EMOTIONAL PERCEPTIONS IN MICE:

STUDIES ON JUDGEMENT BIAS AND BEHAVIOURAL HABITUATION

HETTY BOLEIJ

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EMOTIONELE PERCEPTIES IN MUIZEN:

STUDIES NAAR "JUDGEMENT BIAS" EN HABITUATIE VAN GEDRAG

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CHAPTER 1

GENERAL INTRODUCTION

HABITUATION: ADAPTIVE AND NON-ADAPTIVE RESPONSES

Habituation is a response that occurs when an individual is repeatedly exposed to environmental stimuli that are not accompanied by a biologically relevant consequence (Eisenstein and Eisenstein 2006; Thompson and Spencer 1966). This process can be described as the waning of a certain response over time. For example state anxiety that is increased when an animal initially is exposed to a novel environment, such as a testing situation, will habituate over time if the environment is assessed to be safe (e.g. Lister 1990; McNaughton and Gray 2000) by means of exploration (Crusio 2001). Although behavioural habituation is guided by emotional processes (e.g. Blanchard, *et al* 2003; Gray 1982; McNaughton and Gray 2000), the change in behavioural output over time involves cognitive processes as well, in that an animal learns about the emotional value of the environment: for example O'Keefe *et al.* (O'Keefe 1999) hypothesize that a spatial map is formed through exploration of a novel environment and, in consequence, exploratory behaviour decreases over time, an effect that has been referred to as the " cognitive map theory". Another theory, described by Grey *et al* (Gray 1982; McNaughton and Gray 2000) suggests that the initial inhibition of behaviour in a novel environment (and in response to innate fear stimuli) is mediated by the behavioural inhibition system (BIS). The function of this system is to evaluate potential threat by comparing environmental stimuli with previously acquired information. Based on both theories it has been hypothesized that adequate adaptation towards a novel stimulus is reflected by a decrease in inhibition and an increase in exploration when no threat is encountered (Gray and McNaughton 1983; O'Keefe 1999). Thus one might get an indication of an animals' adaptive capacities via the measurement of behavioural habituation towards a novel environment.

Previously it had been suggested that internal neuronal set-points that regulate behaviour are static and that that a static equilibrium would be restored after temporal challenges (homeostasis (Bernard 1865)). Over the last decades, more dynamic concepts have been introduced in that adaptation of an organism involves changes of internal set-points to meet environmental demands, a mechanism called allostasis (stability through change)(Koolhaas, *et al* 2011; Korte, *et al* 2007; Sterling and Eyer 1988). Allostatic mechanisms act within a certain range through which an animal can adequately respond to challenges (Koob and Le Moal 2001; McEwen and Wingfield 2003). However, a new set-point or allostatic state is characterized by a narrower regulatory range (Koob and Le Moal 2001) and increases the chance to be overstimulated (McEwen and Wingfield 2003), which might result in failures adequately to respond to environmental stimuli, such as in the ability to habituate (Koolhaas, *et al* 1999; Korte 2001; Korte, *et al* 2005). For example, animals with a history of stressful experiences that might have adapted to a previous stressor by increasing their basal hormonal stress response, will be more vulnerable when exposed to subsequent stressors and may develop maladaptive responses as a result (De Kloet, *et al* 2005; Koolhaas, *et al* 1997; e.g. Veenema, *et al* 2003).

ADAPTIVE CAPACITIES AND ANIMAL WELFARE

Interestingly, it has been suggested to take such adaptive capacities into account in animal welfare considerations (Barnett and Hemsworth 1990; Korte, *et al* 2007; Ohl and van der Staay 2012). There is a wide range of approaches to define animal welfare, still, it difficult to explain what animal welfare may be a simple sentence. It is generally agreed that animal welfare consists of more than physical health

alone (Mills 2008) and it is recognized that the animals internal emotional states (sometimes referred to as feelings) are an important aspect of animal welfare (Duncan 1993; Fraser and Duncan 1998). Animal welfare today is considered a continuum between positive/good welfare and negative/bad welfare (Dawkins 2008; Yeates and Main 2008). A widely used practical approach to animal welfare has been described in the form of the five freedoms (Brambell 1965) which states that welfare can be safeguarded when an animal is free from negative states such as pain, fear, hunger and distress and is free to perform normal behaviour. However, this focus on negative affective states neglects the importance of the presence of positive affective states and the fact that the experience of negative emotions has value for the individuals survival, i.e. animals have evolved to optimize their ability to cope with and adapt to environmental challenges (Barnard and Hurst 1996).

Thus the expression of a negative emotion such as anxiety might actually protect the animals' welfare by avoiding potentially harmful situations (Ohl and van der Staay 2012), while only prolonged negative emotional states might eventually exceed the animals capacity to adapt and compromise welfare (Korte, *et al* 2007; McEwen and Wingfield 2003).Therefore, as long as an animal is able to adapt to (changing) environmental conditions and in that way is able to achieve an internal state that is perceived as positive welfare may be guaranteed (Ohl and van der Staay 2012). Since habituation can be considered as a process reflecting adaptive capacities, via this approach it might be investigated if an animal is able to adapt and what this implies for the animals emotional perception and, thus, its state of welfare.

