respect to either behaviour (all P > 0.10).

Individual Consistencies across Age in Vocalisation and Locomotor Activity

(a) Domestic cat

Individual differences in the number of calls emitted by kittens (n = 33) during the 3-minute separation tests were repeatable across all 4 age classes (intra-class repeatability: R = 0.491, $CI_{95\%}$ = [0.299, 0.645], P = 0.001; Fig. 2a–c), and even when only considering week 1 and week 4 (R = 0.298, $CI_{95\%}$ = [0.041, 0.663], P = 0.015; Fig. 2d). Individual differences in the time that kittens spent in locomotor activity were also repeatable across the first 4 postnatal weeks (R = 0.169, $CI_{95\%}$ = [0.002, 0.349], P = 0.025; Fig. 2e–g), although this was not the case when only testing for repeatability between week 1 and 4 (R = 0, $CI_{95\%}$ = [0, 0.327], P = 0.71; Fig. 2h).

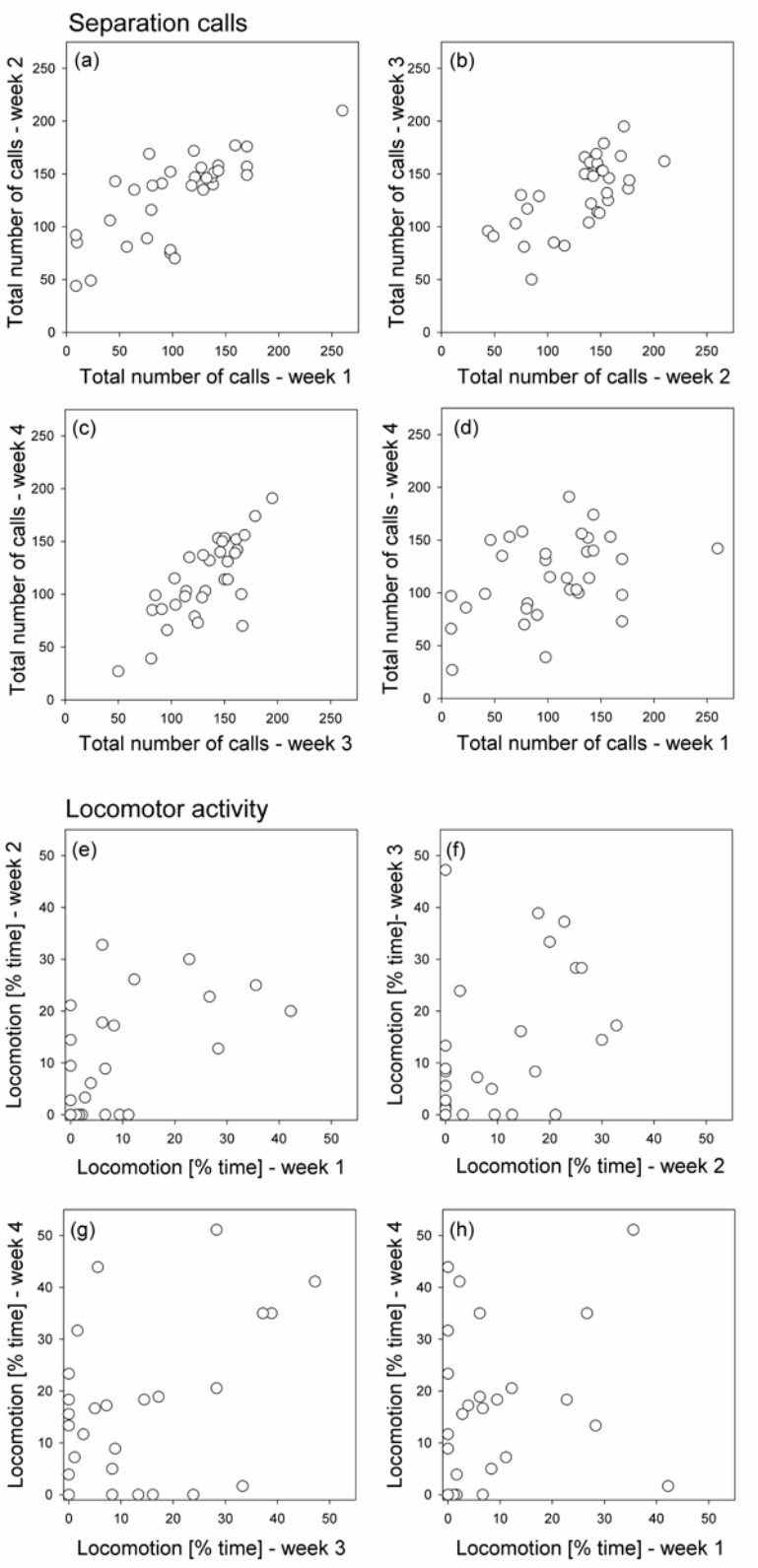

Fig. 2. **Domestic kittens: individual differences in separation calls and locomotor activity across age.** (a–d) Separation calls. Consistent individual differences in the number of calls during four, 3-minute separation tests across the first postnatal month. (e–h) Locomotor activity. Weaker consistency in individual differences in the % time the animals spent showing locomotor activity during the four tests, with no significant correlation (h) between individual differences in the first and the final week. Each circle gives the score for an individual kitten (*n* = 33 / 8 litters). Note that the association depicted in (h) is not statistically significant; see text for details on statistics.

We also obtained significant results when considering individual differences in behaviour with respect to litter siblings, calculated as the repeatability (among weeks 1,2,3 and 4) of the percentage deviation from the litter mean in vocalisation (R = 0.370, $CI_{95\%}$ = [0.180, 0.540], P = 0.001) and in locomotor activity (R = 0.179, $CI_{95\%}$ = [0.011, 0.347], P = 0.011). And again, when only taking into account data obtained during weeks 1 and 4, within-litter differences were significantly repeatable for the frequency of vocalisation (R = 0.298, $CI_{95\%}$ = [0, 0.581], P = 0.043) but not for within-litter differences in locomotion (R = 0.141, $CI_{95\%}$ = [0, 0.477], P = 0.209).

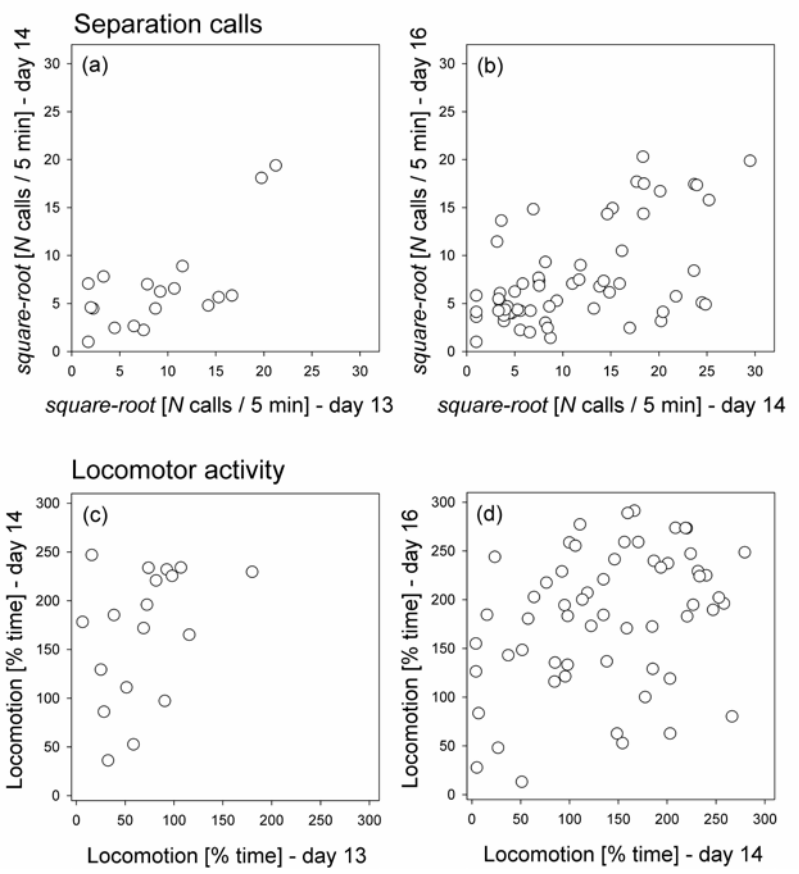

Fig. 3. Mound-building mice: individual differences in separation calls and locomotor activity across age. (a,b) Consistent individual differences during two 5-minute separation tests in the number of separation calls, and (c,d) in the % time the animals spent showing locomotor activity. The frequencies of vocalization are square-root transformed for presentation. Data from repeated measures during postnatal days 13 and 14 (a,c: n = 18), or during days 14 and 16 (b,d: n = 59) are presented. Each circle gives the score for an individual mouse. Note that the association depicted in (c) is not statistically significant; see text for details on statistics.

(b) Mound-building mouse

Individual differences in the number of calls emitted by young mound-building mice during the 5-minute separation tests were repeatable between postnatal days 13 and 14 (intra-class repeatability: R = 0.635, n = 18, $CI_{95\%}$ = [0.220, 0.877], P = 0.007; Fig. 3a) as well as between days 14 and 16 (R = 0.504, n = 59, $CI_{95\%}$ = [0.332, 0.745], P = 0.001; Fig. 3b). The time that the animals spent in

locomotor activity during the separation test was also repeatable between postnatal days 14 and 16 (R = 0.248, $CI_{95\%}$ = [0.001, 0.488], n = 59, P = 0.036; Fig. 3d), but not between postnatal days 13 and 14, when only 18 animals were tested (R = 0, $CI_{95\%}$ = [0, 0.415], n = 18, P = 0.79; Fig. 3c).

Also here, individuals showed repeatable differences with respect to their littermates. Within-litter intra-class repeatability with respect to the frequency of vocalisation was R = 0.605 (n = 18, $CI_{95\%}$ = [0.212, 0.826], P = 0.005) between postnatal days 13 and 14, and R = 0.547 (n = 59, $CI_{95\%}$ = [0.337, 0.701], P = 0.001) between days 14 and 16. With respect to locomotor activity, the within-litter repeatability was R = 0.421 (n = 18, $CI_{95\%}$ = [0, 0.721], P = 0.034) between postnatal days 13 and 14, and R = 0.420 (n = 59, $CI_{95\%}$ = [0.210, 0.631], P = 0.002) between days 14 and 16.

Lack of Associations between Vocalisation and Locomotor Activity

(a) Domestic cat

We did not find significant associations between individual frequencies in the number of calls and the percentage time individual kittens (n = 33) spent in locomotor activity during testing on week 1 (GLMM for count data: χ_1^2 = 0.01, P = 0.97), week 2 (χ_1^2 = 0.06, P = 0.80), week 3 (χ_1^2 = 1.10, P = 0.30) or week 4 (χ_1^2 = 2.78, P = 0.10).

There were also no significant associations between kittens' vocalisation and locomotor activity during any of the 4 weeks when considering relative differences among littermates, i.e. the percentage deviation in these two behaviours from the litter mean (P > 0.10).

We tested for interactions with body mass and with sex in order to detect potentially body mass-specific or sex-specific relationships between vocalization and locomotor activity. However, all interactions were non-significant (all P > 0.10).

(b) Mound-building mouse

Also in mice we did not find significant correlations between individual differences in the number of calls emitted and the percentage of time individual pups spent in locomotor activity, either on postnatal day 13 (n = 18; χ_1^2 = 0.08, P = 0.78), day 14 (n = 77; χ_1^2 = 0.01, P = 0.91), or day 16 (n = 59; χ_1^2 = 0.28, P = 0.59).

Also here, there were no indications of a significant association between the number of calls emitted and the pups' locomotor activity relative to their littermates, either during the tests on postnatal days 13, 14, or 16 (all P > 0.10).

We again tested for interactions with sex and with body mass, but these were non-significant in all cases (P > 0.10).

Discussion

In fulfilment of one of the defining criteria for the existence of animal personality (Sih *et al.* 2004b; Briffa & Weiss 2010; Dingemanse & Wolf 2010; Stamps & Groothuis 2010), we found stable individual differences in the behavioural responses of young kittens and mice to repeated separation from their mother, nest, and littermates. In both species some individuals consistently responded by emitting a larger number of separation (distress) calls than others, and some with greater locomotor activity. Such individual consistencies in vocalisation as well as in locomotor activity were also apparent when only considering relative differences in these two behaviours among littermates. Furthermore, as revealed by our analyses, these individual differences in behaviour were not just a consequence of variation in body mass, which might be considered to potentially affect vocal or locomotor performance (Muciño *et al.* 2009; Rödel & Meyer 2011).

In both species, however, consistent individual differences were more evident for the emission of separation calls than for locomotor activity as shown by comparatively higher repeatabilities in calling compared to locomotion. This consistency in differences in the frequency of separation calls is in accord with similar reports in a wide range of mammals (Blum *et al.* 2002; Müller & Schrader 2005; Ligout *et al.* 2011; Scheumann *et al.* 2012; Grandin & Deesing 2014). Thus, vocalization behaviour would seem to offer a useful means of testing for individual differences in emotionality or temperament, and particularly as in many mammals, including the cat (own observations), animals respond to separation from companions or familiar environments with distress calls across the whole lifespan (Grandin & Deesing 2014: for examples in other domestic animals).

Moreover, our findings seem reliable and possibly to reflect mechanisms underlying the development of individual differences in behavioural phenotypes more generally. The two study species were housed under very different conditions, and they were tested on different schedules. Kittens were even maintained and tested under “noisy” everyday conditions, including considerable fluctuations in ambient temperature. Nevertheless, the pattern of results was highly similar for both species.

Although it is not known if the individual differences reported here are due to genetic (van Oers *et al.* 2005; van Oers & Mueller 2010) or experiential factors (including in utero: Bánszegi *et al.* 2009; Bautista *et al.* 2015), their existence from such an early age in kittens suggests that there could be a genetic contribution (Raihani *et al.* 2014). In support of an endogenous (epi)genetic component was the difference in the number of cries emitted by male and female kittens, with males consistently emitting fewer cries across all four ages tested.

Unexpected, however, was our failure to find a correlation between individual differences on the two behaviours measured, and again in both species. Consistent individual differences across behaviours are also often considered part of the definition of personality (Bell 2007; Réale *et al.* 2007; Dingemanse & Wolf 2010). The unexpected lack of such a relation in the present study was all the more surprising given that vocalization and locomotor activity were measured in the same context, in repeated open-field separation tests.

At one level such a disjunction might be accounted for by the problem (common in the study of behaviour) of knowing what exactly our tests, and even those as well-established as the open field, actually measure (Archer 1973; De Passillé *et al.* 1995; Hudson & Distel 2013; Hudson 2014). Animals may behave in seemingly similar ways for different reasons, and in seemingly different ways for the same reason. This may be particularly true for complex behaviours such as locomotion to which a wide range of neural and motivational systems contribute. Thus, some of our animals might have shown little locomotor activity from fear ("freezing") or alternatively, from a lack of motivation to move around or explore. Others might have shown high levels of activity also from fear ("panic") or alternatively, from a motivation to explore. Choosing non-arbitrary behavioural measures of evident adaptive significance might be one way to help avoid such ambiguity. Indeed, this might help explain the more robust and consistent results obtained for measures of individual differences in vocalization than in locomotor activity. Vocalizations, such as separation calls in young individuals, often have a clear functional meaning, and as mentioned in the Introduction, are the products of neural processes closely related to emotional and motivational systems (Jürgens 2009; Briefer 2012).

Additionally, the lack of relation between individual differences in separation calling and locomotor activity might have been due to differential maturation of the two systems. Whereas many altricial mammals emit separation calls with facility from birth, locomotor behaviour develops more slowly and not necessarily at the same pace for all individuals, a phenomenon sometimes referred to as developmental heterochronicity (Smith-Gill 1983). This would also potentially explain the lack of a significant correlation between individual differences in locomotor activity of kittens between weeks 1 and 4, in contrast to a significant correlation in individual differences in frequency of separation calls over the same developmental time span.

A remaining issue is whether or to what extent individual differences in behaviour during early development translate into or are predictive of differences in later life (Sachser 1993; Stamps & Groothuis 2010; Rödel & Meyer 2011; Kanda *et al.* 2012; Herde & Eccard 2013; Petelle *et al.* 2013; Guenther *et al.* 2014; McCowan & Griffith 2014; Würz & Krüger 2015). The results of the present study, in agreement with the growing literature indicated in the Introduction, suggest that vocalizations, and particularly separation calls, might be a particularly good candidate for investigating this (see Blum *et al.* 2002 for a study in human infants). A wide variety of mammals,

including many domesticated and laboratory species emit separation calls during juvenile age or even across the whole life span. In addition, vocalizations provide readily quantifiable measures ranging from the simple frequency counts used here to detailed analyses of the physical properties of individuals' calls (Schrader & Hamerschmidt 1997; Bradbury & Vehrencamp 2011). Furthermore, because vocalizations often have a known functional (adaptive) significance, they may better reflect an individual's behavioural, physiological and psychological state than the somewhat arbitrary test paradigms sometimes used in studies of animal personality.

Conclusions

The separation calls emitted by many mammalian young (and in some species also by adults) when isolated from their mother, other caregivers or companions, seem to provide a particularly useful behavioural indicator for studying the ontogeny of personality. Kittens given brief separation tests once a week for the first four postnatal weeks (until the start of weaning) showed stable individual differences in the frequency of emitting such calls. This was also the case for mound-building mice although tested across a shorter developmental period. A second widely used indicator of individual differences in personality, locomotor activity, gave less consistent evidence of stable individual differences. We suggest that separation calls are particularly reliable indicators of personality because of the close neural connections between vocal and emotional systems of the brain, and because of their clear functional meaning (adaptive significance). It remains to investigate whether such differences persist into adulthood, at least in the cat which responds life-long to separation from companions or familiar surroundings with persistent isolation (distress) cries.

Material and methods

Study Animals and Sample Sizes

(a) Domestic cat

We collected data from 33 kittens (19 males, 14 females) of eight litters from five multiparous, crossbreed mothers kept at a private home in Mexico City (Hudson *et al.* 2009; Raihani *et al.* 2009, 2014). The mothers had mated with local free-ranging males, which from observations were usually several different individuals for each female. Once a day they were fed commercial canned cat food and fresh meat. Water, milk, commercial dried cat food and litter trays were always available. Mothers shared the house with other intact male and female cats and were free to leave the house at will. Except when kittens were being tested (see below) mothers had free access to their young. When 8 weeks old (weaning), kittens were transferred to the cat facility at the Laboratorio de Psicobiología de Desarrollo, Instituto de Investigaciones Biomédicas, UNAM, Mexico.

(b) Mound-building mouse

We collected data from 77 pups (60 males and 17 females) of 23 litters, each stemming from a different parental pair (further details under *Experimental procedure* below). Studies on mound-building mice *Mus spicilegus* were carried out in the Laboratoire d'Ethologie Expérimentale et Comparée at the Université Paris 13 in France. The animals of the breeding stock maintained in this laboratory were descendants ($16^{th,}$ 17^{th} and 18^{th} generation) of animals caught from the wild at different sites in Hungary in 1999. We ensured the genetic variation of the breeding stock by adding some new individuals every 2–4 years, captured at the same Hungarian collection sites. Animals were kept on a 14:10 h light / dark cycle (lights off and red light on at 12:00 am) in standard polycarbonate cages (26 × 14 × 16 cm, Iffa Credo, Lyon, France), containing wood shavings as bedding. Animals had *ad libitum* access to rodent standard diet (Special Diets Services, Ext. M20, Witham, Essex, UK) and water. Temperature in the housing rooms was maintained at 21 ± 0.5°C, and relative humidity at approximately 50%. Except during experiments, all pups used in this study were kept with their parents and siblings in their home cage. Several cotton balls (diameter: approx. 5 cm) were always provided, which the animals used for nest building in a corner of their cage.

Experimental Procedures

(a) Domestic cat

Mothers gave birth in foam rubber beds 70 cm × 40 cm, lined with flannel and located in a quiet part of the house. Several hours later (and daily thereafter) we weighed the kittens on digital scales to the nearest gram. We recorded their sex, fitted each with a differently coloured neck ribbon for individual identification, and returned them to their mother until the start of testing two days later (see below). We considered the day of birth as postnatal day 1. Starting on postnatal day 3, we tested each kitten once a week with an inter-test interval of about 7 days until the end of the first postnatal month (4 test sessions per kitten, 132 sessions in total; see below). During testing, we confined the mother in a familiar transport cage in a separate room to her litter, and in random order we placed each kitten individually for 3 min in the centre of a 1-m diameter arena located in a room away from the rest of the litter. We recorded kittens' behaviour in the late morning, including vocalizations, using a digital video camera equipped with a microphone (Sony HDR-CX 100) and mounted 1.5 m above the centre of the arena. Kittens' separation calls range from about 2 to 7 kHz and are clearly audible to the human ear (Scheumann *et al.* 2012; Simmons *et al.* 2013). Immediately after testing, each kitten was returned to its littermates, suspended by the scruff of its neck to mimic the method of retrieval by the mother.

Throughout the study, animals were kept and treated according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, USA, and the National Guide for the Production, Care and Use of Laboratory Animals, Mexico (Norma Oficial Mexicana NOM-062-200-1999). The experimental procedures had no apparent effect on the general behaviour of mothers and young, kittens showed normal weight gain and all survived to the end of the study.

(b) Mound-building mouse

Mothers gave birth in their home cage. As we checked the nests daily for new born pups, we could determine the day of birth with an accuracy of 24 h, and considered this as postnatal day 1.

Pups repeatedly (2 times per individual, see below) underwent a 5-min separation test, where we placed them singly into an arena and recorded their vocalization and locomotor activity. Three to four pups per litter were randomly chosen and were individually marked with a permanent non-toxic hair dye (Nyanzol-D, Greenville Colorants, New Jersey, USA) on their backs in the morning (around 4 hours before the end of the white light period) of the first day of testing. Tests were carried out around 6 hours later, during the early red light period. For this, we placed each pup in a defined corner of a test arena, consisting of a rectangular plexiglas box (14.5 × 9.5 × 8.5 cm). The frequency of separation calls emitted by pre-weaned laboratory mice (i.e. in a closely related species of the same genus) usually ranges from 40 to 90 kHz (Branchi *et al.* 1998; Hahn & Lavooy 2005). Therefore, we recorded pups' ultrasonic calls by the use of a bat detector (Batbox Baton, Batbox LTD, Steyning, UK) fixed 5 cm above the centre of the test arena. The frequency range of this detector was between 20 and 120 kHz. Recordings were automatically transformed by a division factor of 10 (i.e. 50 kHz were reduced to 5 kHz) to make them detectable for the human ear. During the experiments, recordings were saved on file for later analysis. We also recorded the pups' locomotor behaviour by a video camera (Sony HDR-XR 200), filming the arena from the side through the plexiglas as the bat detector was mounted over the arena and thus filming from above was not possible. After testing, subjects were removed from the arena, weighed to an accuracy of 0.01 g, and returned to their home cage with their parents and siblings.

As reported above, experiments were based on 77 pups born in 23 litters, each stemming from a different parental pair. A subset of pups was tested at postnatal day 13 and again at day 14 (n = 18 / 4 litters). Remaining subjects were tested at postnatal day 14 and at day 16 (n = 59 / 19 litters; 2 sessions per pup, 154 test sessions in total). We used two independent sets of animals with slightly different age classes to obtain information on the generality of our results at least across a short span of juvenile life. Our preliminary tests revealed that mound-building mouse pups show ultrasonic vocalization after separation until at least postnatal day 16. However, we did not test them

before day 13 because, in contrast to the larger and well-furred kittens, isolated mouse pups cool quickly when very young, affecting their ability to vocalize (Hahn & Lavooy 2005). Moreover, we needed to mark them individually prior to testing, which is only feasible in a minimally invasive way after they have fur at the end of the second week (Sokolov *et al.* 1998).

Animals were kept and treated according to the ethical and animal care guidelines of France (where the project was carried out) and the institutional guidelines of animal welfare. Experimental procedures were approved by the local authority for laboratory animal care and use (Comité d'Ethique en Expérimentation Animale 'Charles Darwin'; authorization codes: Ce5/2011/068; Ce5/2012/212; 00809.02). Also in the mound-building mice, the experimental procedures had no apparent effect on the general behaviour of parents and offspring. The pups showed normal weight gain and all survived to the end of the study.

Behavioural Measures

(a) Vocalisation

In both study species we measured the total number of calls emitted by each individual during each separation test; four 3-min sessions in kittens, two 5-min sessions in mouse pups.

(b) Locomotor activity

We defined locomotion as displacement of the whole body, including all four paws. In both species the occurrence of this behaviour was quantified in seconds (transformed into % observation time for statistical analysis) by analysis of video footage for each individual in each session.

Statistical Analysis

Statistical analyses were carried out using the program R, version 3.1.1 (R Core Team 2014). First, we tested for changes across age with respect to the number of emitted calls and locomotor activity. This was done by using linear mixed-effects models (LMM) to analyse locomotor activity (dependent variable), and generalized linear mixed-effects models (GLMM) for count (i.e. Poisson distributed) data for the analysis of the number of emitted calls (dependent variable). Vocalizations of kittens corresponded well to a normal distribution and thus we used an identity link. For data analysis of young mice, we used GLMM with a square-root link in order to adjust for the right-skewed distribution of the data. GLMMs and LMMs were calculated with the R package *lme4* (Bates *et al.* 2014). Models included multiple independent variables. In the case of kittens we tested for changes across the 4 ages (factor with 4 levels), and in the case of young mound-building mice we ran 2 different models as we had 2 different data sets, each with 2 ages (postnatal days 13 vs. 14 and 14 vs.