THE BIOLOGICAL FUNCTION AND EMOTIONAL VALUE OF ANXIETY

Anxiety is an emotion that has evolved to help animals survive potentially dangerous situations by helping the animal to escape from danger and adapt to environmental challenges (Gross 1999; Livesey 1986). Anxiety prepares the individual to react appropriately by for example displaying offensive or defensive behaviours (Blanchard, *et al* 2003; e.g. Boissy 1995). This behavioural output is regulated via the BIS (Gray 1982) comprising, at the central nervous level, the septo-hippocampal system and the basolateral amygdala. The BIS compares input from sensory systems with previously stored information and evaluates the emotional value of the situation. In effect, the BIS estimates threat on basis of previous acquired information or innate threatening stimuli (McNaughton and Gray 2000). Activation of the BIS leads to an inhibition of on-going behaviour and to an increase in attention and arousal (Gray 1982). Anxiety-related behaviour, thus, is the result of a highly adaptive process that regulates an individuals' interaction with its changing environment. As such, the adaptive value of anxiety-related responses may represent a useful measure of the animals' functioning under given environmental circumstances.

Behavioural expressions of anxiety and additional physiological parameters can be used to estimate state anxiety in (laboratory) animals and a range of behavioural tests has been developed to measure these in e.g. rats and mice (see Lister 1990 for a review). Common tests are for example the light/dark box, the elevated plus maze and the open field test (Hogg 1996; Lister 1990; Pellow, *et al* 1985) in which animals initially tend to avoid "unsafe" areas, i.e. higher levels of anxiety are reflected by higher amounts of avoidance related behaviour. Other parameters, such as a reduction in locomotion (Hall 1934) and exploration (Archer and Birke 1983; File and Wardill 1975), an increase in

defecation (Silva and Calil 1975) and grooming (Kalueff and Tuohimaa 2004) and food intake inhibition (Britton and Britton 1981) are indicative of anxiety as well. However, applying such a selective approach (i.e. examining only one parameter) might not give a complete picture of the animals emotional state and can thus lead to misinterpretations, e.g. locomotor activity can be influenced by other motivational systems, grooming also occurs in relaxed situations and food intake and defecation are influenced by the amount of food consumed before the animals are tested. To tackle this problem it might be suggested to apply a more complex approach by e.g. using a testing environment in which multiple behavioural dimensions can be examined in parallel, for example by observing a larger range of behaviours in the home cage (De Visser, *et al* 2006; Kas and Van Ree 2004) or in mildly aversive environments, for example in the elevated plus maze (Lee and Rodgers 1990; Rodgers, *et al* 1992), the modified hole board (mHB) (Ohl, *et al* 2001) and the Mouse Defence Test Battery (Blanchard, *et al* 2003). However, anxiety is not a unitary phenomenon and for example a distinction often is made between anxiety as an intrinsic characteristic of an individual which does not vary from moment to moment (general anxiety level, 'trait anxiety'), and anxiety as a state as experienced by an individual at a particular moment in time and induced by an anxiogenic environmental stimuli (at a specific moment in time, 'state anxiety') (Lister 1990). In animals a combination of physiological and behavioural measures can be used indirectly to estimate levels of anxiety. Still, the existing behavioural tests for anxiety are only able to measure the state of anxiety in a specific test, while trait anxiety in animals is not that easy to estimate (Belzung and Berton 1997; but see Griebel, *et al* 1993) while in humans it can be done via questionnaires.

Although increased state anxiety often reflects a biologically adequate emotional response, high trait anxiety is thought to be of high risk for the development of emotional dysfunctioning (e.g. Leonardo and Hen 2008). Differentiating between adaptive and non-adaptive anxiety, respectively, may be of use as well for a better understanding of what may constitute 'pathological' anxiety in animals. As related to the consideration of biological functioning of anxiety, 'pathological' anxiety in animals may be defined as "...a persistent uncontrollable, excessive, inappropriate and generalised dysfunctional and aversive emotion, triggering physiological an behavioural responses lacking adaptive value" and pathological anxiety-related behaviour has been defined as"...a response to the exaggerated anticipation or perception of threats, which is incommensurate with the actual situation" (Ohl *et al* 2008). While pathological anxiety is likely to be quantitatively or qualitatively different from anxiety within the normal range (Belzung and Griebel 2001), most research in animals on anxiety and drug screening for possible treatment of anxiety disorders, is based on test situations in which actually normal adaptive anxiety-behaviour is measured, i.e. avoidance of (potential) danger, behavioural inhibition and an increase in arousal can be considered as highly important for survival.

ADAPTIVE VERSUS NON-ADAPTIVE ANXIETY IN MICE

The development of anxiety circuits in the brain is partly mediated by genetic components (Hettema, *et al* 2001; Sullivan, *et al* 2000). However, the development of a pathological condition in humans is determined by a combination of the genetic risk and environmental factors (Kendler, *et al* 2003; Roy, *et al* 1995). Likewise, in laboratory animals a combination of genetic vulnerability and aversive stimulation in critical developmental periods increases anxiety related

behaviours (Dirks, *et al* 2002; Schmidt, *et al* 2002). Further, some inbred strains have been characterized as being innately highly anxious (e.g. the BALB/c mouse (Beuzen and Belzung 1995; Makino, *et al* 1991)) and the Wistar-Kyoto rat (Goto, *et al* 1993), and genetic manipulations (for example serotonin receptor 1A knockout mice (Ramboz, *et al* 1998) and selective breeding (for example Low Avoidance Behaviour (LAB) and High Avoidance Behaviour (HAB) rats (Landgraf and Wigger 2002) and mice (Muigg, *et al* 2009)) can also strengthen anxiety traits. Apparently, 129P3 mice as well possess a genetic susceptibility for the development of non-adaptive anxiety, since these mice are characterized by an impaired habituation (Salomons, *et al* 2010; 2010a; 2010b). In contrast, the BALB/c inbred strain, which reveals a high initial state anxiety response (Belzung and Berton 1997; Belzung and Griebel 2001), shows rapid habituation towards a novel environment (Salomons, *et al* 2010; Salomons, *et al* 2010a; Salomons, *et al* 2010b), a profile that is considered adaptive. Such differences in adaptive capacities might be of translational value for pre-clinical research on affective disorders in humans and, in addition, may be of relevance for research on aspects of animal welfare.

EMOTIONAL PERCEPTIONS: COGNITIVE BIAS

To investigate the interaction of cognitive and emotional processes in animals and, especially, to understand how an animal might perceive its own emotional state one might consider the use of cognitive bias tests. Cognitive processes are closely linked to emotional states as they are for example necessary for the appraisal of environmental cues and for the "production" of emotions (Lazarus 1982; Mathews and MacLeod 1994). It has been suggested that anxiety states are caused by mismatches between the information already stored in the brain and perceived environmental information and that pathological anxiety might be caused by cognitive dysfunctions that result in inappropriate emotional responses (Gray 1982; McNaughton 1997). Further, emotional states also influence information processing in the brain, which helps the animal to react appropriately within a certain context (Mathews, *et al* 1997). Emotional influences on cognition are defined as cognitive biases, of which three types can be distinguished: attention biases, memory biases and interpretation or judgement biases (see Paul, *et al* 2005). Attention bias occurs in threatening situations as a result of an anxious emotional state and is characterized by an increased attention to negative and threatening cues (Mathews and MacLeod 1994; Mogg and Bradley 1998). Memory bias refers to the fact that events, associated with positive or negative emotions, are more readily remembered than neutral events, and includes memory storage, consolidation and retrieval processes (Cahill and McGaugh 1996; Hamann, *et al* 1999), although the effects on memory are probably caused by high arousal and not that much by the valence of the emotion (Bradley, *et al* 1992). Judgement bias or interpretation bias (from now on referred to as judgement bias) refers to the influence of emotions on the interpretation of ambiguous information (Eysenck, *et al* 1991; Mathews, *et al* 1989; Mathews, *et al* 1997; Richards and French 1992).

Negative emotional states induce a more negative interpretation of ambiguous information (negative judgement bias) (see for example Eysenck, *et al* 1991; Mathews, *et al* 1989), whereas positive emotional states induce a more positive interpretation of ambiguous information (positive judgement bias) in humans (see for example Nygren, *et al* 1996). Not only the emotional state at a particular moment in time, but also trait anxiety has an effect on cognitive biases. People with high trait anxiety are characterized by more negative attention- and

judgement bias than people with low trait anxiety (Chan and Lovibond 1996; MacLeod and Byrne 1996; MacLeod, *et al* 1997; MacLeod and Byrne 1996) and people with anxiety disorders show a more negative judgement bias than healthy controls (Eysenck, *et al* 1991; MacLeod, *et al* 1997; Mathews, *et al* 1989). Since cognitive biases include cognitive components of emotions an estimation of emotions via cognitive biases might provide us information on emotional perceptions in humans as well as animals (MacLeod and Byrne 1996).

COGNITIVE BIAS IN ANIMALS

Measurement of cognitive biases in animals adds to existing behavioural and physiological measures of emotions for several reasons. For example, physiological indicators do not clearly indicate the valence of emotions (Dawkins 2006; Paul, *et al* 2005; Rushen 1991) and existing behavioural tests are traditionally focused on measuring negative emotional states (Boissy, *et al* 2007b), such as anxiety. Considering that cognitive biases (judgement bias in particular) are influenced by both positive and negative affective states the evaluation of judgement bias might help to identify the valence of emotional states of animals (Mendl, *et al* 2009; Paul, *et al* 2005).
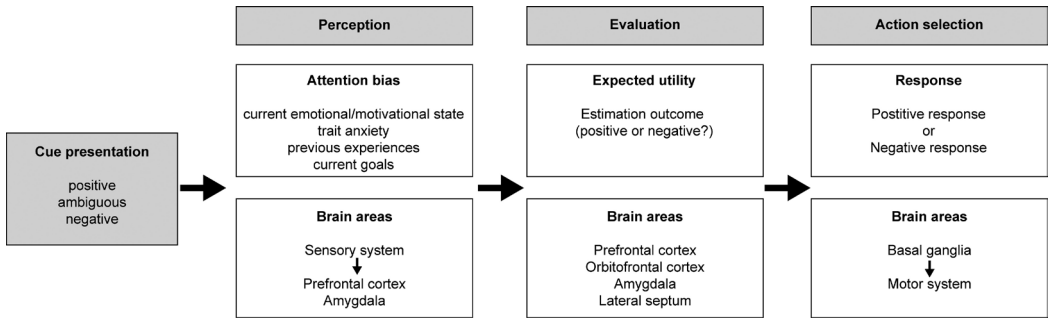
As found in humans (Chan and Lovibond 1996; MacLeod and Byrne 1996), cognitive biases in animals might be related to trait anxiety and might thus be used as "indirect" indicators of trait anxiety. Thus, the development of reliable judgement bias tests in (laboratory) animals provides a basis for further pre-clinical research on this phenomenon and its mechanisms, i.e. it is of value for translational purposes. Further, cognitive bias tests may allow for an assessment of how animals experience certain environmental stimuli (e.g. under certain housing or experimental conditions) via getting an indication of the valence of their emotional perceptions (Mendl, *et al* 2009).