16; factors with 2 levels). In addition, we tested for potential differences among males and females (factor with 2 levels) and models also included individual body mass (covariate; measured directly after each test). The latter variable was considered as previous reports on pre-weaned small mammals highlight the effects of individual body mass on behavioural responses in different test situations (Bautista *et al.* 2010; Rödel & Meyer 2011). Statistical models on kittens included random factors coding for litter identity and maternal identity, as several subjects were litter siblings (in total 8 different litters) or originated from the same mothers (in total 5 different mothers). In mice, we only used litter identity as a random factor (in total 23 different litters), as each litter stemmed from a different parental pair. In all cases random effects were random intercepts. In the case that differences among groups (Fig. 1a) were significant, we used GLMM with the same setting of random factors for pairwise post-hoc comparisons between the different time steps. Alpha levels were corrected for multiple comparisons by a sequential Bonferroni correction (Holm 1979).

In a last section of the results, we tested for associations between individuals' frequency of vocalizations and locomotor activity (both independent variables), again using GLMM for count data. Also here we used multiple independent variables, including sex (factor with 2 levels) and body mass (covariate). Again, litter identity and maternal identity (in the case of kittens) were used as random factors. These analyses were done separately for different ages, i.e. during postnatal weeks 1, 2, 3 and 4 in kittens and on postnatal days 13, 14 and 16 in young mice. For all models we tested for interactions among the predictor variables. Non-significant interaction terms or independent variables were sequentially removed from the models before these were re-calculated. We calculated variance inflation coefficients (VIF) for all models with multiple fixed factors / covariates in order to check for (multi)colinearities among them (Zuur *et al.* 2010). VIF were always lower than 1.5, indicating no interfering effects of multicolinearities. We verified homogeneity of variances by plotting residuals versus fitted values for all models (Faraway 2006). Whenever GLMM for Poisson distribution showed signs of overdispersion we included case-level random effects (Browne *et al.* 2005). When using LMM we made sure that the model residuals were normally distributed by visually checking normal probability plots. *P*-values were extracted by Wald chi-square tests (type III).

Furthermore, we tested for repeatability of vocalization and of locomotor activity during the test sessions across age classes. We (a) analysed the total frequency of emitted calls and time that the animals showed locomotor behaviour during the experiments. In addition, we (b) calculated relative differences among littermates with respect to these 2 behaviours as the individual percentage variation from the litter mean. This was done in order to test for within-litter repeatabilities in behaviour. To this end we applied intra-class correlations calculated as the proportion of phenotypic variation that can be attributed to between-subject variation (Lessells &

Boag 1987). For kittens, this was done using repeated measurements of *n* = 33 individuals across 4 tests conducted during the first 4 postnatal weeks (once per week). In young mice this was done separately in two different samples: *n* = 18 individuals were tested on postnatal days 13 and 14, and another *n* = 55 individuals were tested on days 14 and 16. We used (G)LMM-based calculations of repeatability with the aid of the R package *rptR* (Nakagawa & Schielzeth 2010). For testing the repeatability of the frequency of vocalization we applied an intra-class correlation based on GLMM for count (Poisson distributed) data. For data on frequencies of vocalization of kittens we used GLMM with an identity link, according to the distribution of the model residuals. Repeatabilities of locomotor activities (% time, for kittens and young mice) were calculated using an intra-class correlation based on a LMM with restricted maximum likelihoods. In mound-building mice we used an intra-class correlation based on a GLMM with a square-root link in order to adjust for the right-skewed distribution of the vocalization data base obtained. Within-litter deviation in vocalisation as well as in locomotor activity was also analysed using a LMM with restricted maximum likelihoods. For all intra-class correlations we assessed 95% confidence intervals by 1000 bootstrap steps. Individual identity was used as a random factor. *P*-values were calculated by 1000 permutations.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

RH and HGR initiated the study. RH, HGR and MR designed the experiments, and MR and AS performed them. HGR, RH and MR analysed the data. RH, HGR, MR and OB contributed in writing the manuscript. All authors read and approved the final manuscript.

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Manuscript III – in preparation for submission

Individual differences in separation calls and social interactions with siblings as part of a behavioural syndrome in a rodent of wild origin

Abstract Consistent individual differences in behaviour in social contexts are well known to be an important part of an animals' personality type. However, studies focussing on social personality traits in young animals, including the long-term associations of such individual differences across contexts are still scarce. We explored such associations in a rodent of wild origin, the mound building mouse (*Mus spicilegus*). To this end, we quantified individual differences in distress calling of pre-weaned individuals during brief social separation, and assessed the sociability of individuals by recording their interactions with siblings around 3 weeks after weaning. We tested whether such social traits were associated with traits expressed in repeated standard tests in non-social contexts (elevated plus maze, open field, novel object test) during the pre-weaning and post weaning period, and around maturity. Animals showed consistent individual differences in the behavioural traits measured in social as well as in non-social contexts, thus fulfilling the condition of animal personality. Interestingly, these consistencies across time were apparent with respect to the absolute response values, and – in particular during early life stages – also with respect to relative differences among litter siblings. Individual differences quantified in non-social contexts clearly supported the existence of a behavioural syndrome, as indicated by the positive associations between behaviours reflecting anxiety and neophobia, and the negative associations of these with the animals' exploratory activity. Most importantly, individuals phenotyped as being less anxious were more sociable in interactions with their siblings as they consistently showed more initiations of more positive social contacts – although this association was only apparent when considering within-litter differences in behaviour. Moreover, animals who repeatedly emitted more distress calls after social separation were less sociable in sibling interactions. These results suggest that social traits, even when measured during early life stages, can be considered an important part of a behavioural syndrome in the mound building mouse and thus of the individual personality type. In addition, our findings highlight the importance considering within-litter differences in the study of personality in young animals.

Keywords: mound-building mouse, *Mus spicilegus*, temperamental traits, social interactions, sibling group

Introduction

The study of consistent individual differences in behaviour (Sih *et al.* 2004, Réale et al. 2007), frequently referred to as animal personality, currently receives much attention in different disciplines such as in behavioural biology, evolutionary biology and biomedical sciences (Cavigelli 2005; Briffa & Weiss 2010; Carere & Maestripieri 2013; Dall & Griffith 2014). The consistency of individual behaviour across time is an essential condition for the existence of animal personality (Stamps & Groothuis 2010), and such patterns have been shown in a large number of species across a broad range of taxa (Gosling 2001; Freeman & Gosling 2010; Kralj-Fišer & Schuett 2014). In addition, animal personality typically includes individual consistencies in behaviour across different contexts, further defining the behavioural phenotype of an individual. Such correlated suites of behaviour across multiple situations are usually referred to as behavioural syndrome (Sih *et al.* 2004a).

Despite the large number of papers on animal personality published during the last decade, the attention to the study of the ontogeny of this phenomenon has only increased recently (Stamps & Groothuis 2010; Hudson *et al.* 2011a; Trillmich & Groothuis 2011; Trillmich & Hudson 2011). Although individual differences in behaviour in young animals are frequently found to be less stable and individuals still appear more plastic than during later life stages (Trillmich *et al.* 2015; Wuerz & Krüger 2015), various studies confirm that such consistent individual difference already exist during early life stages (e.g. mammals: Gracceva *et al.* 2011; Rödel & Meyer 2011; Herde & Eccard 2013; Petelle *et al.* 2013; Guenther *et al.* 2014; birds: Carere *et al.* 2005; Favati *et al.* 2015; Wuerz & Krüger 2015). Changes in behaviour are often thought to be associated with changes in their physiology or changes in the social niche of the animals (Stamps & Groothuis 2010;Trillmich & Groothuis 2011).

However, so far, few studies on the ontogeny of personality actually integrate tests focussing on behavioural traits measured in a social context – although such social traits, as for example sociability and aggression are clearly an important component of animal personality (Cote *et al.* 2008; Réale *et al.* 2010b; Webster & Ward 2011; Montiglio *et al.* 2013). Tests focussing on responses in social contexts – such as contexts referring to mother-offspring interactions or interactions with siblings – might be particularly meaningful to assess individual differences in young animals. For example, young domestic rabbits *Oryctolagus cuniculus* during the early post-natal period have been shown to display consistent differences in their efforts to reach thermally advantageous, central

positions within the litter huddle, with strong implications for their growth, development and survival (Bautista *et al.* 2015). Furthermore, consistent individual differences in the frequency of offensive interactions of juvenile European rabbits towards siblings and other juveniles of their social group were reported in a study under semi-natural conditions (Eccard & Rödel 2011). Recent studies in pre-weaned domestic kittens *Felis silvestris catus* and in mound-building mouse pups *Mus spicilegus* have shown individual consistencies in distress calling and locomotor activity in responses to brief social separations from mother and littermates. The display of such vocalisation responses constitutes a highly adapted behaviour in various species of altricial mammals, as it usually leads to the arrival of the caregiver carrying back the young to the nest, shelter or burrow (Haskins 1977; Briefer 2012).

Generally, the development and use of such biologically meaningful tests for young animals in social contexts might help to overcome the general problem: tests, that are often developed for adults might not always perform well when being applied during early life stages (Rödel & Meyer 2011). However, still little is known about the long-term associations of consistent individual differences in behaviour displayed in social contexts during early life and personality traits measured during later life stages. One of the few examples suggesting such long-term consistencies comes from a study on zebra finches *Taeniopygia guttata*, in which the activity of nestlings during begging was positively associated with individual activity levels during adulthood (McCowan & Griffith 2014). Furthermore, a study in domestic rabbits indicates long-term consistencies across contexts with respect to the relative position that the young occupied within their litter huddle (Reyes-Meza *et al.* 2011), a feature which has been shown to be repeatable across the first postnatal days (Bautista *et al.* 2015). That study reported that individuals occupying more peripheral positions in the litter huddle, compared to their siblings in more central ones, were more responsive in a jump-down test around weaning and showed less anxiety-related behaviour in response to conspecific distress calls as subadults.

A further difficulty in the study of individual differences during early life stages can arise when animals stemming from different litters show strong variation in developmental trajectories, such as in growth or physiological parameters (Hudson *et al.* 2011b; Rödel & Meyer 2011; Rödel & Monclús 2011), potentially affecting their performance in different test situations. Such effects, which typically affect litter siblings in a similar way (e.g. litter size effects or maternal effects; Rödel *et al.* 2008), might have the potential to attenuate or even mask general rank consistencies in behavioural responses across time and context. For instance, maternal or parental effects, the causal influence of the parental phenotype on the offspring phenotype (Mousseau & Fox 1998) have the potential to affect the offspring phenotype, as they can modify the early environment experienced

by the offspring both behaviourally and physiologically. As not all siblings experienced the same early-life environment, considering within-litter variation among siblings can be an appropriate method to unravel the existence of stable individual differences, even under conditions where the variation among litters is high and non-constant over time. For example, this method has been successfully applied in studies on individual differences in behaviour of domestic kittens and young mound building mice (Hudson *et al.* 2015) and of domestic rabbit pups (Bautista *et al.* 2015).

The general goal of this paper, carried out in a rodent of wild origin, the mound-building mouse, was to explore if social personality traits measured during early life were associated with the animals' responses in other contexts during different life stages. To this end, we tested for general associations across time and context, but also for associations considering within-litter variation of individual differences among siblings. We (*i*) studied individual consistencies in behavioural responses displayed in two different social contexts: (*a*) the emission of distress calls after brief separation from parents and siblings during early postnatal life, and (*b*) social interactions with their siblings in their home cage after weaning. Furthermore, we (*ii*) quantified whether the animals showed consistencies in their behavioural responses within and between different standard tests (open field, elevated plus maze novel object test), which we applied during different life stages. Most importantly, we (*iii*) explored whether and how social traits measured early in life were part of a behavioural syndrome in the mound building mouse by testing for associations of these with individual differences in exploratory activity, neophobia and anxiety-related behaviour as quantified by batteries of standard tests.

Material and methods

Animals and Housing Conditions

Mound building mice *Mus spicilegus* of our breeding stock were descendants of 80 animals caught from the wild at different sites in Hungary in 1999, kept in the animal facilities of our unit for 16 generations. To maintain genetic variation, additional individuals were captured at the same Hungarian collection sites every 2-4 years. This has been done recently in 2011, 1 year prior to the onset of the study in 2012, when 3 wild-caught females and 1 male were added to our breeding stock.

For production of experimental animals, we used adult virgin male-female breeding pairs. We kept the animals on a 14:10 h light / dark cycle (lights off at 12:00 h) in standard polycarbonate cages (26 × 14 × 16 cm, Iffa Credo, Lyon, France), containing wood shavings as bedding, and with free

access to rodents standard diet (Special Diet Services type M20, Witham, Essex, U.K) and water. Temperature was maintained at 21 ± 0.5°C, and relative humidity at approximately 50%. Cages were checked daily for the presence of litters, and we considered this postnatal day 1. All animals, including the focal animals later on, were transferred to clean cages once a week, and we always provided a large cotton ball (diameter: approx. 5 cm), which the animals (including juveniles) used for nest building in a corner of their cage.

Outline of the Study and Sample Sizes

In total, 181 focal animals from 50 litters were used for testing. At postnatal day 14, focal animals were weighted to the nearest 0.01 g and marked with different symbols on their back using a black, permanent non-toxic hair dye (Nyanzol-D, Greenville Colorants, New Jersey, USA) and were re-marked around 3 weeks later to recognize them individually within their sibling groups. We also determined their sex by external genital inspection at this time. At 28 days, focal animals were weaned and kept in cages of 3-4 litter siblings (on average: 3.5 individuals per sibling group) of different sexes ($N_{females}$ = 52, N_{males} = 131) until postnatal day 55. From this day on, before mound building mice reach maturity (at around an age of 70 days, Busquet *et al.* 2009), all individuals were transferred to new cages and were housed singly.

The general aim of the study was to test for consistencies in individual behavioural responses across time and context, integrating tests by which the animals' responses in a social context could be quantified. To this end, we run repeated tests at different age classes. During the pre-weaning period, social separation tests were conducted on postnatal days 14 and 16, and a first battery of standard tests (open field, novel object, and elevated plus maze test) was carried out from postnatal days 17 to 20. A second battery of these standard tests was carried out during the post-weaning period from postnatal days 40 to 44 and a further, third one around the time when the animals reached maturity, from postnatal days 70 to 74. In-between these last two batteries of standard tests, the social interactions among siblings within the different groups were recorded on postnatal day 50. All experiments and observations were carried out during the animals' activity period, that is, during the dark (red light) phase between 01:00 pm and 05:00 pm. Individuals were always weighted to the nearest 0.1 g and were returned in their home cages immediately after each test – except for the recording of sibling interactions, which were carried out in the animals' home cages. We cleaned the different test apparatuses with water and soap (Cleansinald, Johnson Diversey, Fontenay-sous-Bois, France) between the different trials.

Behavioural Tests

Social separation test: Pups individually underwent a 4-minute separation from their parents and littermates on postnatal day 14 and again on postnatal day 16. For this, individuals were transferred singly into a defined corner of a small test arena consisting of a rectangular plexiglas box (14.5 × 9.5 × 8.5 cm). Their ultrasonic vocalizations were recorded by the use of a bat detector (Batbox Baton, Batbox LTD, Steyning, UK) fixed 5 cm above the centre of the test arena. Recordings were saved on file for later analysis, consisting in the measure of the number of each pup's ultrasonic calls for each 4-minute test. See Hudson *et al.* (2015) for more details on this test procedure. A total of 134 individuals stemming from 38 litters underwent this test. Out of this sample, 56 animals (from 14 litters) were only tested one time (on postnatal day 14), and therefore repeatabilities across time could only be calculated using a subset of 78 individuals stemming from 24 litters. Thus, for later testing of consistencies across context, we did not use the averaged values over days 14 and 16 but always used subjects' responses during the first test to use the maximum of available data.

Open field test: This test was carried out three times, on postnatal day 17 (pre-weaning), day 40 (post-weaning) and on day 70, when reaching maturity. The open-field was made of white polyethylene consisting of a circle arena surrounded by high walls. The one used for animals at pre-weaning age had a diameter of 48 cm surrounding by walls of 50 cm, and the one used later on had a diameter of 120 cm surrounded by walls of 69 cm. Each individual was placed singly on a defined position at the edge of the arena, and its behaviour was recorded for 5 min by use of a digital video camera mounted 120 cm above the centre of the arena. During later analysis, the distance covered by the animals were automatically quantified by the aid of Ethovision XT7 (Noldus Information Technology, Wageningen, The Netherlands). A total of 181 individuals underwent these tests; although due to failures of video recordings, the sample size which could be used for analysis was sometimes lower (Tables 1, 2).

Novel object test: This test was carried out in the same apparatus than the open field test; that is, even during the first time of testing, the arena was not novel to the animals. As for the novel object test, each individual placed in a define position in the edge of the arena. After 10 minutes of habituation, a novel object was introduced in the centre of the arena. Tests were done on postnatal days 17, 42 and 72 using different novel objects: brown plastic teddy bear (length: 3.1 cm, height: 3.6-3.8 cm) on day 17; a coloured, artificial hamburger made of soft, plasticized PVC (diameter: 8.5 cm, height: 4.5-5.0 cm) on day 42; and a 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) on day 72. Immediately after the introduction of the novel object, behaviours were recorded with a digital video camera mounted 120 cm above the centre of the arena for 5 minutes. We quantified the latency

until the animals sniffed the novel object for the first time, and the summed-up time the animals spent exploring the object by means of sniffing the object, touching it with the front paws and climbing on it. A total of 181 individuals underwent these tests; although due to failures of video recordings, the sample size which could be used for analysis was sometimes lower (Tables 1, 2).

Elevated plus maze test: Also this test was carried out three times, on postnatal day 20, day 44, and on day 74. The apparatus was made of white, rigid PVC consisting of four arms, 5 cm wide and 30 cm long, arranged at 90° angles, and mounted 70 cm above the floor by a stable wooden and metal construction. Two opposite arms enclosed by 30 cm high walls and two open arms without walls were connected by a 10 × 10 cm central platform. Each animal was placed on the central platform facing an open arm and its behaviour was recorded for 5 min by use of a video camera mounted 120 cm above the centre of the maze. We scored the percentage of time an animal spent in the open arms, defined as crossing the respective thresholds with more than 50% of its body length. A total of 181 individuals underwent these tests; although due to failures of video recordings, and due to animals jumping from the apparatus during testing (which were discarded from the analysis), the sample size which could be used for analysis was always lower (Tables 1, 2).

Social interactions in sibling groups: Only groups of 4 litter siblings underwent this test in order to control for the number of potential interactions partners (n = 100 individuals stemming from 20 litters). Behaviours were recorded on postnatal day 50, when focal individuals were kept in groups of four. A few hours prior to the start of the recording, we removed a part of their nest (made of cotton balls) and the grid cover of the cage was replaced by a flat, transparent plastic glass cover with vent holes at the periphery. Water and food were then put directly inside the cage – as feeding racks and water bottles usually fixed at the grid cover were removed. These modifications allowed us to identify individuals by their colour marks on their backs and to record their social interactions by a video camera mounted 30 cm above the centre of the cage.

Three periods of 10 min length were recorded for each sibling group. Recordings were started at around 02:00 pm for 10 minutes and were then automatically re-started for two times at the next two full hours. For each of the 3 periods; we quantified the number of approaches, and of nasal contacts (naso-nasal and naso-anogenital). Approaches were defined as events where the initiator was moving towards and into a 5-cm distance range around a sibling without touching it, whereat the initiator did or did not change its initial direction. Nasal contacts were defined as the initiation of brief contacts of the nasal or the anal region of the receiver. Such interaction were only counted when they were receded by an initial approach of the initiator, but not when the two interaction partners were previously engaged in huddling together. Differences in the frequency of approaches and nasal contacts have been frequently used as measures of sociability, for example in

young laboratory mice *Mus musculus domesticus* (Brodkin *et al.* 2004; Fairless *et al.* 2008). Agonistic interactions by means of events of chasing and displacement did not occur during the times of recording.

Experiments and observations were recorded with video cameras in night vision mode (Sony HDR-XR 200) for later analysis. Behavioural recordings were done by the aid of EthoLog, version 2.2.5 (Ottoni 2000).

Ethics Note

Animals were kept and treated according to the ethical and animal care guidelines of France (where the project was carried out), and experimental procedures were approved by the local authority for laboratory animal care and use (Comité d'Ethique en Expérimentation Animale Charles Darwin; Ce5/2011/068; Ce5/2012/212; 00809.02).

Subjects were kept singly from postnatal day 55 until the end of the experiments. Being solitary is not an unusual situation in this species, as solitary females and males have been frequently found under natural conditions (Simeonovska-Nikolova 2012). Furthermore, as it has been shown in one of our previous studies on this species (Rangassamy *et al.* 2015), the growth rates of the animals during the last 2 weeks before being separated from their siblings (mean ± SE = 0.69 ± 0.19 g) did not differ significantly from those during the first 2 weeks of single housing (0.82 ± 0.15 g; paired *t* test: $t = 0.72$, $P = 0.47$). This indicates that the animals did not suffer from obvious effects of separation stress.

After the experiments, animals were killed off in accordance to French animal law. This procedure was carried out in two steps: subjects were first anesthetized by putting them into a closed transparent plastic box filled with isoflurane gas of a concentration of 3% (IsoFlo, Axience, France), administered by an automatic system (Univentor 400 Anaesthesia Unit, Uno Roestvststaal BV, The Netherlands). Then, the anesthetised individuals were killed with a high dose of CO_2 gas (delivered by a compressed gas cylinder) for at least 5 minutes. Subjects were observed until all muscle activity and other signs of life had been absent for at least 30 s. After removal, we carried out cervical dislocation in order to ensure death. This whole procedure was conducted by a qualified and experienced person.

Statistical Analyses

Statistical analyses were done with R, version 3.1.1 (R Core Team 2014). Analysis were based on the individual behaviours recorded in the different tests. In addition, we re-ran all analyses while taking into account individual differences within litter with respect to siblings. That is, we calculated the

percentage deviation for each pup from the litter mean for all variables included in the analysis. This method has been frequently used to capture within-litter variation of behaviour in young mammals (e.g. Bautista *et al.* 2015; Hudson *et al.* 2015).

We checked for repeatabilities across time in the animals' behavioural responses in the different tests by intra-class correlation, calculated as the proportion of phenotypic variation that can be attributed to between-subject variation (Lessells & Boag 1987). This was done by repeatability using (generalized) linear mixed-effects models implemented in the R package *rptR* (Nakagawa & Schielzeth 2010), using individual identity as a random factor. See Table 1 and 2 for the different link-functions used. We assessed 95 % confidence intervals by 1000 bootstrap steps. *P*-values were calculated by 1000 permutations.

Furthermore, we applied (generalized) linear mixed-effects models based on restricted maximum likelihood estimates using the package *lme4* (Bates *et al.* 2014) to test for the associations of different behavioural variables within tests and across contexts. Therefore, the behavioural responses of the animals (the absolute values as well as their deviation from litter means during the different trials) were averaged. Litter identity was included as a random factor (random intercept) to adjust for the same origin of litter siblings tested. Furthermore, models included sex as a fixed factor, and we tested for the interactions with sex in order to verify whether the associations between the different behavioural responses considered differed between males and females. However, this was never the case, and thus the interaction and also the main effect of sex was omitted from the models and these were re-calculated. *P*-values were extracted by Wald Chi-square tests (type II). We assure that the model residuals were well adjusted to a normal distribution by visually checking normal probability plots. Furthermore, we verified homogeneity of variances by plotting residuals versus fitted values (Faraway 2006). For all significant linear mixed-effects models, we calculated Nagelkerke's $pseudoR^2$, which can be interpreted as a proxy of the proportion of explained variation (Nagelkerke 1991).

Results

Consistent Behavioural Responses across Time

General Consistencies: Individual differences in the animals' frequency of distress calls emitted after social separation on postnatal days 14 and 16 were significantly repeatable across time (R = 0.368, $CI_{95\%}$ = [0.150, 0.547], N = 78, P = 0.003; Fig. 1a). Furthermore, all behaviours quantified during the three consecutive observations of sibling groups (T_1, T_2, T_3), such as the initiation of approaches

(R = 0.321, $CI_{95\%}$ = [0.267, 0.531], N = 100, P = 0.002) and of naso-nasal (R = 0.346, $CI_{95\%}$ = [0.290, 0.546], N = 100, P = 0.002) and naso-anal contacts (R = 0.376, $CI_{95\%}$ = [0.336, 0.596], N = 100, P = 0.002) were significantly repeatable across the 3-hour observations (associations between T_1 and T_3 are given in Fig. 1f-h).