Over the last decade the investigation of judgement biases in animals has gained popularity, both in the field of animal welfare science as in more fundamental neuroscience research using animal models to investigate depression and anxiety-related diseases (Anderson, *et al* 2012a; Anderson, *et al* 2012b; Bateson and Matheson 2007; Bateson, *et al* 2011; Bethell, *et al* 2012; Brilot, *et al* 2010; Brydges, *et al* 2011; Burman, *et al* 2008; Burman, *et al* 2009; Burman, *et al* 2011; Douglas, *et al* 2012; Doyle, *et al* 2011; Enkel, *et al* 2010; Harding, *et al* 2004; Hymel and Sufka 2012; Matheson, *et al* 2008; Mendl, *et al* 2010a; Müller, *et al* 2012; Pomerantz, *et al* 2012; Richter, *et al* 2012; Salmeto, *et al* 2011; Sanger, *et al* 2011; Wichman, *et al* 2012). Studies on judgement biases in humans mainly use linguistic stimuli, for example "homophones" (words that can have multiple meanings but are pronounced similarly) (see for example Eysenck, *et al* 1991; Mathews, *et al* 1989) which is not possible in animals. The pioneering study from Harding *et al*. (2004) solved this problem by introducing an operant conditioning paradigm using tones as conditioned stimuli predicting positive or negative outcomes, i.e. when a tone of 2 kHz was presented rats could obtain a food reward by pressing a lever and when a tone of 4 kHz was presented the same rats could avoid an aversive noise by not pressing a lever. Subsequently, after a training period reactions towards intermediate tones of 2.5, 3 and 3.5 kHz (the ambiguous cues) were investigated. Chronically stressed rats showed a more negative bias, that is, they were less likely to respond positively towards the ambiguous cues, indicating that this method might be suitable to investigate judgement biases in rats. All following judgement bias studies were based on a similar training and testing principle, using stimuli of different sensory modalities

and different test set-ups depending on the investigated species. Environmental manipulations assumed negatively to affect the animals' emotional state, such as unpredictable housing conditions in rats (Harding, *et al* 2004) and removal of shelter enrichment in rats (Burman, *et al* 2008) and starlings (Bateson and Matheson 2007), as well as testing under bright light conditions (acute increase in state anxiety) in rats (Burman, *et al* 2009) have been shown to result in negative judgement biases, while assumed positive manipulations such as environmental enrichment in rats (Brydges, *et al* 2011), pigs (Douglas, *et al* 2012) and starlings (Matheson, *et al* 2008) have been shown to induce positive judgement biases. In laboratory mice no experiments on judgement bias have been described so far. It is however of high interest to develop a judgement bias test for mice, since mice are frequently used in laboratory research, in (pathological) anxiety research and are often subject of transgenic studies.

BRAIN MECHANISMS

Central nervous processes regulate behavioural expressions and, thus, differences in emotional characteristics are expected to be reflected on the brain level as well. Emotional processes are mainly mediated via areas in the limbic system and prefrontal areas (e.g. Etkin 2010; Millan 2003; Panksepp 1998; Pratt 1992). Approach or avoidance behaviour that is shown in potentially threatening situations is thought to be mediated via the septo-hippocampal system, basolateral and central amygdala (Gray 1982; McNaughton and Gray 2000),while cognitive control over emotional reactions is regulated via areas in the prefrontal cortex (Goldman-Rakic 1995). In the previously characterized mouse strains showing adaptive (BALB/c strain) and non-adaptive (129P3) habituation profiles (see section "Adaptive versus non-adaptive anxiety in mice" above), (Salomons, *et al* 2010; Salomons, *et al* 2010a; Salomons, *et al* 2010b), differences in neuronal activation patterns were found in the prelimbic cortex and lateral septum, indicating that the non-adaptive profile of 129P3 might lie in the appropriate integration cognitive with emotional information (Salomons, *et al* 2010; Salomons, *et al* 2010a). Judgement bias, being a cognitive process induced by emotional states (Mathews, *et al* 1997; Mogg and Bradley 1998), is likely to be regulated by prefrontal and septal circuits as well. Moreover, the amygdala might be of relevance for judgement bias processes since this area is involved in the attachment of values to distant cues (Davis and Whalen 2001). Although the central-nervous regulation of judgement bias has not yet been described for animals, human literature might give some indications on the subject: For example the review of Mendl *et al*. (2009) gives a comprehensive overview of the brain structures that might be involved in judgement bias (see also fig 1). Human experiments indicate that the selective attention towards threat (increased anxiety) is regulated by a decreased top-down control from the medial prefrontal cortex or increased amygdala response to threat (Bishop 2007), while cognitive control areas such as the orbitofrontal cortex and prefrontal cortex are involved in ambiguous cue evaluation (Padoa-Schioppa and Assad 2006; Rolls 1992). The combination of emotional values processed by the amygdala and cognitive control exerted by the prefrontal areas then might be integrated and regulated in the (lateral) septum (see Sheehan, et al 2004 for a review). Further, action selection mechanisms are thought to be regulated by the nuclei within the basal ganglia and lead to a behavioural response (Bogacz 2007).

Perception	Evaluation	Action selection

Cue presentation

positive
ambiguous
negative

Attention bias

current emotional/motivational state
trait anxiety
previous experiences
current goals

Expected utility

Estimation outcome
(positive or negative?)