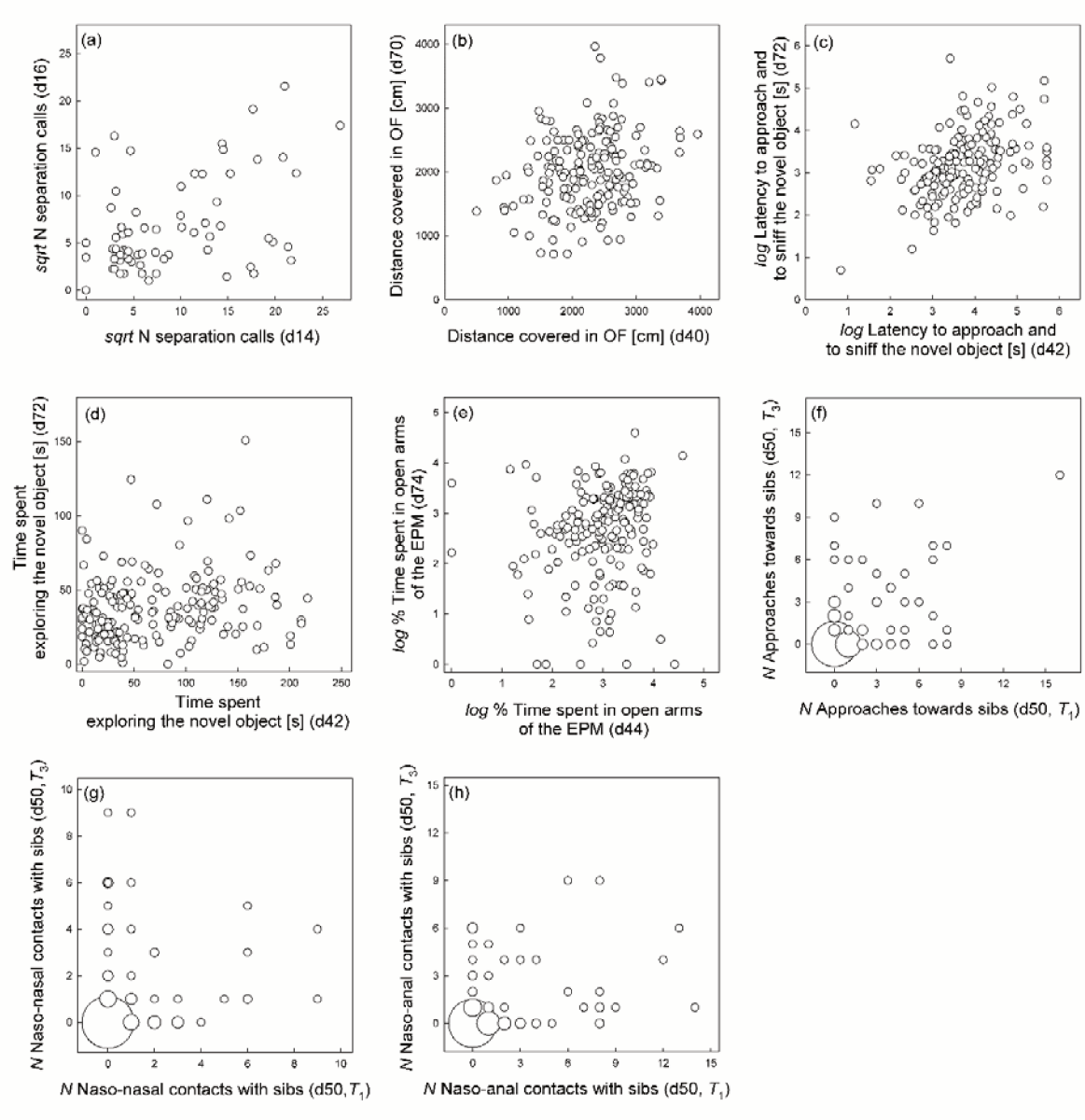

Fig. 1. Consistencies across time in individual behavioural responses of mound building mice during different tests. (a) The frequency of calls emitted after brief separation from parents and litter mates prior to weaning, (b) the distance covered in the open field (OF), (c) the latency to sniff the object and (d) to explore it in a novel object test (NO), (e) the time spent exploring the object in the percentage time spent in the open arms of the elevated plus maze (EPM), and (f-h) the frequency of approaches and nasal contacts with litter siblings (groups of 4), initiated by the focal animal in consecutive recordings with 1 hour time lag. The days of testing are always given in the figure legends. The number of overlapping data points in (f-h) is reflected by different-sized bubbles. All associations shown are significant (tested by intra-class correlations), see Table 1 for statistics and sample sizes.

The animals' behavioural responses quantified in the open field, the elevated plus maze and the novel object test were also significantly repeatable between the post-weaning period and around

the time when the animals reached maturity (statistics in Table 1; Fig. 1b-e). However, when testing for behavioural consistencies during early life stages, that is, between the pre-weaning and the post-weaning period, no significant repeatabilities were apparent for the distance covered in the open field and the percentage time spent in the open arms of the elevated plus maze (Table 1).

Consistency within Litters: Individual differences with respect to litter siblings, by means of the % deviation from the litter mean, were significantly repeatable with respect to the frequency of separation calls in pre-weaned pups (R = 0.367, $CI_{95\%}$ = [0.139, 0.566], N = 78, P < 0.001). The same applied to within-litter individual differences in the initiation of approaches (R = 0.308, $CI_{95\%}$ = [0.239, 0.504], N = 100, P = 0.001), naso-nasal (R = 0.389, $CI_{95\%}$ = [0.236, 0.519], N = 100, P = 0.001) and naso-anal contacts with litter siblings (R = 0.328, $CI_{95\%}$ = [0.161, 0.475], N = 100, P < 0.001).

Table 1. Intra-class correlations across time of different behavioural responses of mound building mice in repeated open field tests, elevated plus maze tests and novel object tests. Repeatabilities (R) between the pre-weaning period (tests during postnatal days 17-20), and post-weaning period (tests during postnatal days 40-44) and between the post-weaning period and the time around maturity (tests during postnatal days 70-74) are presented. N_I represents the number of individuals, and N_L the number of litters included in the analysis. Significant effects are given in bold.

	Test	Behaviour	$T_{pre\text{-}weaning}$ vs. $T_{post\text{-}weaning}$			$T_{post\text{-}weaning}$ vs. $T_{maturity}$		
			N_I/N_L	R [$CI_{95\%}$]	P	N_I/N_L	R [$CI_{95\%}$]	P
(a)	OF	Distance covered	171/51	0.043 [0 /0.138]	0.951	181/50	0.207 [0.061/0.336]	**0.003**
(b)	EPM	% Time spent in open arms	162/49	0.124 [0 /0.265]	0.065	175/49	0.217 [0.061/0.348]	**0.003**
(c)	NO	Latency to object	174/50	0.201 [0.062/0.342]	**0.007**	181/50	0.220 [0.080/0.354]	**0.010**
		Time spent exploring object	174/50	0.238 [0.092/0.375]	**0.002**	181/50	0.224 [0.079/0.347]	**0.011**

All behaviours considered in the open field, the elevated plus maze and the novel object test were significantly repeatable between the pre-weaning and post-weaning period as well as between the post-weaning period and the time around which the animals reached maturity (Table 2). All behaviours were also repeatable across tests during the post-weaning and early adult period, or when considering all three test responses within a single model (all P < 0.05).

We obtained similar (statistically significant) results when both sexes were analysed separately with respect to general consistencies as well as with respect to individual variation relative to litter siblings. Thus, the results presented in Tables 1 and 2 derive from pooled data of females and males.

Table 2. Intra-class correlations across time of the within-litter individual differences to siblings (i.e., % differences to the litter mean) of different behavioural responses of mound building mice in repeated open field tests, elevated plus maze tests and novel object tests. Repeatabilities (*R*) between the pre-weaning period (tests during postnatal days 17-20), and post-weaning period (tests during postnatal days 40-44) and between the post-weaning period and the time around maturity (tests during postnatal days 70-74) are presented. N_I represents the number of individuals, and N_L the number of litters included in the analysis. Significant effects are given in bold.

	Test	Behaviour	$T_{pre\text{-}weaning}$ vs. $T_{post\text{-}weaning}$			$T_{post\text{-}weaning}$ vs. $T_{maturity}$		
			N_I/N_L	R [$CI_{95\%}$]	P	N_I/N_L	R [$CI_{95\%}$]	P
(a)	OF	Distance covered	171/51	0.180 [0.020/0.320]	**0.011**	181/50	0.229 [0.088/0.355]	**0.001**
(b)	EPM	% Time spent in open arms	162/49	0.147 [0.007/0.291]	**0.019**	175/49	0.238 [0.085/0.368]	**0.001**
(c)	NO	Latency to object	174/50	0.255 [0.108/0.390]	**0.002**	181/50	0.285 [0.156/0.414]	**0.001**
		Time spent exploring object	174/50	0.251 [0.108/0.386]	**0.001**	181/50	0.172 [0.031/0.304]	**0.006**

Associations between different Social Interactions of Siblings

Individuals showing higher frequencies of approaches towards their siblings also displayed higher frequencies of naso-nasal (χ_1^2 = 47.19, *pseudo*R^2 = 0.338, N = 100, P < 0.001) and naso-anal contacts (χ_1^2 = 36.12, *pseudo*R^2 = 0.285, P < 0.001) with them. As a consequence, individual frequencies of naso-nasal and naso-anal contacts were also positively associated (χ_2^2 = 145.00, *pseudo*R^2 = 0.587, P < 0.001). Such positive correlations between these 3 behavioural variables were also apparent when considering individual differences among siblings within litter (all P < 0.001).

Associations across Contexts

Associations among Responses in Standard Tests

The percentage time the animals spent in the open arms of the elevated plus maze was positively correlated with the distance covered in the open field, indicating that animals, which showed lower levels of anxiety-related behaviour in the elevated plus maze showed more exploratory activity in the open field (χ_1^2 = 9.75, *pseudo*R^2 = 0.066, N = 175, P = 0.002; Fig. 2a). Both, the animals' responses in the open field (χ_1^2 = 9.08, *pseudo*R^2 = 0.054, N = 181, P = 0.003; Fig. 2b) and the elevated plus maze (χ_1^2 = 9.92, *pseudo*R^2 = 0.061, N = 175, P = 0.002; Fig. 2c) were negatively and significantly associated with the latency to approach and sniff the novel object: Animals, which spent more time in the open arms of the elevated plus maze and were more active in the open field, approached and sniffed the novel object quicker. However, the time the animals explored the novel object was not significantly associated with the animals' responses quantified in the open field and in the elevated plus maze (both P > 0.10). Finally, there was a significant association between the two different

behaviours recorded during the novel object test: Animals, which approached the object quicker spent a longer time exploring it (χ_1^2 = 37.68, *pseudoR*^{2} = 0.172, *N* = 181, *P* < 0.001).

We found the same pattern of significant associations between the behaviours quantified in the different tests when considering individual differences among siblings; that is, the percentage deviation of each individual from its litter mean. Within-litter differences in the distance covered in the open field and in the time spent in the open arms of the elevated plus maze were significantly and positively associated, and both were negatively and significantly associated to the latency to sniff the novel object (all *P* < 0.010).

Associations between Separation Calling and Responses in other Tests

The frequency of calls emitted during a brief separation from parents and litter mates on postnatal day 14 was significantly and negatively correlated with the frequency of approaches (χ_1^2 = 5.05, *pseudoR*^{2} = 0.057, *N* = 94, *P* = 0.025; Fig. 2d) and with the frequency of naso-nasal contacts (χ_1^2 = 7.64, *pseudoR*^{2} = 0.081, *N* = 94, *P* = 0.006; Fig. 2e) that the animals initiated towards their siblings in their home cages. There was no significant association with the frequency of initiated naso-anal contacts (*P* > 0.10).

The animals' vocal responses during the separation test did not show any significant association with their responses in the open field, novel object test or elevated plus maze test (all *P* > 0.10).

Associations between Social Interactions with Siblings and Responses in other Tests

Associations between the animals' responses in the different standard tests and their social interaction with siblings were only apparent when considering individual variation within litters by means of the percentage deviation from the litter mean. This within-litter variation in the frequency of initiated naso-nasal contacts was significantly and positively associated with within-litter variation in the distance covered in the open field (χ_1^2 = 4.35, *pseudoR*^{2} = 0.046, *N* = 100, *P* = 0.037; Fig. 2f) and in the time spent in the open arms of the elevated plus maze (χ_1^2 = 4.61, *pseudoR*^{2} = 0.051, *N* = 89, *P* = 0.031; Fig. 2g).

When testing for associations across context, we always considered interactions with sex in order to verify whether there were differential associations in males and in females. However, this was never the case (all *P* > 0.10), and thus this non-significant interaction was always removed from the models before these were recalculated.

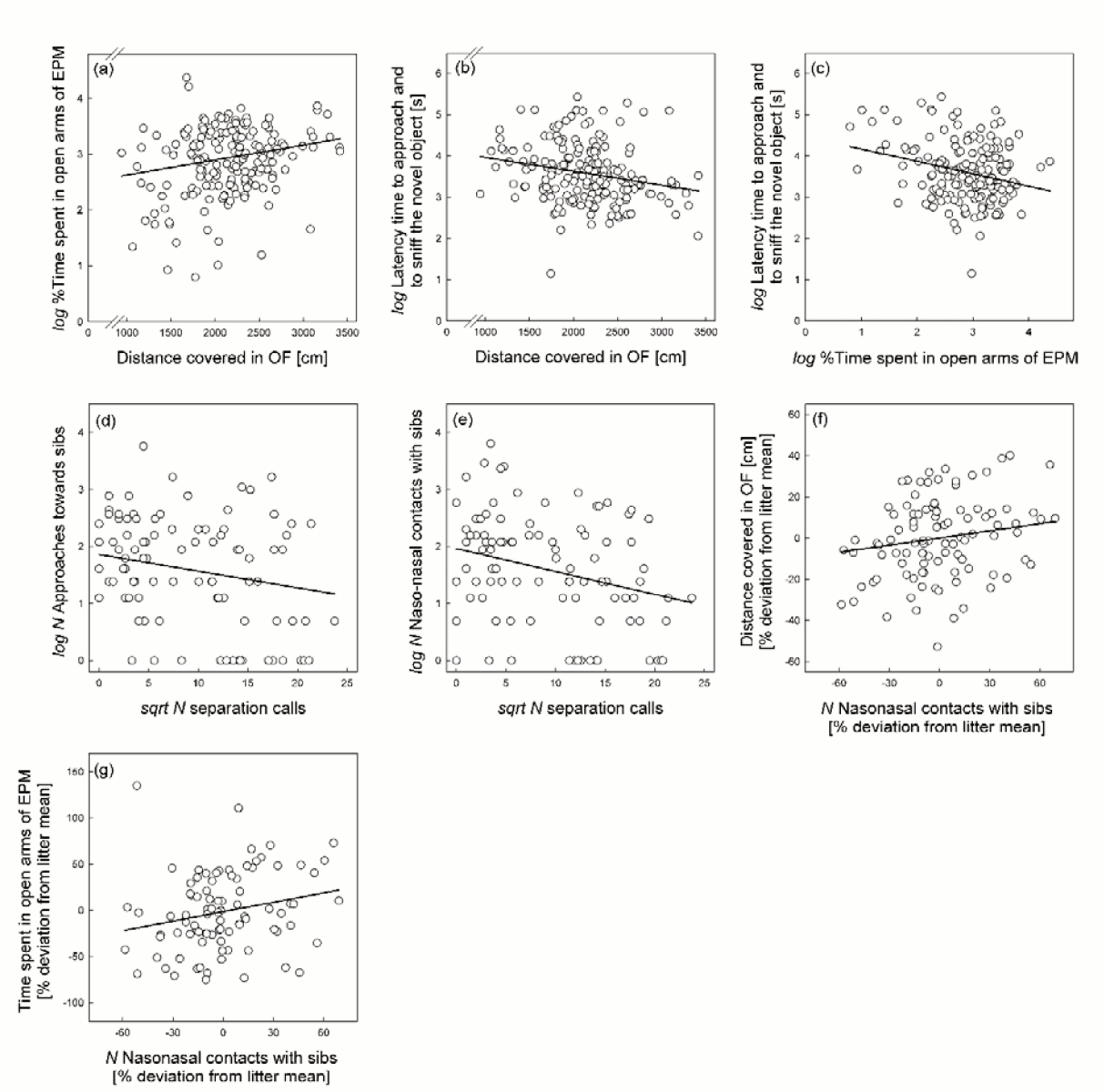

Fig. 2. Associations between individual behavioural responses in different tests. Individual values from each test represent averages over repeated tests, as shown in Fig. 1. Distance covered in the open field (OF; averages across tests on postnatal days 40 and 70) and (a) the percentage time in the open arm of the elevated plus maze (EPM; averages across tests on postnatal days 42 and 72), or (b) the latency to approach the object in a novel object test (NO; averages across tests on postnatal days 44 and 74). (c) Percentage time spent in the open arm of the EPM and the latency to approach the object in the NO test. (d) Number of calls emitted after brief separation from parents and litter mates (test responses on postnatal day 14) and approaches towards siblings during recordings of social interactions (averages across three 10-minute test periods on postnatal day 50) or (e) the frequency of naso-nasal contacts initiated by the focal animal during that test. Association between within-litter differences in the initiation of naso-nasal contact with litter siblings and (f) within-litter differences in the distance covered in the OF, or (g) in the percentage time spent in the open arms of the EPM. All associations shown are significant; regression lines are based on parameter estimates obtained by (generalized) linear mixed effects models; see text for statistics and sample sizes.

Discussion

The results of our study indicate the existence of personality types in young mound building mice by means of individually consistent differences in behaviour across time and across social and non-social contexts (Sih *et al.* 2004b; Stamps & Groothuis 2010). In particular, such consistencies across time were already apparent during early postnatal life, as revealed by our analysis of separation distress calls. There were also consistent individual differences with respect to positive social interactions with siblings at least measured across a short time span, reflecting sociability. Furthermore, repeated test batteries during different life stages showed individually consistent behavioural responses in the elevated plus maze reflecting anxiety-related behaviour, in the novel object test reflecting neophobia (but see Greggor *et al.* 2015 for a detailed analyses of that trait), and in the open field reflecting exploratory activity. In addition, the results suggest the existence of a behavioural syndrome in the mound building mouse, based on various associations across context, integrating social traits quantified during early life stages (Fig. 3 for an overview). Interestingly, some associations were only apparent when looking at within-litter differences, i.e. at individual differences with respect to litter siblings.

The study of the ontogeny of personality in young animals is inherently difficult to conduct, as animals are expected to undergo various major ecological and physiological changes before reaching adulthood; which might affect consistencies of their behavioural responses (Stamps 2007; Biro & Stamps 2008; Careau *et al.* 2008; Stamps & Groothuis 2010). Studies on animal personality in young animals frequently report rather low repeatabilities of individual behaviour or even the absence of these when compared to studies carried out during later life stages (Caspi *et al.* 2005; Fratkin *et al.* 2013; Brommer & Class 2015). Other studies showed differential consistencies between contexts across different life stages (Sinn *et al.* 2008; Wuerz & Krüger 2015) or changes in the absolute value of personality traits between major life transitions (Gyrius *et al.* 2012). Such patterns are frequently explained by a higher plasticity in behavioural responses during early life (Stamps & Groothuis 2010; Trillmich *et al.* 2015). Nevertheless, in our study we found clear individual consistencies across time in all behavioural parameters tested regarding the animals' responses in non-social as well as in social contexts. That is, animals showed repeatable individual differences in neophobia and anxiety-related behaviours between the post-weaning period and the around maturity, and also between the pre- and post-weaning period – but in parts only when considering relative individual differences among litter siblings (Tables 1, 2). Furthermore, the frequency of distress calls after separation from parents and litter mates prior to weaning as well as different positive social behaviours with siblings were repeatable across time.

In addition, our findings clearly indicate the existence of a behavioural syndrome in the mound building mouse, as found in other species (Sih *et al.* 2004b; Bell 2007). On the one hand, this was exemplified by the positive associations between behaviours reflecting anxiety and neophobia, and the negative associations of these with the animals' exploratory activity. These findings are in accordance with the results of various other studies, showing similar associations across context (Belzung & Lepape 1994; Heisler *et al.* 1998; Rödel & Meyer 2011; Rödel & Monclús 2011). Most importantly our study demonstrates associations between different social contexts, although the direction of this association was somehow counterintuitive at first sight. Individuals showing a higher frequency of distress calls emitted during social separation were the ones initiating less positive social contacts toward their siblings – thus indicating their lower sociability. This finding of a negative association is in contrast to other studies, for example in sheep *Ovis aries* (Ligout *et al.* 2011). This study showed that animals exhibiting higher frequencies of calls during social separation were the more sociable ones, as measured by their inter-individual distances during their daily activity on a pasture. We propose that the here observed negative association between sociability and the frequency of distress (separation) calling early in life related behaviours might be explained as following: Generally, separation distress calls in young animals are usually considered to be related to emotional reactivity (Pettijohn 1979; Lyons *et al.* 1993; Panksepp 2005), and a higher frequency (number) of calls might express the intensity of the emotional state caused by the separation and the urgency in the response (Banse & Scherer 1996; Manser 2001; Brudzynski 2010). Accordingly, the numbers of calls emitted by the pups during the social separation test applied in our study could be used to characterize how an individual perceives this challenging situation, with high numbers reflecting a state of fear and distress. Later on such pups initiated less social contacts towards litter mates. We hypothesize that an active initiation of a social contact, whatever its nature, could also be considered a potential stressor to such individuals, thus leading to comparatively lower frequencies of initiated approaches and naso-nasal contacts under the condition of our study. Such a mechanism could also help explaining the negative association between anxiety measured in the EPM test and sociability observed in our study, showing that more anxious animals were less prompt to initiate interaction with their siblings.

However, this association between anxiety-related behaviour in the elevated plus maze and the initiation of positive social (naso-nasal) contacts – as well as some associations across time in standard tests during early life stages, as described above – were only significant when considering individual differences among litter siblings. When comparing the absolute values of the complete sample of individuals we did not find a significant association, probably because of a high variation among different litters in the general level of activity. Such a high variation among litters, including

non-constant changes of among-litter variation across time, might have the potential to mask repeatabilities or associations across context at the individual level. This is supported by the fact that variation in behavioural responses within siblings has been frequently shown to be smaller than between litters because siblings have a similar genetic background and experience a highly similar environment during early life (Stamps & Groothuis 2010; Stamps & Krishnan 2014). We recommend the consideration of within-litter variation as a useful tool when exploring individual consistencies across time and context in young animals.

Surprisingly, the frequency / number of separation calls, often considered to reflect a state of distress or anxiety (Gardner 1985; Insel *et al.* 1986; Branchi *et al.* 2001), was not associated with the level of anxiety-related response assessed in our study by means of the animals' open / closed arms activity in the elevated plus maze. Although such positive association between anxiety related behaviours and the emission of distress calls in young animals has been reported (Wigger *et al.* 2001; Brunelli 2005), other studies found contradictory results. For example, studies in laboratory rats showed that pups who emitted lower frequencies of calls when separated from the mother displayed higher levels of calls in a fear-conditioning paradigm, indicating that such animals developed a high-anxiety phenotype as adults (Schwarting & Wöhr 2012). We propose that further studies using challenging or fearful situations, such as social (resident-intruder) confrontations, might help to explore long-term associations of individual differences in distress calling during early life.

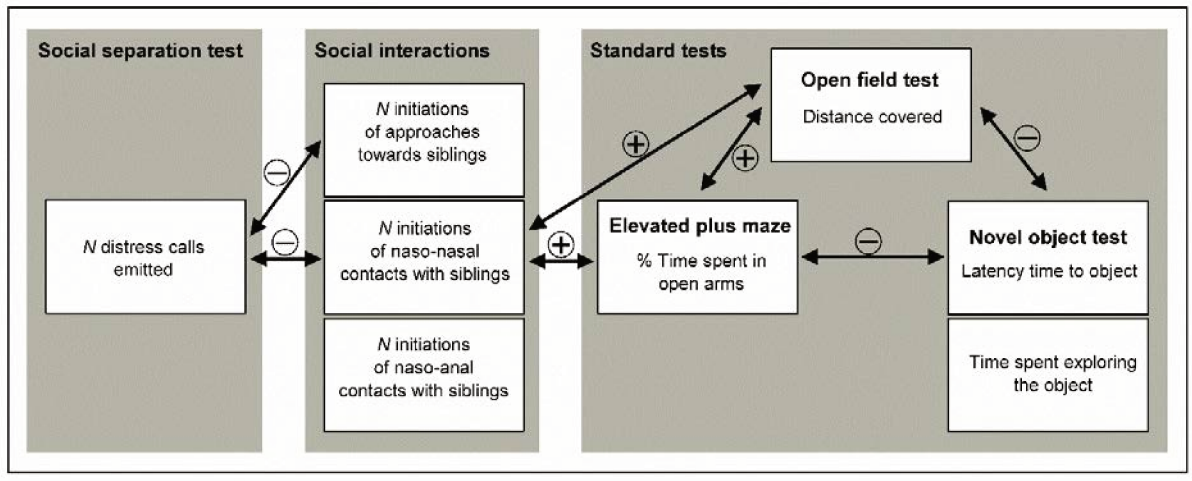

Fig. 3. Schema of the behavioural syndrome in the mound building mouse, including responses in social tests. Multiple associations between the behavioural responses quantified in different tests are summarized; arrows with plus and minus indicate statistically significant bivariate associations, plus indicating positive association while minus indicating negative ones. See Figs. 1, 2 for details and see text for statistics. All behaviours shown are repeatable (Tables 1,2) across the different time scales used, such as across several hours (social interactions), days (social separation test) or weeks to months (standard tests).

In conclusion, our results clearly show constant individual differences with regard to social traits and the presence of a behavioural syndrome in the mound building mouse, integrating social

personality traits. This study also underlines the need to develop specific tests for young animals. Finally, these results emphasize the importance of taking into account within-litter differences into the study of individual differences in young animals.

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Personality modulates proportions of $CD4^+$ regulatory and effector T cells in response to socially induced stress in a rodent of wild origin

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Abstract The way how individuals respond to chronic challenges can vary tremendously, and such differences are closely linked to personality. The few available studies on individual differences in stress-related immunosuppression in non-human mammals have been mainly carried out with laboratory strains. We conducted a study in male mound-building mice (Mus spicilegus) of wild origin. We distinguished between high (HAN) and low anxious / neophobic (LAN) personality types, quantified by subjects' consistent and associated behavioral responses in repeated elevated plus maze and novel object tests. After reaching maturity, parts of the subjects were regularly confronted to different resident pairs over a period of 5 days to provoke a condition of chronic social stress, while others were used as untreated controls. We measured fecal corticosteroid metabolite concentrations (FCM) and different cellular immune parameters from blood and spleen. Socially confronted HAN showed higher increases in FCM concentrations than LAN, indicating a more pronounced physiological stress response in the former personality type. HAN of the experimental group also showed lower percentages of effector T cells (Teff) and higher regulatory T cells (Treg) in the spleen; the latter are known for their immunosuppressive activity. Considering the ratio of Teff /

Treg, animals with higher increases in FCM concentrations during the late period of the experiment showed a stronger shift towards Treg cells, supporting immunosuppressive effects of chronically elevated corticosteroid levels. Summarizing, our results strongly suggest that immunomodulatory effects of socially induced stress were altered by individual differences in anxiety/neophobia, emphasizing the significance of personality in shaping physiological responses to challenge.

Keywords: animal personality, effector T cells, corticosteroid metabolites, immunosuppression, *Mus spicilegus*, regulatory T cells, resident-intruder paradigm, social confrontation, social stress

Introduction

A life under repeated or long-lasting stress, leading to a chronically increased activation of the hypothalamic-pituitary-adrenal HPA axis can have devastating consequences for an individual's health, development, reproductive function and survival (Henry & Stephens 1977; Sapolsky 1992; von Holst 1998). In particular, chronic stress and the concomitant elevation of circulating glucocorticoids can compromise the immune system (Dhabhar 2014). A comprehensive meta-analysis of studies in humans points out that chronic psychological stressors tend to suppress both cellular and humoral immunity (Segerstrom & Miller 2004). Similarly, in laboratory animals, distressed subjects show, for example, an increased number of granulocytes (innate response) but a lower number of adaptive immune cells (Stefanski & Engler 1998; Stefanski 2000) as well as a lowered proliferation response of lymphocytes and splenocytes, including a significant reduction of functional capacities of T cells (Raab *et al.* 1986; von Holst 1998; Sommershof *et al.* 2011). Overall, such suppressive effects on immune functions can weaken the body's resistance to pathogens, thus facilitating the outbreak of infectious diseases (Kelley 1980; Glaser *et al.* 1999; Glaser & Kiecolt-Glaser 2005).

There is great variation in how individuals respond to or cope with stress, leading to individual differences in disease vulnerability and development (Manuck *et al.* 1991; Koolhaas *et al.* 2010; Hodes *et al.* 2014), and understanding the drivers of such individual variation has always been a major question in biomedical research as well as in behavioral neuroscience and evolutionary biology (Marshall 2011; Nesse 2011; Wood *et al.* 2015). Nowadays, human personality traits are frequently considered a key factor in driving the susceptibility and progression of immune-mediated diseases (Cohen & Hamrick 2003; Segerstrom 2003; Zozulya *et al.* 2008; Segerstrom & Sephton 2010; Cohen *et al.* 2012; Ožura *et al.* 2012). However, the exact mechanisms linking immune function and

individual behavioral or personality traits are far from being clear (Friedman 2008; Koolhaas *et al.* 2010; Réale *et al.* 2010a).

Animal models are frequently used to increase our understanding of the patterns and underlying mechanisms in the study of personality and health (Mehta & Gosling 2008). The emerging field of "animal personality", and with a similar rationale the concept of coping style, have opened new perspectives here (Cavigelli 2005; Koolhaas *et al.* 2010). Based on the findings in various taxa, there is now convincing evidence that animals of a given species show individual differences in behavioral phenotypes (Koolhaas *et al.* 1999; Gosling 2001; Briffa & Weiss 2010; Stamps & Groothuis 2010). Animal personality and coping styles are usually defined by the existence of correlated suits of behavioral and physiological responses, being consistent across time and contexts (Stamps *et al.* 2010; Koolhaas *et al.* 1999). Although such a rather simplified view might not always allow a proper comparison with human personality dimensions (Gosling & John 1999), it has been shown that animal personality traits or coping styles are significantly related to to sympathetic-adrenomedullary and adrenocortical activities (Carere *et al.* 2010; Koolhaas *et al.* 2010). For example, submissive (von Holst 1986), reactive or passive (Koolhaas *et al.* 1999; Rödel *et al.* 2006; Øverli *et al.* 2007; Azpiroz *et al.* 2008) and more anxious individuals (Dhabhar *et al.* 2012b) typically show a higher or more chronic HPA activation. In addition, more aggressive individuals have been reported to show a lower adrenocortical activity (Veenema *et al.* 2003; but see Kavelaars *et al.* 1999] and a higher catecholamine reactivity (Sgoifo *et al.* 1996).

Along with this line, several studies have demonstrated that different personality traits or coping styles are involved in altering immune parameters in laboratory animals (Koolhaas 2008; Wood *et al.* 2015). For instance, it has been shown that lymphocyte activity is lower in a psycho-genetically selected laboratory rat *Rattus norvegicus* strain with high avoidance behavior compared to a strain with low avoidance (Sandi *et al.* 1991), and studies in laboratory mice *Mus musculus* suggest the suppression of protective anti-tumor immunity in high-anxious phenotypes (Dhabhar *et al.* 2012b). Domestic pigs *Sus scrofa* with a more (pro)active coping style have been reported to show higher lymphocyte proliferation after experimental immunization (Bolhuis *et al.* 2003), and laboratory mice adopting a passive behavioral profile in response to chronic defeat showed higher levels of interleukin-6 and tumor necrosis factor-α in the spleen compared to subjects with an active profile (Gómez-Lázaro *et al.* 2011).

However, almost all of these studies have been done in domestic or laboratory animals, which can have considerable drawbacks. Inbred strains are strongly selected for traits such as tameness or low levels of aggression, and hence variation in temperamental trait expression is artificially reduced compared to wild animals of the same species (Koolhaas 2008). Thus, there is a

need for exploring the natural variation of personality traits in animals of wild origin. So far, only few such studies are available, for example on greenfinches (*Carduelis chloris*: Sild *et al.* 2011), and rhesus macaques (*Macaca mulatta*: e.g. Sloan *et al.* 2008; Capitanio *et al.* 2011) showing association between personality factors or individual coping styles and immune function.

The major aim of our study was to investigate whether and how animal personality traits modulate immunosuppressive effects of stress. We conducted the study on a rodent of wild origin, the mound-building mouse *Mus spicilegus*, which we behaviorally phenotyped by repeated standard tests to determine individual variation in neophobia and anxiety. The mound-building mouse is a monogamous small rodent with adult body masses of around 14-16 g, occurring in a variety of agricultural and steppe-like habitats in Central and South Eastern Europe (Sokolov *et al.* 1998). Our previous studies have shown that mound-building mice show notable individual differences in certain personality traits, making this species an extraordinarily suitable model for this kind of studies (Hudson *et al.* 2015; Rangassamy *et al.* 2015). To create a condition of enduring, socially induced stress, we repeatedly confronted the subjects with older resident pairs over 5 consecutive days. Such resident-intruder confrontations typically cause physiological stress responses in intruders, and have been reported to exert suppressive effects on immune function (e.g. Raab *et al.* 1986; Stefanski 2000). We monitored the efficiency of this treatment in causing physiological stress responses by repeated, non-invasive measurements of the animals' corticosteroid metabolite levels from feces. We (*i*) expected that animals would show general indications of immunosuppression after undergoing the procedure of repeated social confrontations. In particular, we (*ii*) predicted that more neophobic and more anxious individuals would be more susceptible to stressful conditions and thus will exhibit more pronounced suppressive effects on immune parameters. For this, we quantified the proportions of different subsets of leukocytes in blood and in the spleen. In particular, we measured of a specific T cell population, the regulatory T cells, which have not yet been considered in studies on associations between personality and immunity. These cells are specialized for immunosuppression, and play a central role in the immune system homeostasis and the maintenance of self-tolerance (Shevach 2002; Sakaguchi *et al.* 2008; Sakaguchi 2011).

Materials and methods

Origin and Housing Conditions of Study Animals

Mound-building mice of our breeding stock derived from 80 wild animals caught in different sites in Hungary in 1999. The animals used for this study were the 16th, 17th, 18th generation since we started to breed this species in our laboratory. To maintain genetic variation, additional individuals

were captured at the same Hungarian collection sites every 2-4 years. This has been recently done in 2011 (2.5 years prior to the onset of the present study, which was carried out in 2013 / 2014), when 3 wild-caught females and 1 male were added to our breeding stock. All wild-caught animals were carefully checked for diseases and remained in a quarantine room until their proper health status was confirmed.

Animals were housed on a 14:10 h light / dark cycle (white light / red light) in a temperature-controlled room (temperature 21 ± 0.5 °C; relative humidity at approximately 50%). Mound building mice are nocturnal (Simeonovska-Nikolova *et al.* 2009) and experiments were always conduct between 02:00 pm and 04:00 pm during the animal's activity period (red light "dark" phase). Subjects were kept in standard polycarbonate cages (26 × 14 cm and 16 cm high, Iffa Credo, Lyon, France) containing wood shavings as bedding. Several cotton balls were always provided enabling the animals to build their nests. Food (Special Diets Services, Ext. M20, Witham, Essex, U.K.) and water were supplied ad libitum.

Animals used in this study were weaned at postnatal day 28, when they were transferred into new cages and were housed in groups of 4 until postnatal day 55. From postnatal day 55 until the end of the experiments, subjects were kept singly.

Experimental Procedure

Outline of the Study and Sample Sizes

The general goal of the study was to investigate whether personality modulates physiological stress responses and immune parameters in animals confronted to a repeated stressor. To this end, we first assessed personality types of all study individuals (n = 49 males) by repeated standard tests (novel object test, elevated plus maze) at 2 different age classes (T_1: postnatal days 42 / 44; T_2: postnatal days 72 / 74). As we aimed to focus on individuals with clear differences in personality types (high anxious/neophobic versus low anxious/neophobic), we excluded one quartile (25%) of the animals with personality scores close to the median score of the whole sample from further analysis (see below for details). This resulted in a sample size of n = 37 individuals. After reaching adulthood (in the mound-building mouse at around 80 days, Busquet *et al.* 2009), half of the animals (randomly chosen) were assigned to an experimental group and underwent repeated social confrontations with different resident pairs during 5 consecutive days to cause a condition of chronic social stress (postnatal days 101-105). The other half remained in their home cages as untreated controls (sample sizes in Table 1). Fecal samples were collected from the cages to quantify changes in fecal corticosteroid metabolites as a measure of stress in the experimental animals as well as in the

controls. One day after the last social confrontation (postnatal day 106), animals of both groups were euthanized, and blood and spleen were taken to analyze different cellular immune parameters.

Behavioral Phenotyping

(a) Novel object test

This test, which aims to assess the animal's neophobic responses, was carried out in a white polyethylene circle arena of a diameter of 120 cm and surrounding walls of 60 cm height. The animals were entered into the arena at a defined peripheral position. After 10 minutes of habituation, a novel object was introduced in the center of the area. During the first test at the age of 42 days, a colored, artificial hamburger made of soft, plasticized PVC (diameter: 8.5 cm, height: 4.5-5.0 cm) was presented. During the second test at day 72, a 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) was used. We quantified the latency until the animals sniffed the novel object for the first time, and the summed-up time the animals spent exploring the object by means of sniffing, touching with the front paws and climbing on it.

(b) Elevated plus maze

The elevated plus maze exploits the natural aversion of rodents to exposed fields and it considered a reliable measure of anxiety-related behaviour (Archer 1973). It was made of white PVC, consisting of 2 opposing enclosed arms (by 50 cm high walls) and open arms, each of the arms 30 cm long and 5 cm wide and connected by a central platform (10 × 10 cm). This platform (being relatively larger than the central area of the usual elevated plus maze used for laboratory mice cf. Komada *et al*. 2008) was surrounded by transparent Plexiglas walls with round openings (diameter 5 cm) to all four directions, lowering the chance that subjects would jump off the maze (Rangassamy *et al.* 2015). The whole apparatus was elevated 70 cm above ground by a stable wooden construction. For testing, the focal animal was placed in the central platform facing an open arm. We recorded (a) the number of entries / exits into the closed as well as the open arms, defined as crossing the respective thresholds with more than 50% of the animal's body length. We (b) measured the time the animal spent inside each arm (with all four paws). Subjects underwent this test at an age of 44 days and were retested at an age of 74 days.

Each behavioral test lasted 5 min and was video-recorded with a camera mounted on a tripod 120 cm above the floor. Individuals were returned to their home cages immediately after each test, and we cleaned the apparatuses with water and soap (Cleansinald, Johnson Diversey, Fontenay-sous-Bois, France) between trials.

Social Confrontations

We carried out resident-intruder experiments (Koolhaas *et al.* 2013), where focal animals (intruders) were repeatedly confronted with resident pairs. Experimental subjects were removed from their home cage 2-4 hours after the onset of the activity (red light) period and placed singly into the cage of a 10 month-old resident (male-female) pair for 20 minutes per day. These pairs had reproduced at least once and had been kept together in their home cages for around 7 months. The confrontation procedure was repeated during 5 consecutive days, starting at postnatal day 101 until day 105. To avoid any form of habituation of the intruder to the resident pair, the intruder was confronted to different resident pairs each day. To this end, intruders were placed into a protective cage (17 × 7 × 8 cm) made of metal grid (1 mm mesh width) to avoid any injuries during the confrontations, despite allowing visual contact between intruder and residents. However, on the first, third and fifth day of the experiment, the lid of the protective cage was opened after 15 minutes of confrontation. This brief unprotected confrontation (max. 5 minutes) was always supervised by the experimenter and was interrupted by removal of the intruder from the cage when the first chasing event was observed, and always before chasing resulted in a direct fight. This procedure was done to avoid that intruders habituated to the security provided by the protective cage. Immediately after the confrontations, the focal animals (intruders) were returned to their home cages.

Prior to the start of the experiments, at postnatal day 100, the animals weighed on average 14.7 g (± 2.1 SD), and there were no significant differences in body mass between controls and experimental animals (Fisher Pitman permutation test: $p = 0.51$).

Collection of Feces

Starting 3 days before the first resident-intruder confrontation and until the day after the last social confrontation (day 6), feces of each focal animal (experimental animals as well as controls) were collected every morning from its home cage to habituate subjects to this procedure. We collected the whole fecal output to exclude potential differences in gut passage rates and fecal production among animals (Hayssen *et al.* 2002). During fecal collection, animals always stayed in their nests. The procedure did not exceed 5 minutes and feces in the nest were not removed to avoid excessive disturbance of the animals. Immediately after collection, feces were stored in closed plastic tubes at −20°C until analyses.

We analyzed fecal corticosteroid metabolite concentrations (FCM) of samples collected one day before and in the morning of the day of the first social confrontation (thus reflecting the stress hormone levels prior to the confrontation), and in the morning of the days after the third and last (fifth) confrontation (thus reflecting the levels in response to the confrontations) – and likewise in

the untreated controls. The daily social confrontations were always conducted in the afternoon. For later statistical analysis, FCM concentrations measured 1 day before and in the morning before the start of the confrontations were averaged and used as baseline values for calculation of changes (Fig.2). Collection of feces from the cages started already 3 days before the confrontations to habituate the animals to the procedure and to capture possible treatment effects on the FCM baseline values.

Sampling of Blood and Spleen

The morning following the last resident-intruder confrontation, on postnatal day 106, we took blood and organ samples from the animals of both groups for immune analyses. To this end, animals were anesthetized by an intraperitoneal injection of Avertin (2.5 g of tribromoethanol in 200 ml saline) using a dosage of 0.2 ml per 10 g body mass (Papaioannou & Fox 1993). Blood was taken (0.6-0.8 ml) by heart puncture using a syringe with a fine needle (0.45 mm wire diameter). This procedure never exceeded three minutes; see more details in the ethics note. Blood was transferred into a heparinized 1.5 ml plastic tube and was well shaken to avoid coagulation. Animals were dissected and spleens were taken and transferred into a 1.5 ml plastic tube with a 5% solution of heat-inactivated fetal calf serum (FCS) in phosphate buffered saline (PBS). Tubes with blood and spleen samples were stored on crushed ice for immediate analysis of different immune parameters.

Analysis of Immune Parameters

Cell and Tissue Preparation

Leucocytes from the spleen and blood were prepared using a homogenizer, and red blood cells were lysed in hemolysis buffer (NH4CL, KHCO3, and EDTA). The spleen and blood cell suspensions (400 g) were centrifuged for 10 minutes at 4° C and at room temperature, respectively. Cells were re-suspended in 5 ml PBS- 5% heat inactivated FCS.

Flow Cytometry

Leukocytes isolated from spleen or from blood were stained by PerCP-Cyc5.5-labeled-anti-CD4 (clone RM4-5; BD Biosciences, San José, CA, USA) or PercP-labeled-anti-CD8 (clone 53-6.7; BD Biosciences) and APC-labeled-anti-CD19 (clone 53-6.7; BD Biosciences). To study T regulator cells (Treg) $CD4^+$ $FoxP3^+$, and T effector (Teff) cells CD4+ $FoxP3^-$, after cell staining with PercPCyc5.5-labeled-anti-CD4 (clone RM4-5; BD Biosciences), the APC-labeled anti-FoxP3 (clone FJK-16s) Staining Set (eBioscience, San Diego, CA, USA) was used for intracellular staining according to the manufacturer's recommendations. For quantification of neutrophils and monocytes, the surface of leukocytes

isolated from blood was stained with FITC-labeled-anti-Ly6C (clone AL-21) and PE-labeled-anti-Ly6G (clone1A8) and PerCP-Cyc5.5-labeled-anti-CD11b (all from BD Science).

The cells were stained at 4° C in PBS containing 5% heat inactivated FCS and 0.01 M sodium azide and incubated for 30 min with appropriate dilutions of each monoclonal antibodies or corresponding isotypes control coupled to FITC, PE, PerCP-Cy-5.5, APC.

Flow cytometry was performed on a four-color FACScalibure flow cytometer (Becton Dickinson, Mountain View, CA, USA). Dead cells were excluded based on forward and side scatter characteristics. Results were analyzed using the software CellQuest Pro (BD Biosciences) and the software WEAZLE (version 2.3, Walter+Eliza Hall Institute of Medial Research, Parkville, Australia) was used for graphical presentation. See Fig. A in the appendix for some representative analyses of different immune cell subsets.

Calculation of Immune Parameters for Statistical Analysis

Cellular immune parameters measured in the spleen or in blood were expressed as percentages on the number of splenocytes and leucocytes, respectively. In several cases, the percentage of Treg cells (which were initially measured among $CD4^+$, before they were re-calculated as %Treg cells in the blood or among splenocytes) was lower than the detection threshold of our methodology (0.5%). This detection limit of 0.5% among $CD4^+$ was denoted as the minimum detection threshold for statistical analysis, thus representing 0.03 – 0.25% of Treg in the blood and 0.05 – 0.17% of Treg among splenocytes. Note that we repeated all statistical analyses by excluding such values and obtained highly similar results, indicating that our findings were not biased by systematic errors in Treg detection.

In some cases the volume of blood obtained was too small to carry out all immune cell analyses: This was particularly the case with respect to the analysis of percentages of monocytes and neutrophils, thus leading to lower sample sizes with respect to these parameters (see Table 1).

Analysis of Glucocorticoid Metabolites

Measurement of Fecal Corticosteroid Metabolites (FCM)

Feces were defrosted and dried at 50 °C. After removing any visible contaminations such as hair, pieces of cotton ball and wood shavings, samples were homogenized with mortar and pestle, and we weighed 0.05 g of each sample with a precision balance. Glucocorticoids were extracted following the method developed by Palme and Möstl (1997). In short, fecal samples were suspended in 80% methanol and after vortexing and centrifuging, we transferred the supernatants into new tubes and

diluted 1:10 with assay buffer. They were frozen at −20°C until analysis. We used an already established 5α-pregnane-3β, 11β, 21-triol-20-one enzyme immunoassay (EIA) specific for laboratory mice, *Mus musculus* (Touma *et al.* 2003). Antibodies were obtained from the Unit of Physiology, Pathophysiology, and Experimental Endocrinology, University of Veterinary Medicine Vienna, Austria. Inter- and intra-assay coefficients of variation were 11.6% and 11.3%, respectively.

Validation of the FCM Assay by ACTH Challenge

We performed a physiological validation of the EIA for quantification of fecal corticosteroid metabolites in the mound-building mouse by an ACTH challenge test. The aim of this test is to validate that the antibody used in the EIA is positively measuring the resulting increase in corticosteroid metabolite levels in feces when animals were injected with a high dose of synthetic ACTH (Touma & Palme 2005). We used 7 adult mound-building mice (3 males, 4 females), which were on average 9 months old. They were housed individually in standard cages for at least one week before the validation. We injected them intra-peritoneally with 0.1 ml of a solution of synthetic ACTH (Synacthen Depot, Novartis, Germany) in saline with a concentration of 7 ng / ml (cf. Touma & Palme 2005). Feces were collected every morning from their home cages, following the same protocol as above. The collection started 3 days before the injection, to habituate the animals to the procedure, and until the second day after the ACTH injection. See above for details on the procedure of feces collection, storage, and analysis.

A successful validation would show an increase of FCM concentrations from day 1, before ACTH injection, to day 2 of the validation experiment, when samples were collected around 16 hours after injection. Note that the gut passage time in the laboratory mouse is around 10 h (Touma *et al.* 2003), most likely being similar in *M. spicilegus*. Furthermore, we expected that FCM concentrations on day 3, when samples were collected around 40 hours after injection, would return to the animal's initial values as quantified on day 1.

As expected, FCM concentrations in all individuals consistently and significantly increased from day 1 (on average 113 ng / g feces ± 41 SD) until day 2 (on average 214 ng / g feces ± 169 SD; Fisher Pitman permutation test: p = 0.016) and decreased from day 2 to day 3 of the validation experiment (on average 153 ng / g feces ± 96 SD; p = 0.014). There were no statistically significant differences between the values at day 1 and 3 (p = 0.29). These results confirm that the assay successfully measures corticosteroid metabolites in the mound-building mouse.

Table 1. Sample sizes with respect to the different immune parameters measured in subjects with high (HAN) and low (LAN) anxiety / neophobia scores within the untreated control group or in experimental group (repeated social confrontation). Mean values including their data ranges for each different immune parameter are given; see Figs. 3,4 and Fig. B in the appendix for details on average values within treatment groups and personality types.

Immune parameters	Origin	Control	Social confrontation	Overall means (min, max)
		N_{total} (N_{LAN}/N_{HAN})	N_{total} (N_{LAN}/N_{HAN})	
% $CD4^+$	Blood	17 (10/7)	19 (8/11)	29.6% (6.2%, 49.6%)
	Spleen	18 (11/7)	19 (8/11)	22.6% (10.8%, 33.0%)
% $CD4^+$ $FoxP3^-$ effector T cells	Blood	18 (10/7)	19 (8/11)	29.0% (5.6%, 48.8%)
	Spleen	18 (11/7)	18 (8/10)	21.8% (10.3%, 32.7%)
% $CD4^+$ $FoxP3^+$ regulatory T cells	Blood	17 (10/7)	19 (8/11)	0.37% (0.07%, 0.99%)
	Spleen	18 (11/7)	18 (8/10)	0.32% (0.06%, 0.85%)
Ratio Teff / Treg	Blood	17 (10/7)	19 (8/11)	123.9 (11.7, 199.6)
	Spleen	18 (11/7)	18 (8/10)	118.0 (19.4, 199.7)
% $CD8^+$	Blood	13 (7/6)	17 (6/11)	9.7% (2.5%, 26.7%)
	Spleen	17 (11/6)	17 (8/9)	6.4% (2.5%, 17.0%)
% CD19 B	Blood	15 (9/6)	18 (7/11)	25.5% (4.6%, 57.1%)
	Spleen	18 (11/7)	19 (8/11)	35.8% (5.5%, 58.5%)
% Neutrophils	Blood	8 (3/5)	15 (5/10)	5.3% (1.2%, 16.0%)
% Monocytes	Blood	11 (3/5)	19 (5/11)	4.6% (0.8%, 13.5%)

Ethics Note

Animals were kept and treated according to the ethics and animal care guidelines of France (where the project was carried out), and experimental procedures were approved by the local authority for laboratory animal care and use (Comité d'Ethique en Expérimentation Animale Charles Darwin; Ce5/2012/212; 00809.02). Focal animals were sacrificed during blood sampling by heart puncture after anesthesia. Prior to heart puncture, we ensured the absence of any pedal withdrawal reflexes. Blood sampling never exceeded three minutes and none of the animals showed any signs of muscular activity during the procedure. Immediately after, we carried out cervical dislocation in order to ensure death of subjects.

Resident pairs were also sacrificed after the end of the experiments. To this end, they were first anesthetized by putting them into a closed transparent plastic box filled with isoflurane gas of a concentration of 3% (IsoFlo, Axience, France), administered by an automatic system (Univentor 400 Anaesthesia Unit, Uno Roestvststaal BV, The Netherlands). Immediately after, animals were euthanized with a high dose of CO_2 gas, delivered by a compressed gas cylinder. We always observed the animals until all muscle activity and other signs of life had been absent for at least 30 s. Finally,

we carried out cervical dislocation in order to ensure death. All procedures were conducted by a qualified and experienced person.

Statistical Analysis

Analyses were done with the program R, version 3.1.1 (R Core Team 2014). Statistical comparisons and correlations were done by permutation tests allowing the handling of non-normally distributed data of moderate sample sizes (Good 2005). *P*-values of all permutation tests performed were based on 10,000 replicates.