Response

Postitive response
or
Negative response

Brain areas

Sensory system
↓
Prefrontal cortex
Amygdala

Brain areas

Prefrontal cortex
Orbitofrontal cortex
Amygdala
Lateral septum

Brain areas

Basal ganglia
↓
Motor system

Fig 1. Simplified overview of the processes and brain areas that might be involved in the processing of stimuli in a judgement bias task. More detailed information is provided in the text. The figure is partly based on a schematic diagram in Mendl *et al*. (2009)

UNDERSTANDING EMOTIONAL PERCEPTIONS IN MICE

The expression of a negative judgement bias is influenced by state as well as trait anxiety characteristics (Bateson, *et al* 2011). An adaptive anxiety response includes the expression of negative cognitive biases, i.e. in a threatening situation it is more adaptive to avoid an ambiguous stimulus (Bateson et al 2011). Therefore, the measurement of judgement bias might not necessarily say something about the adaptive capacities of animals, but rather about the animals' perception of its own emotional state and/or trait (Mathews and MacLeod 1994; Mogg and Bradley 1998). Still, it seems reasonable to assume that challenges that exceed the adaptive capacities of an animal will result in a shift towards a more negative judgement bias, while it already has been shown that the measurement of behavioural habituation can provide information on the adaptive value of responses (Salomons, et al 2010a). Thus, both aspects are of interest for a better understanding of how animals might perceive and cope with environmental challenges.

AIM AND OUTLINE

The main aim of this thesis was to develop more understanding on how mice perceive their own emotional state.

In **Chapter 2** habituation profiles in four 129 substrains (129P2/OlaHd, 129X1/J, 129S2/SvPasCrl and 129S2/SvHsd) are compared in order to evaluate whether delayed habituation towards novelty is a structural characteristic in 129 substrains, based on a previous study that found this characteristic in 129P3/J mice. It appears that in all tested 129 substrains repeated exposure to a testing environment results in an increase in anxiety-related behaviour over time, suggesting a that this lack of ability to habituate has a genetic background. Still, some of the substrains reveal potentially confounding characteristics in overall locomotion, which limits the validity of those substrains for behavioural testing.

Chapter 3 further validates that a lack of behavioural habituation towards novelty as found in 129 substrains may indeed constitute a genetically based, non-adaptive behavioural profile that cannot simply be induced by environmental challenges. Apparently the application of chronic social stress (CSS) during the adolescent and early adulthood in outbred CD1 mice had no effects on habituation, while during the procedure mice did show clear signs of chronic stress. Further, there are high individual differences in stress-response, pointing towards an inter-individual variance in stress coping profiles in mice that should

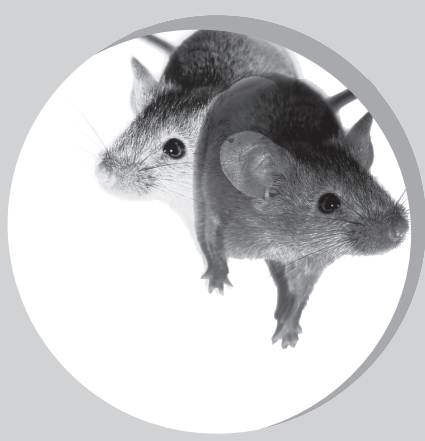be taken into account when planning future CSS-experiments.

In **Chapter 4** a method to identify judgement bias in mice is introduced and two inbred mouse strains, known to react either adaptively (BALB/cJ mice) or non-adaptively (129P3/J mice) towards novelty are compared. The behavioural response in BALB/cJ towards an ambiguous stimulus indicates a negative judgement bias that is sensitive to changes in state anxiety. Further, area-specific analysis of neuronal activity implicates an involvement of the lateral septum and amygdala in these behavioural responses. 129P3/J animals though seem not to differentiate between positive and negative stimuli.

Chapter 5 continues with the judgement bias test using quinine, and further investigates whether the relationship between state anxiety and negative judgement bias may be causal. To this aim anxiety in BALB/cJ mice is modulated by treatment with the anxiolytic drug diazepam before exposing the animals to the judgement test procedure. Treatment with diazepam seemed to ameliorate the negative judgement bias induced by aversive test conditions. Behavioural results support this notion of a causal relation between state anxiety and judgement bias. However, experimental groups of mice reveal responders as well as non-responders to the negative stimulus, an effect that weakens the statistical analysis.

In **Chapter 6** a distinct methodological aspect of the judgement test set-up is investigated. From our first tests we got the impression that part of the mice might have been using the taste additive on the food reward to differentiate between the positive and the negative stimulus, respectively. The comparison between quinine and denatonium benzoate as taste additive to almond pieces in the judgement bias training procedure shows that denatonium benzoate is less suitable, because mice appear to habituate towards the taste of denatonium benzoate, but not to quinine.

In **Chapter 7** the effects of testing environmental on judgment bias in BALB/cJ mice is investigated. A novel testing environment increases overall behavioural inhibition in mice, thus obscuring more specific effects on judgement bias. Testing the animals in their home cage therefore can be concluded to be more effective. Further, extra home cage enrichment seems to affect negative judgement bias in mice slightly positively. However, overall judgement biases in all experimental groups rather indicate a positive bias than a negative bias in that responses towards the ambiguous stimulus are more comparable to that towards the positive stimulus than the negative one.