We explored associations between the different behavioral variables quantified during the repeated novel object tests and elevated plus maze tests by linear regression permutation tests using the *lmPerm* package (Wheeler 2010). By using principal component analyses PCA (package *prcomp*) we captured the information of the different behavioral responses in the novel object test and in the elevated plus maze in a single score. First, this was done separately for each time step (T_1, T_2), based on the animals' percentage time spent in the closed arms of the elevated plus maze, the frequency of exits from this arm, and the latency to sniff, as well as the percentage time to explore the novel object. During both time steps, the first component used as score variable had an eigenvalue > 1, indicating that this component accounted for more variance than any of the original variables of the standardized data. Note that all behavioral variables were associated in the same way (either positively or negatively) with the resulting score variable during both time steps. These score variables (anxiety / neophobia), but also each of the behavioral variables quantified during the novel object and elevated plus maze tests were tested for repeatability across time (T_1, T_2) using intra-class correlations, calculated as the proportion of phenotypic variation that can be attributed to between-subject variation (Lessells & Boag 1987). To this end, we used LMM-based calculations of repeatability with the R package *rptR* (Nakagawa & Schielzeth 2010). Also here, *P*-value calculation was based on 10,000 permutations, and 95% confidence intervals of repeatabilities were assessed by 10,000 bootstrap steps. Individual identity was used as a random factor. For further analysis (see below) we then run a PCA over both time steps. Our goal was to identify animals with high or low anxiety/neophobia scores. To this end, we discarded one quartile (25%) of animals (12 out of 49 individuals of the original sample size), which were rather ambiguous with respect to this trait. That is, 12.5% of the individuals above and 12.5% below the median anxiety / neophobia score were excluded from further comparisons. The remaining animals with anxiety / neophobia scores above this threshold interval were referred to as HAN (high anxious/neophobic; n = 19), and subject below this threshold interval as LAN (low anxious / neophobic; n = 18) individuals.

The interactive effects of treatment (socially confronted animals versus controls) and personality type (HAN versus LAN) were analyzed by a 2-way ANOVA with permutation, using the *lmPerm* package (Wheeler 2010). In case the interaction between treatment and personality type was statistically significant, we performed post-hoc comparisons between HAN and LAN within the different treatment groups or cross-wise comparisons of personality types between treatments with non-parametric Fisher-Pitman permutation tests using the *coin* package (Hothorn *et al.* 2008). Correlations between changes in FCM concentrations and immune parameters were calculated by parametric linear regression permutation tests (*lmPerm* package).

Results

Determination of Behavioral Types

Associations of Different Behavioral Responses within and across Tests

The latency to sniff the novel object (on average: 48 s, min: 4 s, max: 281 s) and the percentage time the animals spent exploring the object by sniffing, touching it with the front paws and climbing on it (on average: 21.0%, min: 0.3%, max: 62.1%) were significantly and negatively correlated during the first test T_1 (linear regression with permutations: $R^2 = 0.275$, $\beta = -0.156$, $p < 0.001$) and showed the same tendency during the second test T_2 ($R^2 = 0.061$, $\beta = -0.056$, $p = 0.084$). That is, animals that were quicker to approach the object (tended to) spent more time exploring it. Moreover, the two behavioral responses recorded in the elevated plus maze, i.e. the percentage time the animals spent in the closed arms of the maze (on average: 60.4%, min: 29.0%, max: 94.4%) and the frequency of exits from these arms (on average: 8 min: 2, max: 22) were significantly and negatively correlated during both tests (T_1: $R^2 = 0.226$, $\beta = -2.760$, $p < 0.001$; T_2: $R^2 = 0.437$, $\beta = -2.942$, $p < 0.001$).

Several of the animals' behavioral responses were significantly repeatable across time, such as the latency to approach and sniff the novel object (intra-class repeatability: $R = 0.437$, $CI_{95\%} = [0.183, 0.683]$, $p = 0.005$), the percentage time the animals spent in the closed arm of the elevated plus maze ($R = 0.418$, $CI_{95\%} = [0.149, 0.613]$, $p = 0.008$) and, following the same tendency, the frequency of exits out of the closed arms ($R = 0.178$, $CI_{95\%} = [0, 0.423]$, $p = 0.10$).

Furthermore, there were indications for associations between the variables measured in the different tests. The averaged values (across T_1 and T_2) of the latency to sniff the novel object were significantly and positively correlated with the percentage time spent in the closed arms of the elevated plus maze (linear regression with permutations: $R^2 = 0.084$, $\beta = 1.026$, $p = 0.042$). In addition, the latency until sniffing the novel object tended to be negatively correlated with the

frequency of exits out of the closed arms of the elevated plus maze (R^2 = 0.068, β = –4.996, p = 0.073). Other associations were not statistically significant with $p > 0.10$.

Consistencies in Anxiety / Neophobia Scores across Time

Using PCA, we obtained a component (the first axis of the PCAs for T_1 and T_2, respectively) integrating the animals' behavioral responses in the two different, repeated tests. During both times of testing, higher individual scores of this component were always associated with higher levels of anxiety-related behaviors such as a higher percentage time spent in the closed arms (factor loadings T_1: 0.500; T_2: 0.629) and a lower frequency of exits out of this arm (T_1: –0.483; T_2: –0.563), and of behaviors indicative of neophobia such as a longer latency to sniff the novel object (T_1: 0.595; T_2: 0.377) and a lower percentage time of exploring the object (T_1: –0.404; T_2: –0.382). Overall, the components, which we used as the anxiety / neophobia score, explained 41.4% of the variation of the raw data during the first tests T_1 and 48.4% of the variation during the second tests T_2.

Anxiety / neophobia scores were significantly repeatable across time, i.e. individuals with higher scores in relation to the other animals tested during T_1 also tended to have higher scores during T_2 (intra-class repeatability: R = 0.501, $CI_{95\%}$ = [0.246, 0.677], p = 0.001; Fig. 1). For further analyses, we re-calculated the anxiety / neophobia score by the first component of a single PCA over both time steps. Animals with anxiety / neophobia scores of at least half a quartile above the median are hereafter referred to as HAN (high anxious / neophobic), and animals with scores of at least half a quartile below the median are referred to as LAN (low anxious / neophobic); see details in methods section.

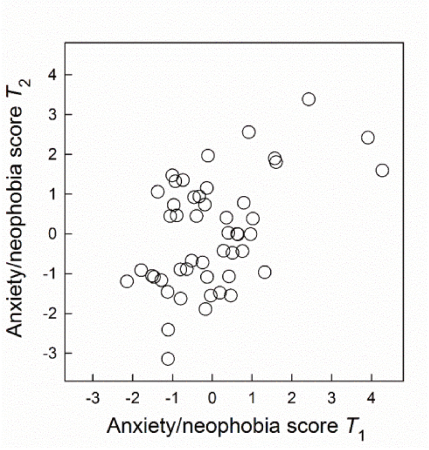

Fig. 1. Consistencies across time in anxiety / neophobia scores. Calculation of scores was based on subjects' behavioral responses during repeated novel object NO and elevated plus maze EPM tests (NO: T_1: postnatal day 42, T_2: day 72; EPM: T_1: day 44, T_2: day 74). Higher scores indicate more pronounced anxiety-related/neophobic behaviors during the tests, respectively. The score was significantly repeatable based on the proportion of phenotypic variation attributed to between-subject variation (intra-class correlations); see text for details on statistics.

Modulation of Responses in Fecal Corticosteroid Metabolites to Social Confrontation by Personality Traits

Prior to the start of the experiments, at an age of 100 days (= day 0 of the experiment), FCM baseline concentrations were on average 128.8 ng / g ± 47.6 SD and did not differ significantly between the animals assigned to the 2 treatment groups (experiment=social confrontation and control), or within the 2 treatment groups between HAN and LAN individuals (Fisher Pitman permutation test: all $p > 0.10$).

During the first 3 days of the experiment, the increase in fecal corticosteroid metabolites in the animals of the experimental group was significantly higher than in the control group (2-way ANOVA with permutation: $p < 0.001$; Fig 2A). There was no significant interaction between treatment and personality type, and also no general significant difference between HAN and LAN individuals (all $p > 0.10$).

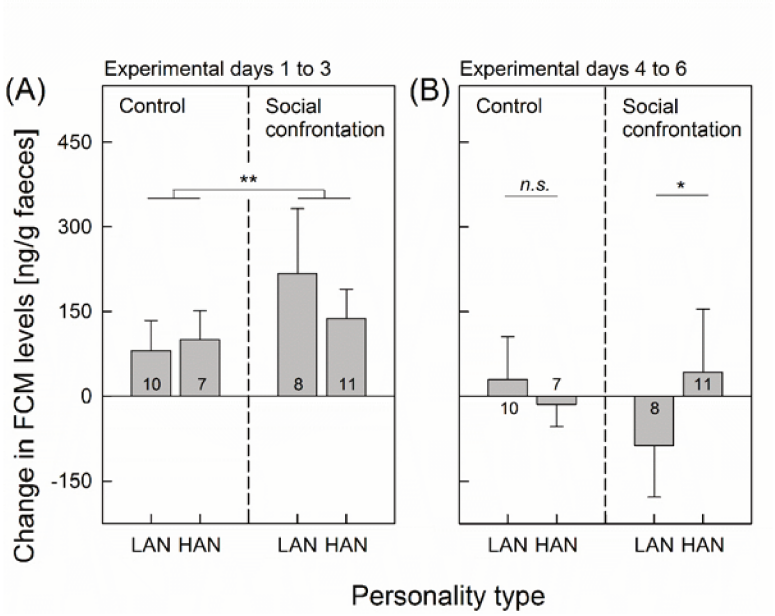

Fig. 2. Changes in concentrations of fecal corticosteroid metabolites during the early and late experimental period. Mean values (+ SD) for individuals of the control and experimental group with high (HAN) or low (LAN) anxiety / neophobia scores are given. Animals of the experimental group were daily confronted to different resident pairs over a period of 5 days. Statistical analysis by 2-way ANOVA revealed significantly higher FMC levels in experimental animals than in controls during (A) the early period (changes between day 0 and day 3; ** $p < 0.010$). During (B) the late period (changes between day 4 and day 6), the interaction between treatment and personality type was significant; see text for details on statistics. Post-hoc comparisons by Fisher Pitman permutation tests between different personality types are shown (Bonferroni-corrected: * $p < 0.025$). Sample sizes are given inside or next to the bars. Baseline concentrations of fecal corticosteroid metabolites measured on day 0 are provided in the text.

However, when considering the changes in FCM levels from days 4 to 6, there was a significant interaction between treatment and personality type ($p = 0.005$). Post hoc comparisons revealed significant differences between animals with different personality types in experimental

group but no significant differences within the controls (statistics in Fig. 2B). Within the experimental group, HAN animals showed a marked increase in FCM levels, whilst LAN individuals rather showed constant or decreased values (Fig. 2B).

Modulation of Responses in Immune Parameters to Social Confrontation by Personality Traits

Effector T cells and Regulatory T cells

Significant interactions between treatment and personality type were apparent for the percentages of $CD4^+$ cells (2-way ANOVA with permutation: $p = 0.028$), effector T cells ($p = 0.008$) and of regulatory T cells ($p = 0.040$) in the spleen, but not in blood (all $p > 0.05$). Note that Teff cells represented the vast majority among $CD4^+$ cells in the spleen of on average 97.8% ± 1.5 SD and on average 97.7% ± 1.7 SD in blood. Treg cells represented on average 1.8% ± 1.4 SD of $CD4^+$ cells in the spleen and 1.5% ± 1.2 SD of $CD4^+$ in blood.

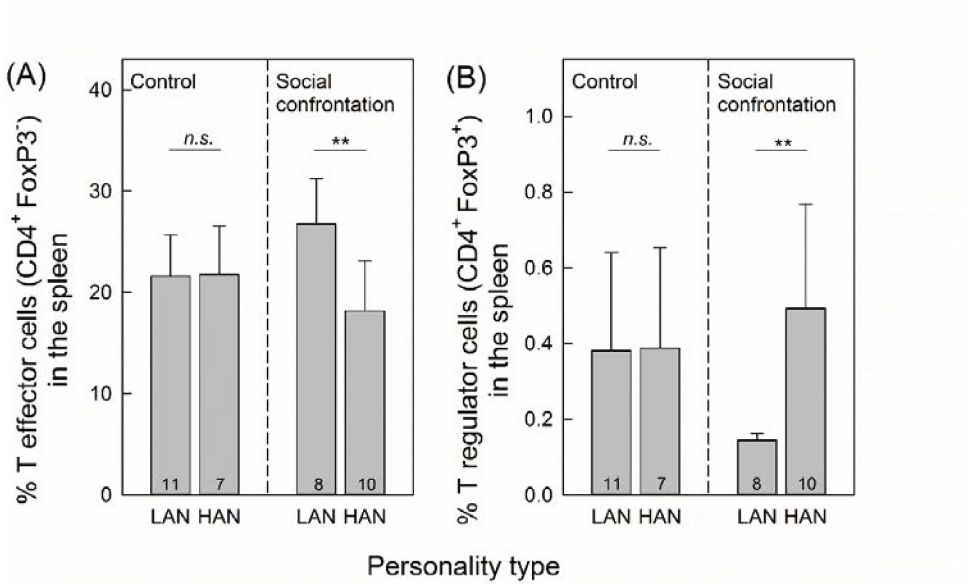

Fig. 3. Percentages of (A) $CD4^+$ $FoxP3^-$ effector T cells and (B) $CD4^+$ $FoxP3^+$ regulatory T cells in the spleen. Mean values (+ SD) of individuals of the control and experimental group (daily social confrontation with changing resident pairs) with high (HAN) or low (LAN) anxiety / neophobia scores are given. Pair-wise comparisons between animals with different personality types (post hoc to significant interactions between personality type and treatment; see Table 1 for statistics) were calculated by Fisher Pitman permutation tests (Bonferroni-corrected: $*p < 0.025$, $**p < 0.005$). Sample sizes are given in the bars.

Post hoc comparisons revealed that the percentage of $CD4^+$ cells as well as the percentage of Teff cells among splenocytes (Fig. 3A) were significantly lower in HAN compared to LAN individuals within the experimental group (Fisher-Pitman permutation test: $CD4^+$: $p = 0.002$; Teff: post-hoc statistics in Fig. 3A), but not in controls ($p > 0.05$). In contrast, percentages of regulatory T cells ($CD4^+$ $FoxP3^+$) among splenocytes were significantly higher in HAN compared to LAN animals of the experimental group (post hoc statistics in Fig. 3B). There was also tendency of an interaction between treatment

and personality type (p = 0.066) with respect to the percentage of Treg cells in blood. Also here, when pair-wise comparisons were applied post-hoc to this non-significant interaction, statistical results indicated significantly higher Treg levels in HAN than in LAN animals within the experimental group (Fisher Pitman permutation test: p = 0.002), whereas there were no significant differences in the controls (p = 0.53; see Fig. B in the appendix).

It should be noted that the average percentages of Teff and Treg cells in the spleen (Fig. 3), and also in Treg cells in the blood (see Fig. B in the appendix) of animals of the control group tended to be at an intermediate level between the values of HAN and LAN individuals of the socially stressed treatment group. For Teff cells in the spleen, LAN animals of the treatment group showed significantly higher values than the controls (Fisher Pitman permutation test: p = 0.011), whereas the values HAN of the treatment group showed a statistical tendency to be lower than the controls (p = 0.058). The percentage of Treg cells in the spleen were significantly lower in LAN of the treatment group animals than in controls (p = 0.014), whereas values of treatment group HAN did not differ significantly from controls (p = 0.29).

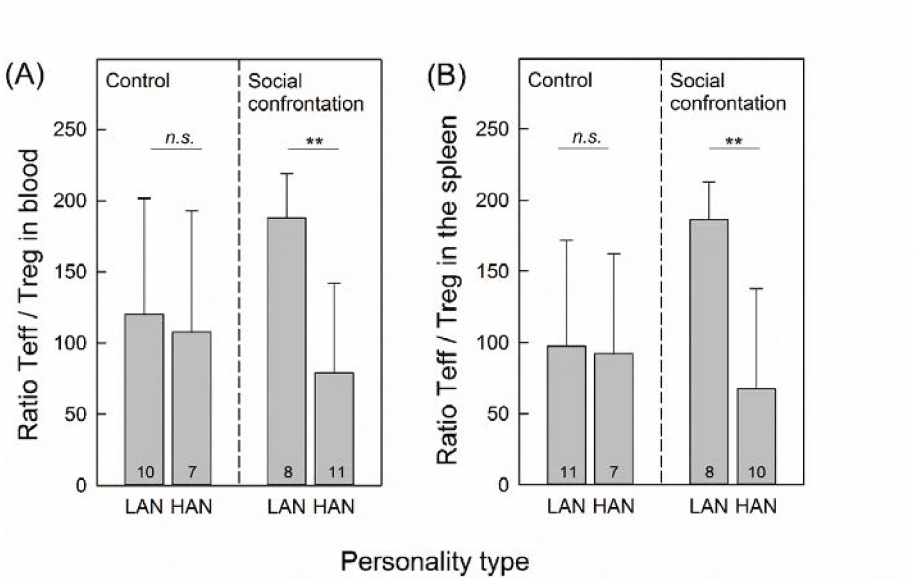

Fig. 4. Ratios between $CD4^+$ $FoxP3^-$ effector T cells and $CD4^+$ $FoxP3^+$ regulator T cells (A) in blood and (B) in the spleen. Mean values (+ SD) of individuals of the control and experimental group (daily social confrontation with changing resident pairs) with high (HAN) or low (LAN) anxiety / neophobia scores are given. Pair-wise comparisons between animals with different personality types (post hoc to significant interactions between personality type and treatment; see Table 1 for statistics) were calculated by Fisher Pitman permutation tests (Bonferroni-corrected: $^{**}p < 0.005$). Sample sizes are given in the bars.

Overall, the pattern of contrasting differences between HAN and LAN of the experimental group with respect to the proportions of Teff and Treg cells was further confirmed when considering the ratio of these two cell types. There were significant interactions between treatment and personality type respect to the ratio of Teff and Treg cells in the spleen (2-way ANOVA with permutation: p = 0.016) as well as in blood (p = 0.047). Post hoc comparisons revealed that this ratio was significantly higher in

LAN compared to HAN animals of the experimental group in blood (statistics in Fig. 4A) as well as in the spleen (Fig. 4B), indicating a shift towards Treg cells in such individuals. No such differences were apparent in controls (Fig. 4A,B).

Other Immune Cell Populations Tested

There were no significant interactive effects of treatment × personality type or significant main effects of treatment or personality type on the percentage of $CD8^+$ and CD19 B cells quantified in blood and spleen, or on the percentage of neutrophils in blood (2-way ANOVA with permutation: all $p > 0.10$). However, the percentage of monocytes in blood was generally and significantly lower in LAN (on average: 2.8% ± 1.4 SD) than in HAN animals (on average: 5.2% ± 2.4 SD; $p = 0.015$).

Associations between Changes in FCM Concentrations in the Experimental Group and Immune Parameters Tested

Within the experimental group, changes in FCM concentrations during the late period of the experiment (days 4 to 6) were significantly correlated with the ratio between Teff and Treg cells in blood (linear regression with permutation: $R^2 = 0.236$, $\beta = -0.309$, $n = 36$, $p = 0.029$). The stronger the increase in FCM levels was, the lower was the ratio of Teff / Treg – i.e., the ratio was shifted towards Treg cells. There were no significant correlations between changes in FCM concentrations and the ratio between Teff and Treg cells within the control group ($p > 0.10$). Furthermore, there were no significant associations between changes in FCM concentrations and any other immune parameter measured (all $p > 0.10$).

Discussion

Summary of Findings

In accordance with our hypotheses, our study in a small rodent of wild origin showed clear indications that individuals with different personality types differed in their stress and immune responses to repeated social confrontations with resident pairs. Highly anxious / neophobic individuals (HAN) showed higher increases in FCM concentrations during the later period of repeated social confrontations than low anxious / neophobic ones (LAN), suggesting a chronic physiological stress response in the former group. Most importantly, HAN individuals of the experimental group showed a comparatively lower percentage of effector T cells among splenocytes but a higher percentage of regulatory T cells, an indication of immunosuppression. To our knowledge ours is the

first study suggesting that stress-related differences in regulatory T cell levels depend on an individual's personality type.

Personality and Corticosteroid Levels

Based on individuals' responses during repeated elevated plus maze and novel object tests, we could successfully assign them to personality types with high and low anxiety / neophobia scores. In accordance to our results, behaviors reflecting anxiety and neophobia have been frequently shown to be positively associated in laboratory mice, thus forming part of a behavioral syndrome (Belzung & Lepape 1994; Heisler *et al.* 1998).

Our results obtained in the mound-building mouse also confirm that the experimental setting of repeatedly confronting subjects with different resident pairs (changing residents on a daily basis) led to a significant increase in fecal corticosteroid metabolites (FCM). This is in accordance with studies in other rodents, showing the efficiency of exposing male intruders to brief but repeated social confrontations over several consecutive days to provoke a chronic condition of stress (Tornatzky & Miczek 1993; Mitra *et al.* 2006). Despite the fact that HAN as well as LAN individuals initially showed a marked increase in FCM concentrations during the early period (days 1 to 3) of the experiment, there were clear differences between both groups later on. HAN animals of the experimental group showed a further increase in FCM concentrations during the later period (days 4 to 6) of the social confrontations, whilst the levels remained rather stable or even decreased in LAN individuals (Fig. 2B). Such differences in stress responsiveness between animals with different personality traits or coping styles have been frequently reported in mammals and birds, such as that individuals with shy, slow-exploring, passive or reactive behavioral profiles typically show a higher HPA axis activity than animals characterized as bold, fast-exploring, active, aggressive or proactive (von Holst 1998; Carere *et al.* 2003; Cockrem 2007; Koolhaas *et al.* 2010; Stöwe *et al.* 2010). That is, an animal's stress responsiveness itself can be regarded as an important component of its personality (Carere *et al.* 2010).

Personality and Immune Responses to Stress

Chronic stress can markedly affect an animal's immune response, as glucocorticoids are known to provoke changes in the immune system, for example by altering cell differentiation (Coutinho & Chapman 2011), inducing lymphocyte T apoptosis (Herold *et al.* 2006; Wirth *et al.* 2014) or by affecting phagocytosis (Heasman *et al.* 2013) – showing the general tendency to exert suppressive effects on immune function (see Segerstrom & Miller 2004 for a meta-analysis). For instance, subordinate male laboratory rats subjected to repeated social confrontations with a dominant male showed significant alterations of the immune system as exemplified by decreases in the number of

CD4 cells (Stefanski & Engler 1998; Stefanski & Engler 1999). Such effects did not only manifest in temporary changes of circulating blood cell concentrations, but also consisted in persistent effects on function and cellular composition of the thymus (Engler & Stefanski 2003). However, not all individuals respond in the same way during stressful situations, and different behavioral phenotypes also differ in their immune responses (Bohus *et al.* 1993; von Holst 1998; Bartolomucci *et al.* 2001; Koolhaas 2008; Capitanio *et al.* 2011). For example, passive-reactive individuals of an OF1 laboratory mouse strain developed higher numbers of melanoma pulmonary metastases than active-proactive individuals when subjected to acute social stress (Azpiroz *et al.* 2008). A study in the same strain of laboratory mice also showed differential interleukine activity as well as physiological stress responses according to the coping styles they were exhibiting (De Miguel *et al.* 2011). Studies in domestic pigs *Sus scrofa* have demonstrated that animals displaying more aggressive and resistant behavioral responses to challenging situations had a generally higher cell-mediated immunity than animals characterized by a non-aggressive and non-resistant behavioral style. In contrast, such passive responders showed a comparatively higher humoral immunity (Hessing *et al.* 1995).

With our study, we demonstrate that the assessment of different personality types even prior to the experimental treatment helps explaining individual variation of immune responses to social stress. After repeated social confrontations, animals with higher anxiety and neophobia scores (HAN) showed significantly lower proportions of CD4+ cells in the spleen than individuals with low anxiety / neophobia scores (LAN). Furthermore, we found generally higher proportions of monocytes in blood in HAN compared to LAN individuals. These results confirm previous findings in laboratory mice showing decreases in different subsets of lymphocytes including $CD4^+$, but slightly increased granulocyte and monocyte counts in high-anxious individuals (Rammal *et al.* 2009).

Most importantly, with our study we could shed more light on the proximate mechanisms underlying immunosuppressive effects triggered by an increased HPA axis activity. Our findings suggest that HAN individuals responded to chronic social stress by an increase in regulator T cells – known for their suppressor role of the immune system (Sakaguchi 2011), and by an associated decrease in effector T cells. The combined effects on these two subsets of $CD4^+$ cells in blood and spleen, which were also evident by a negative correlation between the increase in stress hormone levels (measured by FCM concentrations) and the ratio of Teff / Treg cells, strongly indicate a lower immune function in chronically stressed HAN individuals, at least temporarily. This is in line with a study in laboratory mice, showing that chronic immobilization stress significantly enhanced the numbers and function of T regulatory cells, while the proliferation activity of effector T cells was decreased (Kim *et al.* 2012). Taken together, these results can help enlightening how chronic (social) stress - via alterations of Treg cell frequencies - is associated with a defective immune defense, thus

increasing the occurrence of certain diseases. For instance, regulatory T cells play a crucial role in the prevention of autoimmunity and the maintenance of self-tolerance, and low concentrations of regulatory T cells are typically associated to autoimmune disorders and can impair pathogen-specific responses (Sakaguchi *et al.* 2008; Sakaguchi 2011). In contrast, a strongly increased activity of Treg cells can result in chronic infections and are associated to an increased risk of cancer (Smigiel *et al.* 2014). The finding of our study further contribute to the knowledge on individual differences in stress-related alterations of regulatory T cell levels including their potential consequences, as we could show that an individual's personality type has the potential to strongly modulate such effects.

Interestingly, when comparing Teff and Treg control values to the values of HAN and LAN animals of the experimental group, it appears that the proportions of these immune cells in the control group were always on an intermediate level. For example, comparisons of effector T cells levels between controls and socially confronted HAN and LAN indicate that the differences between HAN and LAN within the treatment group were not just due to lower levels in HAN – but also due to increased Teff levels in LAN individuals (and vice versa with respect to Treg cells). These differential responses in HAN and LAN might be explained by the way how animals with different personality perceived the repeated social confrontations. HAN individuals showed a clear physiological response to treatment by means of comparatively stronger increases in FCM levels particularly during the late experimental period (see also Dhabhar *et al.* 2012b), thus leading to immunosuppressive effects, as exemplified by comparatively lower Teff levels. However, LAN individual might have experienced rather mild stress during the brief daily social confrontations. Mild, short-term stress, in contrast to a chronic activations of the HPA axis, has been shown to booster the immune system, for example by the mobilization of various types of immune cells into the blood circulation, including the enhancement of their maturation and function (Dhabhar *et al.* 2012a; Dhabhar 2014).

Concluding Remarks

Inter-individual variation in laboratory animals is frequently considered as 'noise' that plagues experimentation in biomedical sciences – potentially affecting reproducibility of results. Attempts to reduce such unwanted individual differences include the use of inbred and transgenic strains and efforts of high standardization of housing and rearing. However, it has been increasingly noted during the recent years that individual differences in behavior and physiology of laboratory animals, with respect to their behavioral profiles (Lewejohann *et al.* 2011; Rödel *et al.* 2012), due to differences in early environmental conditions (Prager *et al.* 2010), and even in genetically identical individuals (Freund *et al.* 2013) cannot be fully suppressed. However, making sense of such individual differences can be considered an essential step towards the understanding of inter-individual variation in disease susceptibility or treatment responses (Cavigelli 2005; Koolhaas 2008; Mehta &

Gosling 2008). Integrating the study of animals of wild origin in biomedical research has been proposed as a promising, alternative (or complementary) approach in biomedical research (Koolhaas 2008, 2010). This allows to capture the natural variation in personality patterns, which is typically altered in laboratory animals by decades of selective breeding. Our study in the mound-building mouse *M. spicilegus*, a species closely related to the commonly used laboratory mouse *M. musculus* provides an example of such an approach, emphasizing the significance of naturally occurring personality differences in modulating associated hormonal and immunological responses to chronic social stress.

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Electronic Appendix

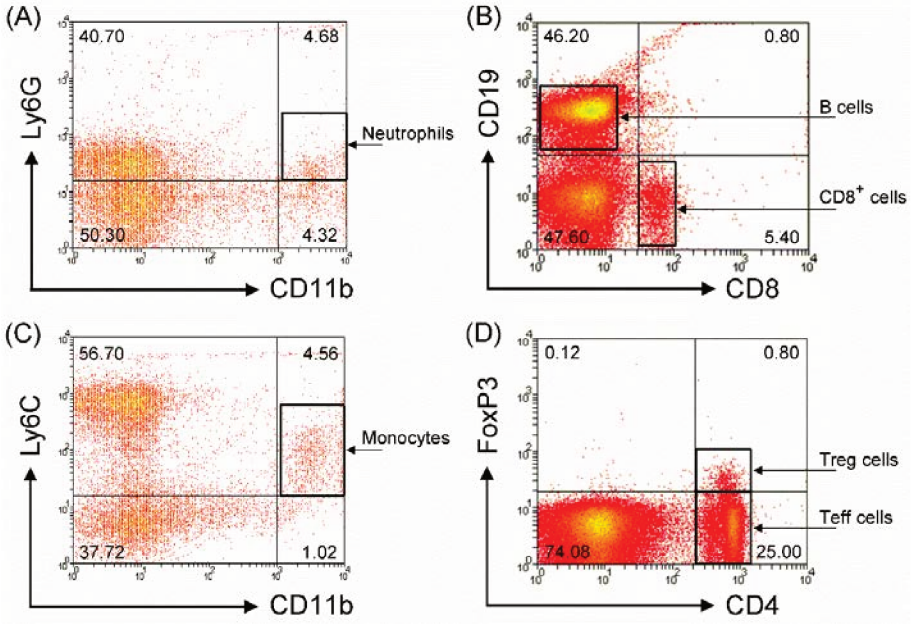

Fig. A. Representative examples of dot plots of the FACS analysis of different immune cell subsets in the blood and spleen of the mound building mouse *Mus spicilegus*. Frequencies of (A) Neutrophils ($CD11b^+$ $Ly6G^+$) and (B) monocytes ($CD11b^+$ $Ly6C^+$) in blood; (C) B cells ($CD19^+$) and $CD8^+$ cells in spleen; (D) $CD4^+$ cells, effector T cells ($CD4^+$ $FoxP3^-$) and regulatory T cells ($CD4^+$ $FoxP3^-$) among splenocytes are shown. Numbers given in the corners of the figures represent the proportional frequencies in the different segments or subsets.

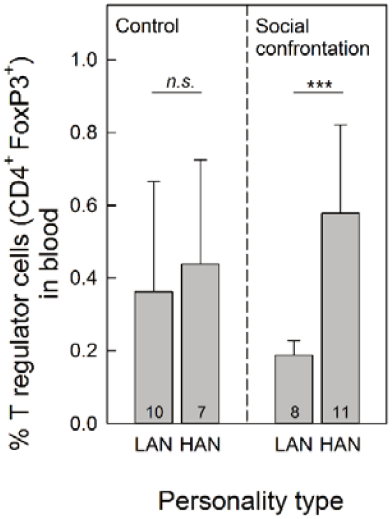

Fig. B. Percentages of $CD4^+$ $FoxP3^+$ regulatory T cells in blood. Mean values (+ SD) of individuals of the control and experimental group (daily social confrontation with changing resident pairs) with high (HAN) or low (LAN) anxiety / neophobia scores are given. Pair-wise post-hoc comparisons between animals with different personality types (although the interaction between treatment and personality type did not reach statistical significance with $p = 0.066$; see Table 1 for details) were calculated by Fisher Pitman permutation tests (Bonferroni-corrected: $^{**}p < 0.005$). Sample sizes are given in the bars.

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Similarity of personalities speeds up reproduction in pairs of a monogamous rodent

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Abstract Animal personality, i.e. stable individual differences in behaviour, is considered to be subject to evolutionary processes, as it has been shown to be heritable and to entail fitness consequences. Different hypotheses have been developed to explain why personality variation is maintained within populations, most likely via processes of balancing selection. One mechanism discussed is that combinations of similar personalities within breeding pairs could increase fitness. We investigated this purported mechanism by studying the association between personality combinations within nulliparous pairs and their onset of reproduction in a monogamous rodent, the mound-building mouse, *Mus spicilegus*. In seasonal iteroparous breeders, reproductive timing is relevant to fitness, as delayed onset of breeding potentially limits the number of breeding occasions and lowers the chance that offspring will start reproducing during the same season. Repeated standardized tests carried out prior to pairing revealed consistent individual differences in subjects' behavioural responses with respect to anxiety and exploratory activity, indicating the existence of personality types. Within-pair similarity in anxiety levels affected the chance of reproduction: pairs with similar anxiety scores had a higher probability of breeding, and were quicker to start, during the observation period, independently of the scores of both partners of the pair. We propose that this association between 'personality matching' and the onset of reproduction could have important life history consequences in this species and might be one of the mechanisms leading to the maintenance of personality variation within populations.

Keywords: assortative pairing, mound-building mouse, *Mus spicilegus*, onset of reproduction, personality matching

Introduction

Animals frequently show consistent individual differences in their behavioural responses across time and context; this phenomenon has been termed temperament, behavioural syndrome, coping style or animal personality (Wilson *et al.* 1994; Koolhaas *et al.* 1999; Bell 2007). To date, a growing body of evidence supports the existence of animal personality across a wide range of taxa, including vertebrates and invertebrates (Gosling & John 1999; Stamps & Groothuis 2010; Kralj-Fišer & Schuett 2014). Furthermore, there is convincing evidence that personality traits are heritable (van Oers *et al.* 2005; van Oers & Sinn 2011) and they have been shown to entail fitness consequences, e.g. by affecting survival, reproduction and longevity (Dingemanse *et al.* 2004; Smith & Blumstein 2008; Réale *et al.* 2009; Wilson *et al.* 2010; Bergeron *et al.* 2013; Rödel *et al.* 2015). Thus, animal personality can be assumed to be subject to natural selection (Dall *et al.* 2004; Sih *et al.* 2004; Réale *et al.* 2007).

The evolutionary mechanisms governing the emergence of this phenomenon, and in particular the processes leading to the generally high variability of personality types within species and populations, are still being debated (reviewed in: Bergmüller 2010; Kight *et al.* 2013). Persistent personality variation within populations may seem surprising at first sight, given that certain personalities, e.g. more aggressive and exploratory individuals, are frequently reported to have fitness advantages (cf. Smith & Blumstein 2008). Such types might then be expected to be favoured by natural selection, thus spreading in their population and reducing overall variability. Different processes of balancing selection are frequently proposed to be involved in maintaining variation in personalities. For example, it has been considered that negative frequency-dependent selection processes, i.e. fitness benefits of rare personalities, could favour the stable coexistence of different types (Dall *et al.* 2004; Wolf *et al.* 2008; Mathot *et al.* 2011). In addition, personality variation could be maintained by differential fitness advantages under heterogeneous environmental conditions (Dingemanse & Réale 2005; Cote *et al.* 2008). Balancing selection could also play a role when the direction of the fitness consequences of personality changes at different stages of an animal's life history (Nettle 2006). For example, a study in North American red squirrels *Tamiasciurus hudsonicus*, showed that the mother's personality was associated with offspring survival prior to weaning and in winter in a contrasting way (Boon *et al.* 2007).

Similar patterns of selection might also arise from fitness differences between certain combinations of personality types within breeding pairs (Schuett *et al.* 2010). This is, for example, supported by studies on great tits *Parus major*, in which pairs with similar personalities, either slow or fast explorers, produced offspring in better body condition and recruited more offspring than pairs

with other personality combinations (Dingemanse *et al.* 2004; Both *et al.* 2005). Further support for potentially fitness-increasing effects of similar (positive assortative) personalities comes from a study on zebra finches *Taeniopygia guttata*. Breeding pairs that were both highly exploratory and aggressive fostered chicks in better body condition than pairs of other behavioural combinations (Schuett *et al.* 2011a), while females actively seem to choose their partners on the basis of personality (Schuett *et al.* 2011b). In addition, a study in eastern bluebirds *Sialia sialis*, reported that parental pairs that behaved more similarly with respect to territorial aggression reared the heaviest offspring (Harris & Siefferman 2014). Apart from these studies in birds, the existence of a link between assortative personality and reproduction finds support by studies in fish (Budaev *et al.* 1999; Ariyomo & Watts 2013) and squid (Sinn *et al.* 2006).

However, to the best of our knowledge, no study has investigated such associations between similarities in personality types and parameters of reproduction in a mammal (Schuett *et al.* 2010). Furthermore, most studies reporting advantages of such 'personality matching' within breeding pairs show associations with offspring parameters (in birds: offspring growth and survival, Both *et al.* 2005; Schuett *et al.* 2011a; Harris & Siefferman 2014), which might have been mainly the result of optimized bi-parental care. However, the timing of reproduction, i.e. the probability of an early start to breeding, can also contribute importantly to parental fitness. This is especially relevant in seasonal environments, in which the number of annual breeding events and favourable conditions for offspring development are restricted by the length of the vegetation period, and thus a prompt onset of breeding can increase fitness (Eccard & Ylönen 2001; Rödel *et al.* 2009). This also applies to the mound-building mouse *Mus spicilegus*, which has a life expectancy of less than 1 year under field conditions. Almost exclusively juveniles survive the winter period, in nest chambers under specially constructed mounds (Garza *et al.* 1997; Poteaux *et al.* 2008; Szenczi *et al.* 2011). These overwintering individuals then start to reproduce in spring after dispersal from the mound and give birth to a few consecutive litters, with early born offspring having the potential to mature and to reproduce within the same season (Milishnikov *et al.* 1998; Gouat *et al.* 2003a).

To shed light on the adaptive significance of assortative personality in breeding pairs of this monogamous rodent (Baudoin *et al.* 2005), we studied the timing and probability of reproduction in pairing experiments of nulliparous mound-building mice under laboratory conditions. Prior to pairing, we attempted to quantify personality types with respect to the animals' consistent behavioural responses in two standardized tests that are frequently used in murine rodents (e.g. Lewejohann, *et al.* 2011; Rödel & Meyer 2011): open field, usually interpreted to indicate activity and exploration tendency, and elevated plus maze, reflecting anxiety-related behaviour (Archer 1973). In accordance with the results obtained in several other taxa, we predicted that pairs of this monogamous mammal

with a similar (i.e. positive assortative) personality would start to reproduce earlier or with a higher probability than pairs with more dissimilar combinations. Furthermore, we also considered the nonexclusive hypothesis that male or female personality might exert distinct effects on reproduction of the pair. For example, such effects have been shown in great tits, in which fledgling size was directly associated with the mother's exploratory behaviour (Both *et al.* 2005).

Methods

Study Animals and Housing Conditions

Our breeding stock were descendants (16th and 17th generation) of mound-building mice caught from the wild at different sites in Hungary in 1999. In addition, we attempted to maintain genetic variation of the breeding stock by regularly (every 2–4 years) adding some new individuals, captured at the same Hungarian collection sites. We kept the animals on a 14:10 h light / dark cycle (lights off at midnight) in standard polycarbonate cages (26 × 14 cm and 16 cm high, Iffa Credo, Lyon, France), containing wood shavings as bedding. Animals always had ad libitum access to rodent standard diet (Special Diets Services, Ext. M20, Witham, Essex, U.K.) and water. Temperature in the housing rooms was maintained at 21 ±0.5 °C, and relative humidity at approximately 50%. All animals, including the focal animals of our study, were transferred to clean cages around every second week but never within 10 days before behavioural tests. We always provided several cotton balls (diameter approximately 5 cm), which the animals (including juveniles) use for nest building in a corner of their cage.

This study consisted of two trials (hereafter referred to as Cohort 1 and Cohort 2), repeating the same experimental procedure with different animals in 2 years (Cohort 1 in 2012 and Cohort 2 in 2013). All focal animals stemmed from unculled litters born to adult virgin male–female breeding pairs (Cohort 1: 10 litters from 10 breeding pairs; Cohort 2: 12 litters from 12 breeding pairs). On postnatal day 14, four pups were randomly chosen from each litter (preferably two females and two males) as focal animals and individually marked with different symbols on their back using a black, permanent nontoxic hair dye (Nyanzol-D, Greenville Colorants, Jersey City, NJ, U.S.A.). For this, the animals were not removed from their home cage, but were held tight at the base of their tail (front paws remaining on the ground) by the experimenter with one hand, while the hair dye was applied to the subject's back with a small brush. This procedure took about 30 s per individual. At this time, we also determined their sex by external genital inspection. The remaining pups were not removed and thus all siblings remained together until weaning (separation from the mother) on postnatal day 28. For this, the four focal individuals per litter were then transferred together into a new cage.

Subjects were re-marked shortly after the first behavioural test (T_1, see below) in order to allow individual recognition within their sibling groups. On postnatal day 55, before reaching maturity (Busquet, Nizerolle & Féron 2009), all animals were transferred into separate cages.

The total number of focal animals used for the study was 82 (41 females and 41 males), with 36 (18 pairs) in Cohort 1 and 46 (23 pairs) in Cohort 2.

Ethics Note

Animals were kept and treated according to the ethical and animal care guidelines of France (where the project was carried out) and the institutional guidelines of animal welfare. Experimental procedures were approved by the local authority for laboratory animal care and use (Comité d'Ethique en Expérimentation Animale 'Charles Darwin'; authorization codes: Ce5/2011/068; Ce5/2012/212).

Subjects were kept singly during postnatal days 55–90. Being solitary is not an unusual situation in this species, as solitary females and males have been frequently found under natural conditions (Simeonovska-Nikolova 2012). Furthermore, the growth rates of the animals during the last 2 weeks before being separated from their siblings (mean ± SE =0.69 ± 0.19 g) did not differ significantly from those during the first 2 weeks of single housing (0.82 ± 0.15 g; paired *t* test: $t = 0.72$, $P = 0.47$), indicating that the animals did not suffer from obvious effects of separation stress. Furthermore, our recent experiments show that single housing of females and males prior to pairing (most probably resembling the natural situation of dispersal) increases their probability of successful reproduction (Féron & Gheusi 2003).

After the experiments, and after the offspring of the experimental pairs were weaned, adults were killed in accordance with French animal law. For this, they were first anaesthetized by putting them into a closed transparent plastic box filled with isoflurane gas at a concentration of 3% (IsoFlo, Axience, France), administered by an automatic system (Univentor 400 Anaesthesia Unit, Univentor Ltd, Zejtun, Malta). Then, anaesthetized individuals were killed by putting them into a closed transparent plastic box filled with a high dose of CO_2 gas (delivered by a compressed gas cylinder) for at least 5 min. They were observed until all muscle activity and other signs of life had been absent for at least 30 s. After removal, we carried out cervical dislocation in order to ensure death. This whole procedure was conducted by a qualified and experienced person. The offspring of the experimental animals were used for further behavioural experiments (not included in this paper).

Procedures and Experimental Settings

All procedures and experiments were carried out with two separate cohorts (Cohort 1 and 2), conducted by two different experimenters. We used the same experimental setting in both cohorts, except for slight differences in the apparatuses used for the battery tests, as outlined below. The animals of the different cohorts were kept in different housing rooms, but with the same conditions of temperature, humidity and illumination (see above).

Repeated Behavioural Tests

To assess individual personality types, we carried out repeated behavioural testing using an open field test and an elevated plus maze test. Two test batteries were done prior to the pairing with a potential mating partner (see below). The first test battery (T_1) was carried out on postnatal days 40 (open field) and 44 (elevated plus maze), when individuals were kept in sibling groups of four, and the second (T_2) on days 70 (open field) and 74 (elevated plus maze), when individuals were housed singly.

Experiments were always conducted between 1400 and 1600 hours during the animals' activity period (red light phase). Individuals were returned to their home cages immediately after each test, and we cleaned the apparatuses with water and detergent (Cleansinald, Johnson Diversey, Fontenay-sous-Bois, France) between trials.

Open field test

The open fields used for both cohorts were made of white polyethylene and consisted of a circular arena surrounded by walls. However, they differed slightly in size. The one used for experiments conducted with Cohort 1 had a diameter of 48 cm and the surrounding walls were 50 cm high. The one used for Cohort 2 had a diameter of 60 cm with surrounding walls of 69 cm.

Each animal was placed on a defined position at the edge of the arena, and its behaviour was recorded for 5 min by use of a digital video camera mounted 120 cm above the centre of the arena. For analysis, we defined a (circular) central zone of the arena with a diameter of 16 cm for Cohort 1 and 20 cm for Cohort 2. For both cohorts, this represented one-third of the total diameter of the open field. We quantified the total distance covered by the animal and the distance covered in the centre of the arena with Ethovision XT7 (Noldus Information Technology, Wageningen, The Netherlands).

Elevated Plus Maze

The apparatuses used for Cohort 1 and Cohort 2 were both made of rigid PVC consisting of four arms, 5 cm wide and 30 cm long, arranged at a 90° angle, and mounted 70 cm above the floor by a stable construction. Two opposite arms enclosed by 30 cm high walls and two open arms without walls were connected by a 10 × 10 cm central platform. This platform was surrounded by clear Plexiglas walls with round openings (diameter 5 cm) to all four directions. Preliminary tests had revealed that such a box around the platform helps to considerably reduce the chance that subjects (which were descendants of wild animals) would jump off the maze. During the experiments with Cohort 1, four of 18 individuals jumped off the apparatus during the first trial but none during the second trial and none during the experiments with Cohort 2. We did not exclude the data from animals that jumped off the maze for later analysis. The apparatuses used for the two cohorts differed slightly in the way the closed arms were constructed. For Cohort 1, the terminal ends of the closed arms were left open. In contrast, the closed arms of the elevated plus maze used for the experiments with Cohort 2 were surrounded by walls on three sides.

Each animal was placed on the central platform facing an open arm and also here its behaviour was recorded for 5 min by use of a digital video camera mounted 120 cm above the centre of the maze. The percentage time spent in the open area of the elevated plus maze and the frequencies of exits from the closed arm were quantified. These were defined as crossing the respective thresholds with more than 50% of the animal's body length. Both measurements have been shown to reflect anxiety-related behaviour in laboratory rodents (Pellow *et al.* 1985; Walf & Frye 2007).

Behavioural tests using an open field as well as an elevated plus maze of similar size and construction have been previously and successfully used in the mound-building mouse to describe its age-related dynamics in risk-taking behaviour (Lafaille & Féron 2014).

Formation of Pairs and Survey of Reproduction

When subjects were about 95 days old (average age ±SE: females: 94.9 ± 1.5 days; males: 94.9 ± 1.3 days), we formed the pairs (18 in Cohort 1; 23 in Cohort 2) and housed them in standard polycarbonate laboratory mouse cages (26 × 14 cm and 16 cm high). Note that this species usually matures prior to an age of 80 days (Busquet *et al.* 2009). Before pairing, animals were weighed to the nearest 0.01 g. We checked the pedigree of each individual and verified that partners stemmed from different direct kin lines for at least two generations. Apart from this condition, partners were randomly chosen.

Experimental pairing of an unfamiliar female and male in this species is usually characterized by an initial increase in chasing behaviour (Patris *et al.* 2002). However, these initial chasing events typically decrease in frequency and appearance within the first 30 min. During this time, we always monitored the new pairs in order to terminate the pairing if the aggression did not stop, but this never occurred during our experiments.

Females were then weighed once per week in order to detect pregnancies. In total, pairs were checked for reproduction during a period of 90 days; this duration corresponds to more than three times the gestation period of this species (20-28 days; Féron & Gouat 2007). Starting at 20 days after pairing, we checked the cages for new litters on a daily basis. In addition to the date of birth of each litter, we noted the litter size. We also weighed the pups on postnatal day 12 (Cohort 1) or day 14 (Cohort 2) in order to obtain a measure of offspring development.

Statistical Analyses

All statistical analyses were done using the program R, version 3.1.1 (R Core Team 2014). Data obtained from experiments with Cohort 1 and Cohort 2 were analysed separately, as they were conducted by different experimenters and there were slight differences in the setting (size of open field arena, construction of elevated plus maze, see above).

Calculation of Personality Scores

To capture the information of the different behavioural responses per test (open field, elevated plus maze) we calculated scores (exploration scores based on open field responses; anxiety scores based on elevated plus maze responses) using the scaled first component as provided by a principal component analysis, PCA (R function: prcomp). This was done separately for T_1 and for T_2, and for Cohorts 1 and 2. These new variables were used to test for repeatability across time. For further analysis (see below), we then averaged the scores over both time steps. In all cases, the first component captured more than 51% (up to 88%) of the variability of the behavioural responses considered (Appendix Table A1 with the explained variations of all first principal components). Furthermore, in all cases the first component had an eigenvalue > 1, indicating that this component accounted for more variance than any of the original variables of the standardized data.

The scores of males and females were compared by linear mixed-effects models LMM, using litter identity as a random factor. This was done with the R package lme4 (Bates *et al.* 2014). *P* values were extracted by Wald chi-square tests (type III).

Repeatability of Behavioural Responses

Repeatability, i.e. the intraclass correlation calculated as the proportion of phenotypic variation that can be attributed to between-subject variation (Lessells & Boag 1987), was calculated for both scores (anxiety, exploration), separately for Cohorts 1 and 2. We used LMM-based calculations of repeatability with the R package rptR (Nakagawa & Schielzeth 2010), and we assessed 95% confidence intervals by 1000 bootstrap steps. Individual identity was used as a random factor. *P* values were calculated by 1000 permutations.

Within-pair Similarity and Reproductive Timing

We tested the effects of sets of different predictor variables on the timing of reproduction of the different breeding pairs using multiple Cox proportional hazard regression (R package: survival), allowing for the analysis of censored data (Therneau & Grambsch 2000). This kind of model tests for the combined timing and probability of occurrence of an event. The latency until pairs had their first litter after pairing and the probability of reproduction, censored after an observation period of 90 days, were used as response variables. Predictor variables were based on the exploration scores and anxiety scores as obtained by PCA (see above).

For each of the two variables, we used the absolute difference between the scores of the male and the female within each pair as a measure of similarity of personality types, calculated as:

Similarity index = ($|male_{pair} – female_{pair}|$).

Low values indicate high similarity and high values indicate low similarity within pairs. The distributions of values for similarity indices calculated for both traits and for both experiments and cohorts are given in Appendix Fig. A1. We also calculated models separately based on the scores of males and of females in order to test for distinct ('direct') effects of the personality type of each partner (cf. Both *et al.* 2005; Schuett *et al.* 2011a). In addition, we considered additive effects of female body mass (covariate) measured on the day of pairing in combination with all other predictor variables as mentioned above. This was done in order to adjust for potential effects of female body condition on reproductive timing and performance (Millesi *et al.* 1999; Rödel *et al.* 2005). We also considered models in which we concurrently included the within-pair similarity of exploration and of anxiety scores (Appendix Table A2).

Information Theory-based Model Selection

For model selection, we constructed sets of 16 candidate models (including a 'null' model, which did not include any slope parameter), separately for each of the two cohorts, where we considered the

above-mentioned combinations of the predictor variables (Burnham & Anderson 2002). The full sets of models are given in Appendix Table A2.

The aim of the AIC-based, information-theoretical approach that we used is to identify the most parsimonious model, i.e. the model of the set considered that represents the data adequately with the smallest possible number of parameters. We used the second-order AIC, the AICc, which includes a correction term for small sample sizes. All models of the set were ordered from 'best' (lowest AICc) to 'worst' (highest AICc). We report AICc differences ($\Delta AICc = AICc_i - AICc_{minimum}$) to compare the support that the different models had for best approximating the data. Burnham and Anderson (2002) suggested that models with $\Delta AIC_i \leq 2$ can be considered to have substantial support, and models with ΔAIC_i of about 4–7 have considerably less support. We also calculated normalized Akaike weights (w_i) for each model, which can be interpreted as a measure of the evidence in favour of model *i* as being the best approximating model of the set (Burnham & Anderson 2002). In addition, *P* values were calculated for the best approximating model of the set using a likelihood ratio test.