In **Chapter 8** the results of judgement bias testing as well as the environmental effects and strain differences on adaptive capacities are discussed and put in a broader perspective.
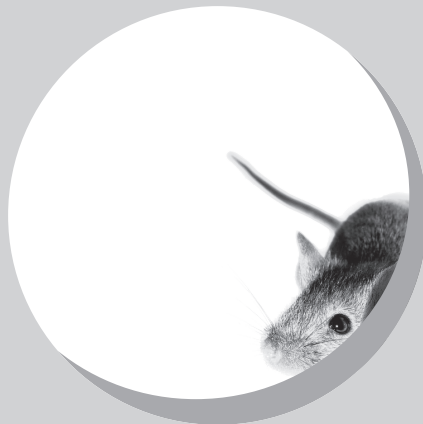
CHAPTER 2

NOT ALL MICE ARE EQUAL: WELFARE IMPLICATIONS OF BEHAVIOURAL HABITUATION PROFILES IN FOUR 129 MOUSE SUBSTRAINS

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ABSTRACT

Safeguarding the welfare of animals is an important aim when defining housing and management standards in animal based, experimental research. While such standards are usually defined per animal species, it is known that considerable differences between laboratory mouse strains exist, for example with regard to their emotional traits. Following earlier experiments, in which we found that 129P3 mice show a lack of habituation of anxiety related behaviour after repeated exposure to an initially novel environment (non-adaptive profile), we here investigated four other 129 inbred mouse substrains (129S2/SvPas, 129S2/SvHsd (exp 1); 129P2 and 129X1 (exp 2)) on habituation of anxiety related behaviour. Male mice of each strain were repeatedly placed in the modified hole board test, measuring anxiety-related behaviour, exploratory and locomotor behaviour. The results reveal that all four substrains show a lack of habituation behaviour throughout the period of testing. Although not in all of the substrains a possible confounding effect of general activity can be excluded, our findings suggest that the genetic background of the 129 substrains may increase their vulnerability to cope with environmental challenges, such as exposure to novelty. This vulnerability might negatively affect the welfare of these mice under standard laboratory conditions when compared with other strains. Based on our findings we suggest to consider (sub)strain-specific guidelines and protocols, taking the (subs)train-specific adaptive capabilities into account.

INTRODUCTION

Animal welfare is an important issue to consider in laboratory animal research and management, from both an ethical point of view and for generating reliable experimental results. Legal frameworks and more detailed guidelines are an important instrument used to safeguard the welfare of experimental animals. Currently the protection of animal welfare is primarily directed on the absence of negative factors such as illness, distress hunger, pain, anxiety and fear (five freedoms (Brambell 1965)) and the promotion of natural behaviour. However, recently the importance of the presence of positive emotions (Boissy, *et al* 2007b; Bracke and Hopster 2006; Ohl and van der Staay 2012; Yeates and Main 2008) as well as the relevance of the animals' adaptive capacities (Korte, *et al* 2007; Ohl, *et al* 2008; Salomons, *et al* 2009) have been discussed, and it has been suggested to consider the animals' freedom to adequately react to prevailing environmental circumstances as an indicator for the individuals' welfare (Ohl and van der Staay 2012).

It is clear that safeguarding animal welfare demands the definition of criteria for both measurable animal based parameters (such as behavioural and/ or physiological parameters) and environmental parameters (such as animal housing and management measures, see for example the Welfare Quality project (Knierim and Winckler 2009)). These criteria have to take animal-specific characteristics into account and today's guidelines usually define minimal welfare demands per animal species. However, one may wonder whether indeed 'a mouse is a mouse' (Webster 1994) when aiming at safeguarding the welfare of the variety of existing laboratory mouse strains, and especially with respect to appreciating the expression of their 'natural' behaviour. Selective breeding programmes in laboratory animals are often focused on physiological characteristics, but selection may have an (unintended) impact on other characteristics, such as emotional traits and related behavioural expressions (see for example (Belzung and Griebel 2001; Clément, *et al* 2009; Jensen 2010; Laarakker, *et al* 2008; Ohl, *et al* 2003; Ohl, *et al* 2008)). Such characteristics then may result in reduced adaptive capacities, which in turn can compromise biological functioning and thus may finally impair welfare in (selectively bred) animals (Ohl, *et al* 2008; Salomons, *et al* 2009).

The adaptive capacity of an animal depends on the genetic background as well as on environmental and epigenetic factors (Crabbe, *et al* 1999; van der Staay, *et al* 2010). Strain comparisons in small rodents reveal that the same environmental stimulation can elicit varying behavioural responses (see for example the effects of environmental enrichment and chronic stress on behaviour of different mouse strains (Abramov, *et al* 2008; Pothion, *et al* 2004; Salomons, *et al* 2010b)). Moreover, gene-environment interactions may influence habituation processes and may affect adaptive capacities, as can be seen in inbred or selectively bred rodent strains. For example, so-called LAB-rats (Low Anxiety Behaviour) and C57BL/6 mice initially show non-anxious behaviour in a novel environment and reveal no further habituation during repeated exposure, while HAB-rats (High Anxiety Behaviour) as well as DBA/2 mice show initially high anxious behaviour, but reveal rapid habituation during repeated exposure (Ohl, *et al* 2002; Ohl, *et al* 2003).

Anxiety is a highly conserved adaptive emotion that occurs in situations of potential danger or threat and is one of the so-called "negative" emotional states that at least all vertebrates are supposed to be able to experience (Livesey 1986). For example exposure to a novel situation or environment induces a

state of anxiety due to the uncertainty of this environment. By exploring a novel environment, it becomes more familiar and anxiety decreases. The process of waning of a certain behavioural response over time is also described as behavioural habituation (Eisenstein and Eisenstein 2006). Hence the ability of an animal to habituate is a reflection of the capacity of this animal to adapt to the situation and vice versa (Salomons, *et al* 2009).

In previous studies we found that the 129P3/J substrain shows a lack of habituation during repeated exposure to an initially novel environment (Salomons, *et al* 2010a). In comparison with the initially highly anxious BALB/c mouse, the 129P3/J substrain shows low initial avoidance behaviour but over time this behaviour increases (Salomons, *et al* 2010a), indicating a fundamental inability to adapt (Salomons, *et al* 2010a). Such differences in adapting to novelty implicate that different mouse-strains may respond very differently to standardized housing-conditions that are defined for mice as a species in general (National Research Council 2010), which actually brings up the question whether one and the same guideline regarding housing conditions may be feasible to safeguard welfare in all laboratory mouse strains, or, as suggested earlier, that it may be necessary to define strain-specific guidelines (Salomons, *et al* 2009).

In extension of earlier strain-comparisons, we here investigated four 129 substrains on their habituation behaviour to evaluate whether structural differences in adaptive capacities have to be taken into account even at a more differential level than the strain-level. This is a relevant question since other studies have demonstrated that there is substantial genetic and phenotypic variation between the 129 substrains (Bothe, *et al* 2004; Bothe, *et al* 2005; Cook, *et al* 2002; Simpson, *et al* 1997; Tang and Sanford 2005). In two independent experiments we repeatedly exposed mice of the 129S2/SvPasCrl (129S2Pas) and 129S2/SvOlaHsd (129S2Hsd) (experiment 1) and mice of the 129X1/J (129X1) and 129P2/OlaHsd (129P2) (experiment 2) to an initially novel testing environment. These specific substrains were chosen because of their frequent use in laboratory research, their relatedness (Simpson, *et al* 1997), and because of the previous findings (see above) in one of the existing 129P3 substrains. On basis of our results we hope to draw conclusions on the general adaptive capacities of the 129 strain and what this might mean for management protocols for safeguarding their welfare under standard laboratory housing conditions.

MATERIALS AND METHODS
ETHICAL NOTE

The experimental protocols (DEC numbers 2007.I.01.007 and 2009.I.10.079) were approved by the Animal Experiments Committee of the Academic Biomedical Centre Utrecht, The Netherlands. The Animal Experiments Committee based its decision on the EC Directive 86/609/EEC (Directive for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes). Furthermore, all animal experiments followed the 'Principles of Laboratory Animal Care' and refer to the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioural Research (National Research Council 2003, see http://www.springerlink.com/content/86881171278wt787/fulltext.html - CR20#CR20).

ANIMALS AND HOUSING

The behavioural experiments were performed at two different locations. In both experiments similar housing conditions and experimental procedures were

applied. The dark period started at 6.00 and lasted until 18.00h (reversed light/dark cycle) and radio music was turned on as background noise during the whole experimental period. During the first two weeks after arrival (habituation period) the animals were handled for ~3 minutes per mouse three times a week (between 9.00 h and 11.00 h) by the experimenter who also did behavioural testing. All testing took place in the animals housing room and equipment was installed before the animals arrived.

EXPERIMENT 1

This experiment took place at the Central Laboratory Animal Research Facility of Utrecht University (location Paviljoen) with 8 male 129S2/SvPasCrl (129Pas, Charles River, Germany) and 8 male 129S2/SvHsd (129Hsd, Harlan, The Netherlands) mice. The animals were 7-8 weeks old at arrival and housed individually in Eurostandard Type II cages (size: 365 x 207 x 140 mm, floor area 530 cm^2; Techniplast, Milan, Italy) with standard bedding material (Aspen chips; Abedd-Dominik Mayr KEG, Köflach, Austria), a tissue (KLEENEX® Facial Tissue, Kimberly-Clark Professional BV, Ede, The Netherlands) a cardboard shelter and some cardboard shredding (Envirodri®, Technilab-BMI BV, Someren, The Netherlands) as cage enrichment. The mice were kept in the test room for 17 days under constant laboratory conditions for acclimatisation to the experimental room with water and food (CRM, Expanded, Special Diets Services Witham, England) available ad libitum. Relative humidity was kept at a constant level of approximately 50% (± 5%), room temperature was sustained at 22 °C ± 2 and ventilation rate was 15-20 air changes per hour.

EXPERIMENT 2

The second experiment took place at the Central Laboratory Animal Research Facility of Utrecht University (location GDL) with 8 male 129P2/J (129P2, Harlan Europe, UK) and 8 male 129X1 (129X, Jackson Laboratory, USA) mice. The mice were 7-8 weeks old at arrival and housed individually in Eurostandard Type II cages (size: 365 x 207 x 140 mm, floor area 530 cm^2; Techniplast, Milan, Italy) with standard bedding material, a tissue, and a cardboard shelter as cage enrichment. The mice were kept in the test room for 17 days (129X1) and 23 days (129P2) under constant laboratory conditions for acclimatisation to the experimental room with water and food (CRM, Expanded, Special Diets Services Witham, England) available ad libitum. Relative humidity was at a constant level of approximately 50%, room temperature was sustained at 22 °C ± 2 and ventilation rate was 15-20 air changes per hour.