Table 1. Set of models (Cox proportional hazards regression) explaining the timing / probability of reproduction (*R*) by means of the birth of a first litter in pairs of nulliparous mound-building mice.

	Model terms	*K*	$\Delta AICc_i$	w_i
Cohort 1	R[Anxiety$_{similarity}$]	2	0	0.266
	R[Body mass$_{female}$]	2	1.57	0.121
	R[Anxiety$_{similarity}$+Body mass$_{female}$]	3	1.80	0.108
	R[Exploration$_{female}$+Body mass$_{female}$]	3	2.01	0.097
	R[Anxiety$_{male}$+Body mass$_{female}$]	3	2.35	0.082
	R[Anxiety$_{similarity}$+Exploration$_{similarity}$+Body mass$_{female}$]	4	2.71	0.069
	R[Anxiety$_{similarity}$+Exploration$_{similarity}$]	3	2.93	0.061
	R[Exploration$_{female}$+Body mass$_{female}$]	3	3.06	0.058
Cohort 2	R[Anxiety$_{similarity}$]	2	0	0.426
	R[Anxiety$_{similarity}$+Body mass$_{female}$]	3	2.47	0.124
	R[Null model]	1	3.27	0.083
	R[Anxiety$_{similarity}$+Exploration$_{similarity}$+Body mass$_{female}$]	4	3.56	0.072

The models are ordered by ΔAICc; only models with ΔAICc < 4 are denoted. The onset of reproduction was monitored for 90 days after pairing (censored data). Predictor variables considered are the similarity (calculated as the absolute score difference between males and females) in anxiety or exploration scores within each pair. Furthermore, distinct effects of score values of males and of females were considered. In addition, female body mass prior to pairing was included in the construction of the models. The number of estimable parameters (*K*), ΔAICc and Akaike weights (*w*) is given. Analyses are based on data from two cohorts (Cohort 1: *N* = 18 pairs; Cohort 2: *N* = 23 pairs).

Model Diagnostics and Graphical Presentation

For model diagnostics, we verified the assumption of proportional hazards by visually checking plots of Schoenfeld residuals versus the transformed time. In addition, we ruled out the existence of

potential nonlinear effects by plotting the Martingale residuals of the models against the different covariates (Fox 2002). There was a certain degree of collinearity between the different anxiety scores and exploration scores (Cohort 1: $F_{1,34} = 1.95$, $R^2 = 0.054$, $P = 0.17$; Cohort 2: $F_{1,44} = 6.42$, $R^2 = 0.127$, $P = 0.015$); however, the level of explained variation (R^2) of these correlations was not particularly high. We calculated variance inflation coefficients (VIF) in order to check for (multi)collinearities among predictor variables (Zuur *et al.* 2010) for all models of the set including more than one predictor variable. VIF were always lower than 1.5, indicating no notable interfering effects of multicollinearities.

For graphical presentation only (see Results), the covariate $\text{Anxiety}_{\text{similarity}}$ (Table 1) was split into three levels, with the two quartiles [0–25% of the data] and [75–100% of the data] representing the groups with high and low similarity, and 25–75% of the data representing the level with intermediate similarity. Note that the effects of within-pair similarity in anxiety scores on reproduction were still statistically significant after the transformation of this predictor variable into three categories (see *P* statistics for Cohorts 1 and 2 in the Results).

Results

Repeatability across Time

Elevated Plus Maze

With respect to data obtained from Cohort 1 as well as from Cohort 2, the PCA revealed similar results during both time steps. Higher scores of the first components were always positively associated with more signs of anxiety, such as a lower percentage time spent in the open arms and fewer exits from the closed arms (Appendix Table A3). These scores did not differ between females and males during any of the time steps in Cohort 1 or 2 (LMM: all $P > 0.10$). Anxiety scores were repeatable across time with respect to Cohort 1 ($R = 0.648$, 95%CI = [0.389, 0.813], $P < 0.001$) and Cohort 2 ($R = 0.519$, 95%CI = [0.286, 0.697], $P = 0.001$; Fig. 1a, c).

Open Field Test

Animals with higher scores, representing the first components of PCAs for the two time steps of Cohorts 1 and 2, showed more signs of exploratory activity, covering a greater total distance and a greater distance in the central zone of the arena. Also here, scores did not differ between females and males during any of the time steps in Cohort 1 or 2 (LMM: all $P > 0.10$). Exploration scores were also repeatable across time in Cohort 1 ($R = 0.510$, 95%CI = [0.225, 0.712], $P = 0.001$) and Cohort 2 ($R = 0.387$, 95%CI = [0.118, 0.606], $P = 0.004$; Fig. 1b, d).

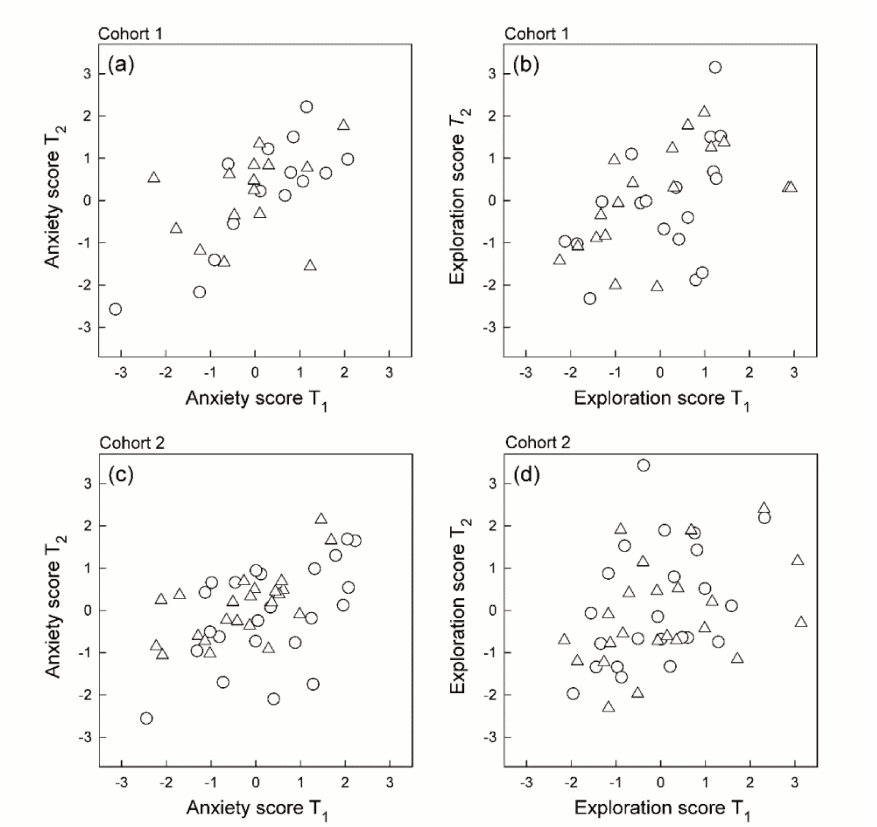

Fig. 1. Consistencies across time in (a, c) anxiety scores (measured in an elevated plus maze) and (b, d) exploration scores (measured in an open field test) of female (circles) and male (triangles) mound-building mice at two times of testing (T_1, T_2). Data from two cohorts are presented. (a, b) Cohort 1: $N_{individuals}$ = 36. (c, d) Cohort 2: $N_{individuals}$ = 46. Higher scores indicate more pronounced anxiety-related or exploratory behaviours during the tests, respectively. All behaviours were significantly repeatable based on the proportion of phenotypic variation attributed to between-subject variation. There were no significant sex-specific differences. See text for details on statistics.

Personality Matching and Onset of Reproduction

Of 18 pairs, 14 (77.8%) started to reproduce in Cohort 1, and of 23 pairs 10 (43.5%) started to reproduce in Cohort 2, within 90 days after pairing. The analysis of the probability of starting to reproduce, taking into account the timing of reproduction by a Cox proportional hazards survival model, revealed highly similar results in both cohorts, despite the variation in reproductively active pairs between them:

The start of reproduction was best explained, by means of the lowest AICc, by the similarity in anxiety scores within pairs (Table 1). According to this model, pairs with more similar anxiety levels (covariate) reproduced significantly earlier / with a higher probability than more dissimilar ones (Cohort 1: χ_1^2 = 7.30, R^2 = 0.342, P = 0.007; Cohort 2: χ_1^2 = 5.68, R^2 = 0.238, P = 0.017). Results were also significant when we split the covariate into three categories (χ_1^2 = 7.05, P = 0.008; χ_1^2 = 4.49, P = 0.034; Fig. 2a, b).

Models considering similarities in exploration score, or the individual scores of either the male or the female partner in anxiety or exploration, did not find notable support by the data, in either Cohort 1 or 2 (Table 1). In both model sets, and in particular in Cohort 1, the effects of the initial female body mass also found some support by the data (ΔAICc < 2; χ_1^2 = 5.73, *P* = 0.016). This result revealed that heavier females tended to reproduce earlier / with a higher probability than lighter ones.

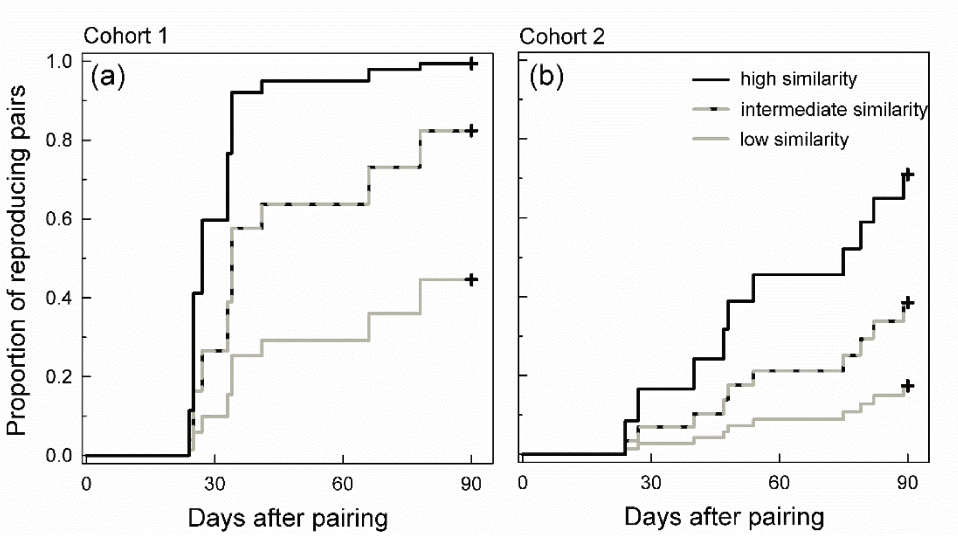

Fig. 2. Kaplan-Meier survival curves for (a) Cohort 1 and (b) Cohort 2 showing the evolution of the proportion of reproducing pairs of mound-building mice, for groups with high, medium and low within-pair similarities in anxiety scores (see text for details of calculation). Note that for statistical analyses (Table 1) the original data on similarities in anxiety scores (covariates) were used; however, analyses based on categorized data were also statistically significant (see text). Day 0 indicates the day of pairing; the census of reproductive activity of each pair was terminated at day 90.

Personality Traits, Litter Size and Pup Body Mass

For pairs that started to reproduce, we tested for correlations (Spearman rank due to moderate sample sizes) of parental temperamental scores and the within-pair similarity of these scores (anxiety and exploration, respectively) with litter size (mean ± SD: 6.4 ± 1.2) or with the averaged individual pup body masses (Cohort 1: mean body mass on postnatal day 12 ±SD: 4.1 ± 0.5 g; Cohort 2: mean body mass on postnatal day 14 ± SD: 5.1 ± 0.7 g).

There were no significant bivariate correlations between these variables (all *P* > 0.10). That is, based on our moderate sample sizes, there was no evidence for an association between either similarities in temperamental traits or parental temperamental traits and litter size or offspring body mass.

Discussion

Behavioural tests revealed consistent individual differences in exploratory activity and anxiety-related responses across time, indicating the existence of distinct personality types in the mound-building mouse. Most importantly, our results suggest fitness-related benefits in pairs with assortative personalities with respect to anxiety, showing a higher probability of starting to reproduce than more dissimilar pairs. These findings were based on coherent results obtained in independent experiments with two cohorts of animals.

To our knowledge, this is the first study reporting effects of personality matching on reproductive activity in a mammal. Our report supports the results obtained in some other taxa. A study in convict cichlids *Amatitlania nigrofasciata*, after spawning revealed a positive correlation in freezing behaviour between partners of reproducing pairs but no such similarity in nonreproductive ones (Budaev *et al.* 1999). Further evidence comes from a recent study in guppies *Poecilia reticulata*, in which females that mated with males with a more similar boldness level had higher parturition success than females with more dissimilar mating partners (Ariyomo & Watts 2013). In a study on dumpling squid *Euprymna tasmanica*, the eggs of bold and intermediate females were predominantly fertilized by males having similar phenotypes; however, reproduction of shy females was not associated with male personality (Sinn *et al.* 2006). Studies on birds, reporting a link between personality matching and offspring body condition or recruitment rates, also showed that male and female behavioural types per se had distinct effects on reproductive parameters such as chick body mass (Schuett *et al.* 2011a), nest survival and the choice of high-quality nestboxes (Both *et al.* 2005). Such independent effects of components of parental personality were not supported by our study. Furthermore, we did not find significant correlations between parental personality matching and parameters of offspring condition or development (i.e. pup body mass). However, conclusions based on this nonsignificant result are difficult to draw as the sample size available for this analysis was rather low. Furthermore, keeping animals under laboratory conditions, and thus protecting them from environmental constraints such as food restriction, might significantly hamper the detection of potential maternal / parental effects on offspring development (Ung *et al.* 2014; Lafaille *et al.* 2015).

Although the percentage of reproductively active pairs differed strongly between the two cohorts (77.8% versus 43.5% of reproducing pairs), we obtained similar results providing evidence for a link between personality matching and the onset / probability of reproduction. In addition, our study emphasizes that potential differences in conditions such as handling (e.g. due to different experimenters) or housing (e.g. different housing rooms) between consecutive trials might strongly

affect the performance of animals kept under supposedly standardized laboratory conditions (see also Richter *et al.* (2011) for variation in behavioural responses to standardized tests among different laboratories).

Different mechanisms might underlie the observed association between within-pair personality matching and the timing / probability of reproduction. In general, assortative mating with respect to different phenotypic traits is not uncommon. A meta-analysis across published data from 254 species showed an overall tendency towards positive assortative mating within populations (Jiang *et al.* 2013). It could be argued that the results of our experiment may in fact reflect mate choice decisions (Schuett *et al.* 2011a; Kralj-Fišer *et al.* 2013). Individuals might adaptively choose partners with a similar behavioural type, as it could improve the coordination of behaviour between partners as compared to non-assortative pairs (Schuett *et al.* 2010). This could be particularly relevant to fitness in species with bi-parental care, such as the mound-building mouse, in which high levels of cooperation between both partners (Patris & Baudoin 2000) could help optimize the development and survival of the young.

On the proximate level, the observed association between personality matching and reproduction might be affected by the quality of the pair relationship. Putting two potential mating partners together does not inevitably lead to the formation of a pair bond (von Holst 1998). We propose that combinations of dissimilar personalities could have resulted in more unstable and disharmonious conditions. For example, disharmonious pairs of monogamous tree shrews *Tupaia belangeri*, as characterized by a comparatively lower degree of positive social interactions and occasionally increased within-pair aggression, show escalated stress hormone concentrations and increased heart rates in comparison to animals in harmonious pairings (von Holst 1998). Generally, chronically increased levels of stress might exert suppressive effects on reproductive function, such as decreases in fertility or higher rates of fetal resorption during early stages of pregnancy, all leading to a lower probability of reproduction (von Holst 1998; Sapolsky *et al.* 2000). In the mound-building mouse, pairwise encounters of unfamiliar adult males and females are frequently characterized by agonistic interactions, initiated by the refusal and defensive behaviour of the female towards approaches of the male (Patris *et al.* 2002). Enduring and frequent aggressive encounters in unstable pairings could entail negative stress effects, which may play a role in suppressing reproduction. However, studies in this species point out that an initial increase in agonistic interactions between partners of unfamiliar pairings is essential in triggering the onset of reproduction (Busquet *et al.* 2009). There is need for further studies exploring to what extent personality differences are involved in mate choice decisions in this monogamous species.

Conclusion

Our findings highlight the mound-building mouse as another example of a mammal providing indications for the existence of personality by means of consistent individual differences in behaviour across time. But most importantly, this study demonstrates for the first time in a monogamous mammal that personality matching provides potential fitness benefits to the partners of a pair by increasing the probability of reproduction. A rapid onset of breeding is clearly an important component of individual fitness in short-lived species. In particular in seasonally breeding iteroparous small mammals, delays in the onset of reproductive activity can limit the number of litters produced per season (Eccard & Ylönen 2001). Furthermore, offspring born earlier during the vegetation period potentially have a higher chance of starting to reproduce during the same season, with positive implications for their parents' inclusive fitness. Assuming there is a genetic basis for anxiety/emotionality (cf. Willis-Owen & Flint 2006), we propose that the observed advantage of positive assortative pairing with respect to this trait could contribute to maintaining personality variation within populations. Thus the results of this study could add to the understanding of the evolution of personality in monogamous mammals.

Acknowledgments

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Electronic Appendix

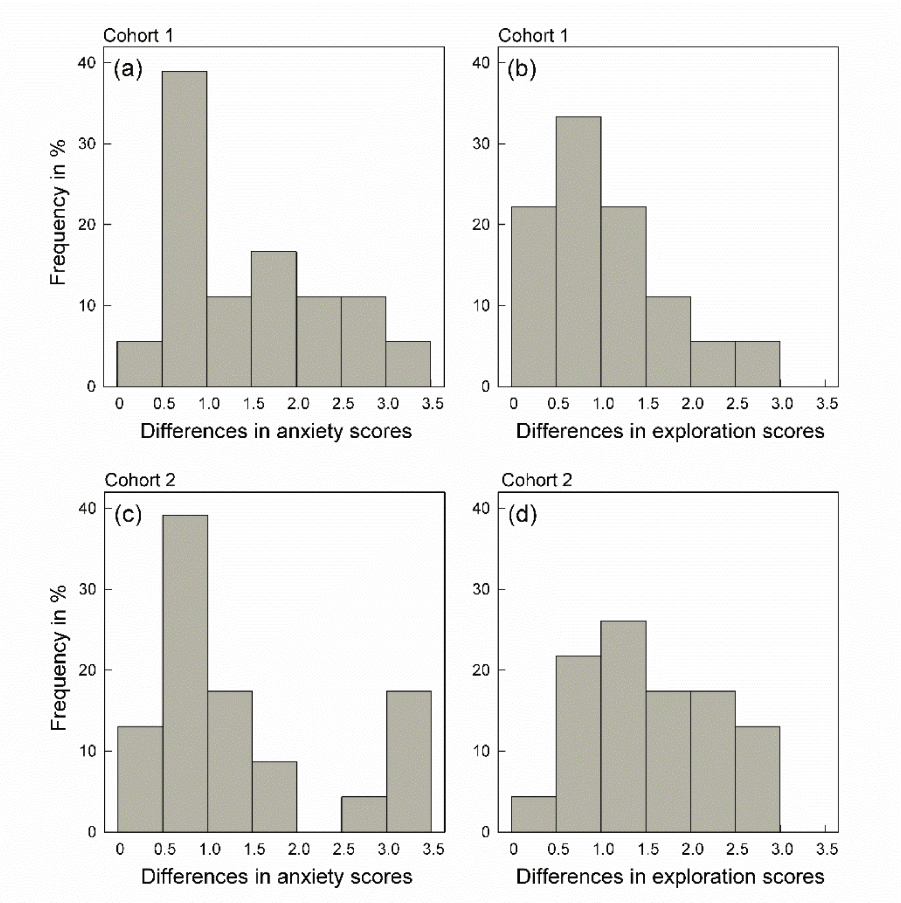

Fig. A1. Distributions of within-pair differences in (a, c) anxiety and (b, d) exploration scores calculated as the absolute score differences between male and female. Data were obtained by experiments with two cohorts. (a, b) Cohort 1: *N* = 18 pairs. (c, d) Cohort 2: *N* = 23 pairs. Values of 0 denote maximum similarity in scores between the partners of a pair.

Table A1. Explained variation of the first component of the PCA

	Cohort 1		Cohort 2	
	Time 1	Time 2	Time 1	Time 2
Elevated plus maze	0.743	0.773	0.743	0.509
Open field	0.877	0.838	0.817	0.857

PCA was based on measures taken during the elevated plus maze tests and open field tests. See input variables in Table A3.

Table A2. Set of models (Cox proportional hazards regression) explaining the timing / probability of reproduction (*R*) by means of the birth of a first litter in pairs of nulliparous mound-building mice.

	Model terms	*K*	$\Delta AICc_i$	w_i
Cohort 1	R[Anxiety$_{similarity}$]	2	0	0.266
	R[Body mass$_{female}$]	2	1.57	0.121
	R[Anxiety$_{similarity}$+Body mass$_{female}$]	3	1.80	0.108
	R[Exploration$_{female}$+Body mass$_{female}$]	3	2.01	0.097
	R[Anxiety$_{male}$+Body mass$_{female}$]	3	2.35	0.082
	R[Anxiety$_{similarity}$+Exploration$_{similarity}$+Body mass$_{female}$]	4	2.71	0.069
	R[Anxiety$_{similarity}$+Exploration$_{similarity}$]	3	2.93	0.061
	R[Exploration$_{similarity}$+Body mass$_{female}$]	3	3.06	0.058
	R[Exploration$_{male}$+Body mass$_{female}$]	3	4.21	0.032
	R[Anxiety$_{female}$+Body mass$_{female}$]	3	4.22	0.032
	R[Null model]	1	4.89	0.023
	R[Exploration$_{male}$]	2	6.01	0.013
	R[Exploration$_{similarity}$]	2	6.24	0.012
	R[Anxiety$_{female}$]	2	6.44	0.011
	R[Exploration$_{female}$]	2	6.99	0.008
	R[Anxiety$_{male}$]	2	7.30	0.007
Cohort 2	R[Anxiety$_{similarity}$]	2	0	0.426
	R[Anxiety$_{similarity}$+Body mass$_{female}$]	3	2.47	0.124
	R[Null model]	1	3.27	0.083
	R[Anxiety$_{similarity}$+Exploration$_{similarity}$+Body mass$_{female}$]	4	3.56	0.072
	R[Exploration$_{male}$]	2	4.36	0.048
	R[Anxiety$_{similarity}$+Exploration$_{similarity}$]	3	4.36	0.048
	R[Anxiety$_{male}$]	2	4.85	0.038
	R[Exploration$_{similarity}$]	2	5.25	0.031
	R[Body mass$_{female}$]	2	5.44	0.028
	R[Exploration$_{female}$]	2	5.58	0.026
	R[Anxiety$_{female}$]	2	5.65	0.025
	R[Exploration$_{male}$+Body mass$_{female}$]	3	6.79	0.014
	R[Exploration$_{similarity}$+Body mass$_{female}$]	3	7.26	0.011
	R[Anxiety$_{male}$+Body mass$_{female}$]	3	7.50	0.010
	R[Exploration$_{female}$+Body mass$_{female}$]	3	8.10	0.007
	R[Anxiety$_{female}$+Body mass$_{female}$]	3	8.11	0.007

The models are ordered by ΔAICc; all models of the sets are denoted. The onset of reproduction was monitored during 90 days after pairing (censored data). Predictor variables considered are the similarity (calculated as the absolute score difference between males and females) in anxiety or exploration scores within each pair. Furthermore, distinct effects of score values of males and of females were considered. In addition, female body mass prior to pairing was included in the construction of the models. The number of estimable parameters (*K*), ΔAICc, and Akaike weights (*w*) is given. Analyses are based on data from two cohorts (Cohort 1: *N* = 18 pairs; Cohort 2: *N* = 23 pairs).

Table A3. Factor loadings of the first components of the PCA.

		Cohort 1		Cohort 2	
	Input variables	Time 1	Time 2	Time 1	Time 2
Elevated plus maze	% Time spent in open arms	–0.707	–0.707	–0.707	–0.707
	Exits from the closed arms	–0.707	–0.707	–0.707	–0.707
Open field	Total distance covered	+0.707	+0.707	+0.707	+0.707
	Distance in centre	+0.707	+0.707	+0.707	+0.707

PCA was based on measures taken during the elevated plus maze tests and open field tests.

3. General Discussion

The foremost goal of this thesis was to elucidate the emergence and the ontogeny of individual differences in behaviour. Studies on the stability of behaviour during early life bring contradictory results depending on the age of the individuals and the traits investigated. Overall, we found that behavioural responses measured in social and non-social contexts during pre-weaning, post weaning and around maturity were consistent across time and across various contexts, highlighting the existence of personality and behavioural syndromes in our study species, even early in life. Environmental social effects shaped personality, as paternal absence resulted in consistent behavioural differences in the offspring later in life, apparent by behaviours reflecting a higher responsiveness in such individuals, although, we did not find support that care behaviour *per se* was the wire mechanism. Moreover, we found personality-associated differences in changes in stress hormones and in immune parameters after chronic social stress, an indication of physiological mechanisms underlying personality variation. In addition, we demonstrated potentially fitness-increasing effects of similar (positive assortative) personalities within breeding pairs, as such pairs had a higher probability to reproduce when compared to other personality combinations.

3.1. How do environmental (non-genetic) factors during early life affect personality later in life?

3.1.1. Parental effects

One aspect of this thesis was to assess whether and how care behaviour displayed by the parents affected personality. Usually, studies in mammals focus on maternal-offspring interactions, mostly because mammalian species are predominantly engaged in uniparental care. In bi-parental species, the absence of the father can affect offspring survival and growth (Wynne-Edwards 1987). Moreover, paternal absence has been reported to potentially affect the offspring behavioural phenotype (review in: Braun & Champagne 2014; Bales & Saltzman 2015) and cognitive development (Bredy *et al.* 2004). For instance, in mandarin voles *Microtus mandarinus* and in prairie voles *Microtus ochrogaster* the absence of the father results in an increase of anxiety-related behaviours and less positive social contacts in the offspring (McGraw & Young 2010; Cao *et al.* 2014). Our results are consistent with such an alteration of the offspring phenotype, at least with respect to behaviours reflecting the responsiveness of the young as exemplified by their latencies to sniff and approach novel objects and to jump from a platform (manuscript I). Paternal absence alters the hormonal profile, and results for instance in an increase in adrenocorticotropic hormone and corticosterone

(Wang *et al.* 2014). Moreover, the absence of the father has been associated with changes in the synaptic composition (Becker *et al.* 2005; Pinkernelle *et al.* 2009), time-specific changes in the dendritic composition (Helmeke *et al.* 2009; Pinkernelle *et al.* 2009) and in neuronal alterations (Jia *et al.* 2011; Cao *et al.* 2014; Wang *et al.* 2014). For instance, in the California mouse *Peromyscus californicus*, a species that shares a similar life history with the mound-building mouse, the absence of the father resulted in disturbances in cortical dopamine and glutamate neurotransmission (Bambico *et al.* 2013). Summarizing, these previous studies suggest the father as an important modulator of the offsprings' behavioural, neuronal and hormonal phenotype.

However, the mechanisms underlying such father effects are actually far from being clear. Indeed, epigenetic inheritance, indirect influence and direct care behaviour through the mother-offspring interactions can play a role (Braun & Champagne 2014). For instance, a study in laboratory mice (CD1) showed that the exposure of the father to a stressor can lead to intergenerational effects by altering offspring as well as grand-offspring behaviour (Saavedra-Rodriguez & Feig 2013). In addition, it has been demonstrated that genetic factors might partly explain between-litter differences in the quantity of care devoted by the parents (Royle *et al.* 2012). Father effects might also affect the offspring via other more indirect pathways. In humans, fathers are usually considered to provide socio-emotional support to the mother (Weinraub & Wolf 1983), potentially improving mother's mood and consequently, the relationship between the mother and their children (Braun & Champagne 2014). In the degu *Octodon degus*, the absence of the father has been shown to reduce maternal care while its presence stimulated parent-offspring contact (Helmeke *et al.* 2009; Pinkernelle *et al.* 2009). Our results did not support such findings, as single mothers and mothers in the presence of the fathers displayed the same quantity of care behaviour (34.1% and 33.9%, manuscript I), which is in accordance with studies in California mouse (Bambico et al. 2013), in mandarin vole *Microtus mandarinus* (Jia *et al.* 2009) and in prairie vole *Microtus ochrogaster* (Ahern *et al.* 2011). Another potential indirect influence might occur through mate choice quality for instance (Burley 1988; Gowaty *et al.* 2007) and females are suggested to increase maternal care when they mate with a high-quality male (Burley 1988; Braun & Champagne 2014). It remains to assess such indirect influence in our model species.

However, the major pathway underlying paternal effects has been proposed to be paternal care, although it has not been tested directly. Such a mechanism appears highly plausible, as paternal behaviour can include all care displayed by the female except lactation (Dewsbury 1985; Oliveras & Novak 1986). For instance, in the degu, paternal care represents 37% of the total parent-offspring interaction (Pinkernelle *et al.* 2009). Such a relatively high contribution was also apparent in the mound-building mouse, as fathers spent on average 32% of their time taking care of their offspring

(manuscript I). However, our findings suggested that differences in behaviour between absent-father pups and pups raised in the presence of the father were not triggered by differences in care behaviour, as there were no such significant correlations within the bi-parental groups, and data even suggested an association in the opposite direction (manuscript I).

Absence of the father not only results in a decrease in care behaviour but can also exert a different environment to the mother (Gubernick & Alberts 1987; Gubernick & Alberts 1989). We suggest that such effects on the mother, caused by the removal of the father, might have also occurred in our study (manuscript I). Mound-building mice display permanent bonds with one partner (Patris & Baudoin 1998; Auffray & Britton-Davidian 2012) and partner removal might lead to a stressful situation for the mother. Moreover, as we removed the father one day before the birth of the offspring, females did not have the opportunity to mate with the male after giving birth. Indeed, mother which were still together with the male after parturition could be rapidly pregnant again due to the post-partum oestrus in this species (Patris & Baudoin 1998). Thus, single mothers were likely to differ in their pregnancy status compared to mothers in which males were present in the home cage. Such modification in maternal state is likely to be transferred to the pups, for example through differences in maternal care behaviour, or through the transfer of hormones and nutrients via the placenta and via the milk mother provide to the pups (Nowak *et al.* 2000; Kaiser & Sachser 2005; Götz & Stefanski 2007). Further studies are needed to increase our understanding on how changes in the environment of the mother, induced by the removal of the father, could shape offspring personality.

3.1.2. Sibling effects

Except parents, other factors in the early social environment can modulate offspring personality. In many polytocous mammalian species, dependent young spend a huge proportion of their time in contact with their siblings compared to their mother, and sibling effects are known to greatly shape the individual phenotype of the animals both behaviourally and physiologically (Hudson *et al.* 2011). Our results showed individual differences within a litter in all personality traits investigated (manuscripts II & III), thus suggesting that early differences in the environment of each pup within a litter might trigger different behavioural trajectories. During the early post-natal period, competition for maternal resources can originate individual differences. For instance, offspring from larger litters receive less milk. This results in a reduction of postnatal growth and in a slower physiological development, with the potential to affect an individual's behavioural performance (Rödel *et al.* 2008; Bautista *et al.* 2010). For example, lighter laboratory rat and domestic rabbit *Oryctolagus cuniculus* pups showed immediate and long-term differences in their motor skills, and in their behavioural style

(Rödel & von Holst 2009; Bautista *et al.* 2010; Reyes-Meza *et al.* 2011; Rödel & Monclús 2011) compared to heavier ones. Such lighter pups performed a higher amount of behaviour directed to reach the central, thermally more advantageous position in the litter huddle, probably caused by a greater effort to reach thermally favourable central positions (Bautista et al. 2010). Personality differences within the litter were not caused by variation in body masses in our study, maybe due to the fact that the mound-building mouse has, on average, a relatively small litter size (± 6.5 pups / litter; manuscript V).

It is necessary to understand the causal effects of the variation in personality within a litter. To this end, it would be interesting to characterize individual differences in huddling behaviour to investigate whether and how the individual position in the litter huddle is associated with personality differences. In addition, interaction with conspecifics early in life might affect personality. In humans for instance, differences in sociability early in life explained 6–26% of the variance in sibling personality scores measured during adolescence (Daniels 1986), where more sociable siblings during infancy exhibited more closeness behaviour during adolescence. In great tits, *Parus major*, sibling competition between chicks within a litter affected personality traits (Carere *et al.* 2005). It is likely that differences in the frequency or in the quality of the interactions among siblings in the mound building mouse might also trigger variation in in their personality.

Sibling and parental effects on personality are not mutually exclusive and a close interaction between both pathways is likely to occur. Indeed, studies in laboratory rats have shown that the quantity in maternal care behaviour is not necessarily evenly distributed but can be strongly skewed towards particular individuals within a litter (Cavigelli *et al.* 2010; Ragan *et al.* 2012). This could then lead to patterns of contest competition between litter siblings.

3.2. How does personality develop over ontogeny?

One of the difficulties in the study of personality in early life comes from the need to find appropriate and meaningful tests in young animals (Réale *et al.* 2007; Stamps & Groothuis 2010; Raihani *et al.* 2014). We proposed in this thesis that social separation from the mother and littermates can be an especially useful test for young animals (manuscript II), as it triggers separation calls. The frequencies (number) of these calls are usually considered to reflect the emotional state of the animals (Jürgens 2009; Briefer 2012). Domestic kittens and mound-building mouse pups showed consistencies across time in distress calls and also in locomotor activity in response to separation, which fulfil the criteria of animal personality (manuscript II). Tested early in life (from postnatal day 3 in cats and day 13 in

the mound-building mouse), social separation tests open up the opportunity to assess long-term consistencies in behaviour over ontogeny. For instance, domestic cats display separation calls from early life into adulthood. Therefore, this variable seems especially useful to be integrated into personality research, and more broadly in any study where the emotional state needs to be elucidated. In the mound-building mouse, as in many other rodent species, separation calls are emitted by the young during a relative short window during the development (between postnatal days 11 to day 16, depending on the species; Branchi *et al.* 1998, Wiaderkiewicz *et al.* 2013). Thus, to investigate long-term consistencies in behaviour, we tested consistencies across context between variation in distress calls and other personality traits measured later in life (manuscript III).

A second issue in the study of the ontogeny of personality arises from the difficulty to study consistencies during early life stages. Most studies to date bring contradictory results (Sulloway 2011; Wilson & Krause 2012; Herde & Eccard 2013; Favati *et al.* 2015; Wuerz & Krüger 2015) and studies on animal personality during early life stages frequently report rather low or even no consistency in behaviour across time (e.g. Caspi *et al.* 2005; Fratkin *et al.* 2013). Animals undergo major ecological and physiological changes before reaching adulthood, and these key events might be responsible for such low consistencies in behavioural responses (Careau *et al.* 2008; Stamps & Groothuis 2010; Trillmich & Hudson 2011; Sachser *et al.* 2013). Indeed, during ontogeny, environmental effects are considered to be of major importance as they have the potential to affect the neural, physiological and behavioural phenotype (Stamps & Groothuis 2010; Trillmich *et al.* 2015). Although personality can be affected by the early environment (manuscript I), mound-building mice were consistent from the pre-weaning period until maturity in all behavioural parameters tested regarding the animals' responses in non-social and social contexts (manuscript III). Moreover, we showed associations across contexts in the animals' behavioural responses, thus providing further evidence of the existence of personality in young mound building mice, often referred to as behavioural syndrome (manuscripts I, III - V). In particular, we found that pre-weaned animals who repeatedly emitted higher frequency of distress calls during the separation test initiated less positive social behaviour in their sibling group during the post-weaning period (manuscript III). These results indicate stability in personality even in early life stages concerning social traits. It thus highlights the usefulness to integrate social tests into the study of animal personality in young animals (manuscript III).

Associations in behaviour across time and across context were even present when considering relative individual differences among siblings within a litter; that is, when considering the percentage deviation of each individual from its litter mean (manuscripts II, III). Thus, in addition to

the problem of non-adequate tests in young animals, we propose that the lack of consistencies across time and context in behaviour could be, at least in parts, due to a high variation in subjects' behavioural responses expressed among different litters. This might have the potential to mask correlations at the individual level. Indeed, variation in behavioural responses with siblings has been frequently shown to be smaller compared to variation in behavioural responses between litters because siblings have a similar genetic background and experience a highly similar environment during early life (Stamps & Groothuis 2010; Stamps & Krishnan 2014). Indeed, we found that variation in the animal's behavioural responses quantified within the litter was lower compared to the variation among litters (manuscripts II, III).

A next step would be then to test animals in the first few days of life to assess whether such consistencies in behaviour across time and context are already present. Indeed, in mammals, social influence mainly occurring through parent-offspring and pup-pup interaction might be especially critical the first few days of life, as they have the potential to shape personality (Champagne *et al.* 2003; Stamps & Groothuis 2010; Hudson *et al.* 2011). To investigate consistencies in behavioural responses over ontogeny from the first days of life might help to disentangle to what extent environmental factors shape or modulate consistencies in personality.

3.3. What are the hormonal and immune correlates of personality variation in response to socially induced stress?

As behaviour is associated with the individual physiology and neural abilities, variation in personality might be caused by multiple internal physical components, such as hormonal and neurobiological factors (Duckworth 2010). It is known that behaviour is associated with metabolic rates (Bautista *et al.* 2013; García-Torres *et al.* 2015), the levels of circulating stress hormones (Champagne & Meaney 2006; Sulloway 2011), the specific neuronal connections and development (Becker *et al.* 2005; Pinkernelle *et al.* 2009), the dendritic composition (Helmeke *et al.* 2009; Pinkernelle *et al.* 2009) and the neurotransmitter synthesis (Jia *et al.* 2009; Cao *et al.* 2014; Wang *et al.* 2014). One of the aims of this thesis was to investigate whether personality variation was associated with physiological differences (manuscript IV). Our results indicate that individuals with different personality types differed in their stress and immune responses to repeated social confrontations with resident pairs. High anxious / neophobic animals had higher levels of faecal corticosteroid metabolites (manuscript IV), which is in line with previous studies showing a higher hypothalamic pituitary adrenal HPA axis

activity in proactive or aggressive animals (e.g. Cockrem 2007; Koolhaas *et al.* 2010). In addition, CD4+ cells in spleen were lower in animals that showed high neophobia / anxiety-related behaviour (manuscript IV). CD4+ cells encompass two major types of cells: regulatory T cells, known for their suppressive role of the immune system (Sakaguchi 2011) and effector T cells. High anxious / neophobic animals had a lower percentage of effector T cells among splenocytes and a higher percentage of regulatory T cells, an indication of immunosuppression (manuscript IV). An increase in regulatory T cells and the associated decrease in effector T cells in high anxious / neophobic animals suggest a proximate mechanism of immunosuppression triggered by an increase in the hypothalamic pituitary adrenal axis activity. To our knowledge, this was the first study that highlights personality-dependent differences in regulatory T cell after exposure to a social stressor.

It is not yet clear how behaviour and hormonal responses are linked (Carere *et al.* 2010) and different hypotheses have been proposed. Physiological responses might determine the behaviour (Koolhaas *et al.* 1999), although the behaviour could also trigger the physiological responses (Koolhaas *et al.* 1999, 2010). Or no causal link might exist and other factors act on both systems simultaneously (Cockrem 2007). The immune environment has been also suggested to drive personality variation (Barber & Dingemanse 2010; Kortet *et al.* 2010). For instance, immune-challenged male mallard ducks *Anas platyrhynchos* during middle stages of development (immediately after the completion of somatic growth), as well as late stages (during the acquisition of nuptial plumage) showed more active behaviour in novel environments as adults relative to developmentally unchallenged birds or those challenged at an earlier developmental time point, emphasizing that immune challenges at specific periods of life have the potential to shape personality (Butler *et al.* 2012). In our study we did not disentangle the mechanisms, but our results (manuscript IV) bring additional insights into the associations between physiological responses and behavioural phenotypes. A next step could be to repeatedly assess whether animals show physiological (immune and hormonal) consistencies in response to repeated challenges over ontogeny. Indeed, stability in physiological responses in the animals might shed light internal constraints in their individual behaviour.

From a technical point of view, a further contribution of this thesis is the use of a wild-type animal in the study of individual differences in behaviour in modulating associated hormonal and immunological responses to chronic social stress. Most of the studies investigating the physiological mechanisms associated with behaviours are carried out in laboratory animals or even in selected strains (Koolhaas 2008; Carere *et al.* 2010). The use of animals of wild origin, still showing a high variation in personality phenotypes, has been therefore suggested as a promising, alternative (or complementary) approach in biomedical research (Koolhaas 2008, 2010).

3.4. Does personality matching within breeding pairs affect their reproduction?

Next to the various studies on the emergence and development of personality carried out in this thesis, we also studied whether and how personality matching within a pair might affects reproduction. Indeed, studies in several taxa show that positive assortative personalities within breeding pairs exert effects on different parameters of reproduction (Budaev *et al.* 1999; Sinn *et al.* 2006; Ariyomo & Watts 2013) and/or offspring development (Both *et al.* 2005; Schuett *et al.* 2010; Harris & Siefferman 2014). However, such patterns have never been studied in a mammal (Schuett *et al.* 2010). The monogamous breeding system of the mound-building mouse makes this species especially suitable to study such associations between assortative personalities and reproduction. In addition, we phenotyped our animals prior to pairing, which has been rarely done (Schuett *et al.* 2011b). Assessing personality only after pairing might potentially lead to biased results, as both partners might adjust their personality to each other (Rhule-Louie & Mcmahon 2007), as it has been reported in a study on cichlid fish (Laubu *et al.* 2016).

And indeed, our results clearly showed that pairs with similar levels in anxiety scores, independently of the scores of both partners, had a higher probability of breeding and were quicker to start (manuscript V). The mound-building mouse has a life expectancy of around 10 months. Juveniles overwinter in nest chambers, reproduce in spring after dispersal and give birth to few consecutive litters (Milishnikov *et al.* 1998; Gouat *et al.* 2003b). An earlier onset of breeding might thus allow to have more offspring through a higher number of litters. In addition, offspring might become mature earlier in the season with the potential to mate in the same season. An interesting follow-up to this study could be to assess whether pairs with assortative personality types indeed raise more litters that than more disassortative ones. In addition, it would be interesting to repeat this study under more natural conditions, where the animals are free to choose their partners. It might shed light constraints and potential trade-off in that pattern. Moreover, it could further inform why similarity in anxiety might be one of the main traits selected in that species.

Although studies report fitness-related advantages of positive assortative personalities within a pair, the mechanism underlying such advantages is not yet known. In bi-parental species, to mate with an adequate partner can greatly affect the reproductive success as mother and father participates in offspring care. It has been suggested that assortative mating is likely to be beneficial in care behaviour because better coordination between parents is more likely to occur in animals with similar personalities (Schuett *et al.* 2010, 2011a). In addition, assortative personality has been argued

to diminish conflicts between the partners of the pair during offspring rearing (Schuett *et al.* 2010). The assessment of differences in the parental behaviour and male-female interaction between pairs with similar and dissimilar personalities might help to understand whether such effects indeed drive such differences in offspring rearing success. Furthermore, mate choice quality has been suggested to increase the quantity of maternal care (Burley 1988; Gowaty *et al.* 2007). In cases, where mating with a partner with similar personality reflects mate choice quality, higher investment of mothers in care behaviour might be expected.

3.5. General Conclusion

This dissertation provides some insights into the ultimate and proximate aspects underlying behaviour. It highlights the existence of personality differences already in very young animals. Indeed, all personality traits assessed in this manuscript were either consistent across time and / or across context over ontogeny, which challenges the assumptions of the absence of consistent individual differences in behaviour during early life (Gracceva *et al.* 2011; Trillmich & Groothuis 2011). These findings might have potential implications for future research on the development and the consequences of stable individual differences to understand what are the constraints that might limit plasticity in behavioural responses, from early life to adulthood. Furthermore, we demonstrated modulatory effects of personality differences on immune parameters under conditions of social stress. A better and broader understanding of the emergence and development of personality can shed light on how personality differences affect the capacities of the animals to cope with environment challenge (Duckworth 2010). In addition, this thesis suggests fitness-related advantages of positive assortative personality within pair in a monogamous mammal species, with implications for our understanding of the maintenance of personality variation within populations.

4. References

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5. Publications

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Oral Presentations

International Conferences

Rangassamy, M., Khaleghparast Athari, S., Monclús, R., Boissier, M.C., Bessis, N. Rödel, H.G. (2016) Personality modulates proportions of CD4+ regulatory and effector T cells, in response to socially induced stress in a rodent of wild origin, the mound-building mouse. *European Conference on Behavioural Biology ECBB*, Vienna, Austria. (*not yet confirmed*)

Rödel, H.G., Seltmann, M.W., **Rangassamy, M.**, Hoffman, K.L., von Holst, D. (2015) Maternal nest building and perinatal offspring survival in the European rabbit. 5th *World Lagomorph Conference WLC*, Tulock, California, USA. (*not yet confirmed*)

Rangassamy, M., Dalmas, M., Féron, C., Gouat, P., Rödel, H.G. (2014) Similarity of personality positively affects the onset of reproduction in pairs of a monogamous rodent. *European Conference on Behavioural Biology ECBB*, Prague, Czech Republic.

Rangassamy, M. (2013) Can I still recognize close kin despite diet changes? *ELTE Spring School on Behavioural Biology*, Bugaz, Hungary.

National Conferences

Rangassamy, M., Hudson, R., Rödel, H.G. (2015) Stable individual differences in separation calls during early development in the mound-building mouse. 11th Student Meeting *Ecology & Behaviour*, Toulouse, France.

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Poster Presentations

International Conferences

Seltmann, M.W., **Rangassamy, M.**, Hoffman, K.L., von Holst, D., Rödel, H.G. (2016) Timing of maternal nest building and perinatal offspring survival in the European rabbit. *European Conference on Behavioural Biology ECBB*, Vienna, Austria.

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6. Déclaration

Je soussignée, Mademoiselle Marylin RANGASSAMY, certifie que le contenu de cette thèse est le résultat de mon travail personnel. Je certifie également que les données, les raisonnements, et les conclusions empruntées à la littérature existantes sont exactement rapportés, cités et mentionnés, en particulier dans la partie Référence. Je certifie finalement que cette thèse, n'a jamais été totalement ou partiellement évaluée auparavant, et n'a jamais été éditée.

Paris, le 18 mai 2016

4. Abstract

« Emergence and development of individual differences in behaviour - a study in the mound building mouse *Mus spicilegus* »

This thesis includes five manuscripts. Two are already published, one is currently under review and two others are in preparation for submission.

Animals frequently show consistent individual differences in behaviour across time and contexts, a phenomenon called animal personality. Animals have been thus described to differ in the expression level of different specific personality traits. However, consistencies in animal personality traits in young animals are especially controversial. One of the main aims of this thesis was therefore to investigate how the early environment experienced shapes the behavioural phenotype and whether the expression of behaviour remains stable over ontogeny. To this end, we used a small rodent of wild origin, the mound-building mouse *Mus spicilegus*, as an animal model. This monogamous mouse occurs in a variety of agricultural and steppe-like habitats in Central and South Eastern Europe, and is characterized by bi-parental care. The main results of this thesis highlight the consistency of personality traits in the mound building mouse from the early postnatal period until around maturity, both in social and non-social contexts. Various personality traits were associated across context, thus forming a behavioural syndrome. Such consistencies across time and context were present when looking at the individual level but also when focusing on the relative differences among siblings within a litter. The early developmental environment proved to be decisive in modulating the emergence personality of the individual, via the presence or absence of the father. Pups growing up in absence of the father showed indications of a higher responsiveness in two different tests compared to pups raised by mothers only. We showed how personality differences are related to physiological parameters. Different personality types coped physiologically different with a chronic stressor, apparent by their hormonal and immunological profiles. Pairs with similar anxiety scores, independently of the scores of both partners of the pair, had a higher probability of breeding, and brought forward the onset of breeding during the observation period, which carries along potential fitness benefits. This dissertation brings thus together some insights into the proximate and ultimate aspects underlying consistent individual differences in behaviour, which is seldom the case in a same model species.

Keywords: personality, emergence, development, within-litter variation, physiology, reproduction, *Mus spicilegus*

« Emergence et développement des différences comportementales individuelles chez la souris glaneuse, *Mus spicilegus* »

Cette thèse comprend cinq manuscrits d'articles. Deux de ces manuscrits sont publiés, l'un est actuellement en révision et deux sont en préparation pour soumission.

Les animaux diffèrent de manière stable au cours du temps et dans différents contextes dans leur comportement, un phénomène souvent nommé personnalité animale. Les animaux diffèrent ainsi dans leur niveau d'expression de différents traits de personnalités. Cependant l'étude de la stabilité des traits de personnalité chez les jeunes animaux apporte des résultats controversés. Les deux principaux objectifs de cette thèse ont donc été d'évaluer comment l'environnement précoce des animaux façonnait leur personnalité et si l'expression de leur comportement était stable au cours du développement. Notre modèle d'étude était un petit rongeur d'origine sauvage, la souris glaneuse *Mus spicilegus*. Cette souris se trouve dans les zones agricoles d'Europe centrale et orientale. Il s'agit d'une espèce monogame et la femelle et le mâle participent aux soins parentaux. Les principaux résultats de cette thèse soulignent la stabilité des réponses comportementales des souris glaneuses dans des contextes sociaux et non-sociaux tôt lors de la période post-natale jusqu'à la maturité. De nombreux traits de personnalité étaient associés à travers différents contextes ; formant ainsi ce qu'il est convenu d'appeler un syndrome comportemental. Cette stabilité dans le comportement était avérée que l'analyse porte sur la totalité de l'échantillon ou qu'elle prenait en compte les différences intra-portées. L'environnement précoce et en particulier la présence du père apparaissent déterminants dans l'émergence et la modulation de la personnalité. Les individus élevés sans père montraient une plus grande réactivité dans deux tests différents par rapport à ceux élevés avec les deux parents. Différentes personnalités étaient associées à des mécanismes physiologiques. Confrontés à un stresseur chronique, les individus exprimant différentes personnalités montraient des différences physiologiques caractérisées par des profils immunologiques et hormonaux distincts. D'autre part les couples possédant des scores similaires d'anxiété, indépendamment du score des deux partenaires du couple, avaient une plus grande probabilité de reproduction durant la période d'observation, que les couples aux scores différents suggérant de potentiels avantages évolutifs. Cette thèse aborde en parallèle les aspects proximaux et ultimes du comportement chez un même modèle biologique ce qui est un but rarement atteint dans une étude éthologique.

Mots-clés: personnalité, émergence, développement, variation intra-portée, physiologie, reproduction, *Mus spicilegus*