MODIFIED HOLE BOARD (MHB)

The mHB consisted of a an opaque grey PVC box (100 x 50 x 50 cm) with a hole board, which was made of the same material as the box, positioned in the middle of the box (60 x 20 x 2 cm), thus representing the unprotected area comparable with the centre of an open field. On the board 20 cylinders (15 x 15 mm) were staggered in three lines. The area around the board was divided by black lines into 10 rectangles (20x15 cm) and 2 squares (20x20 cm), the number of lines crossed were used to get an indication of locomotor activity. The box was illuminated with 1-5 lux (red light), the board was illuminated with a stage light of about 120 lux (white light). For testing, all animals were individually placed in the mHB, always starting from the same corner. Each trial lasted 5 minutes, 4 trials per day over 5 consecutive days (20 trials in total) were performed. For investigation of

food intake inhibition, each animal received a piece of almond daily for three days in its home cage before the start of the experiment. The familiar (almond) and unfamiliar food object (dustless precision pellets, 45mg, Bio-Serv) were also placed in the mHB, always in the same corner, either one positioned at the same distance from the wall.

After each trial, the mHB was carefully cleaned with tap water and a damp towel. All tests were videotaped for raw data storage and behaviour was directly scored by a trained observer using the program Observer 5.0 (Noldus Technology, The Netherlands). The following behavioural parameters were measured and assigned to different behavioural categories according to previous studies (Ohl, *et al* 2001a); avoidance behaviour: the latency until the first board entry, the percentage of time spent on the board and the total number of board entries; risk assessment: the number of stretched attend postures and the latency until the first stretched attend; locomotor activity: the total number of line crossings, the latency until the first line crossing, the total time spent immobile and the latency until the first immobility event; general exploration: the total number of rearings in the box and on the board, the latency until the first rearing in the box and on the board, the total number of hole explorations and the latency until the first hole exploration (a hole was counted as explored when the animal's nose was directed to a hole; direct contact with the hole was not necessary); directed exploration: the total number of holes visited, (a hole was counted as visited when the mouse dipped the nose below the rim of the hole) and the latency until the first hole visit; food intake inhibition: the latency until the first exploration of the unfamiliar and familiar food object; arousal or de-arousal: the percentage of time spent self-grooming, the latency until the first self-grooming event, the total number of self-grooming events and the total number of faecal boli; escape behaviour: the total number of jumps.

CORTICOSTERONE

Basal blood samples were collected four days before the start of the experiment (15.00 h, around the same time the animals started their last mHB trial) to determine basal corticosterone (CORT) plasma levels. Blood sampling and decapitation took place in a room adjacent to the experimental room (in order not to disturb circadian rhythm of the mice, the intermediate hallway and rooms were under red light conditions in both locations). A small blood sample (50 µL) was collected by tail vein incision, and stored in pre-chilled Microvette tubes (CB300, Sarstedt, Numbrecht, Germany) containing lithium heparin. Two-and-a-half hours after the last trial, animals were decapitated and trunk blood was collected in Minicollect tubes (1 ml Lithium Heparin, Greiner Bio-One GmbH, Kremsmünster, Austria). Plasma CORT levels were measured by radioimmunoassay (RIA) according to the protocol of the supplier with an ImmuChem™ Double Antibody Corticosterone kit for rats and mice (MPI Biochemicals, Amsterdam, The Netherlands).

STATISTICAL ANALYSIS

Statistical analyses were performed using the software program SPSS 16.0.1 for Windows (SPSS Inc. IL, USA). Continuous data (plasma CORT, latency and relative duration of behavioural parameters) were represented as mean ± standard error of the mean (SEM), and were first investigated for gaussianity using the Kolmogorov-Smirnov test. Homoscedasticity was tested by Levene's test. Some of these parameters revealed a non parametric distribution and were

rank transformed. Discrete data on the ordinal scale (total number of behavioural parameters) are represented as median with interquartile range (IQR), and were rank transformed. Behavioural data from the mHB experiments were subsequently analysed using linear mixed model analysis. If a certain behaviour did not occur during the trial latencies were set at 300 s (total trial time). Before analyses, the most appropriate test for each parameter was defined by varying the linear mixed model test with or without random intercept and slope. Based on the value of the 2-log likelihood of the Chi-square distribution, the significantly best test (i.e.) was used for analysis for each specific parameter. This included a linear mixed model analysis with fixed effects of strain, trial x strain interaction and a random intercept or random effects of strain, strain x trial interaction and a random intercept. For linear mixed model analyses a probability value less than 0.05 (two-tailed) was considered as statistically significant. CORT analyses were done using paired (basal/non-basal) or unpaired (strain) Student t-tests. The probability value was adjusted for the number of comparisons using Dunn-Šidak correction ($\alpha = 1 - 0.95^{1}/q$, q is number of comparisons).

RESULTS

A summary of the results (comparison 1st and 20th trial, to get an indication of the change over time) from the first and second experiment are listed in table S1.

AVOIDANCE BEHAVIOUR

EXPERIMENT 1: 129S2PAS VS. 129S2HSD

Significant trial and trial x strain interactions effects were found for the latency until the first board entry (trial: $F_{19, 304} = 11.917$, P<0.001; strain x trial: $F_{19,304} = 5.334$, P<0.001), the total time spent on the board (trial: $F_{19, 303.2} = 13.531$, P<0.001; strain x trial: $F_{19,303.2} = 1.694$, P<0.05) and the total number of board entries (trial: $F_{19,303.2} = 5.933$, P<0.001; strain x trial: $F_{19,303.2} = 2.549$, P<0.001). Both strains showed an increase in the latency until the first board entry across the experimental period (Fig 1A), in addition the total time spent on the board and the number of board entries (Fig 2A) decreased across the experimental time period, thereby showing increased avoidance behaviour of the unprotected area.
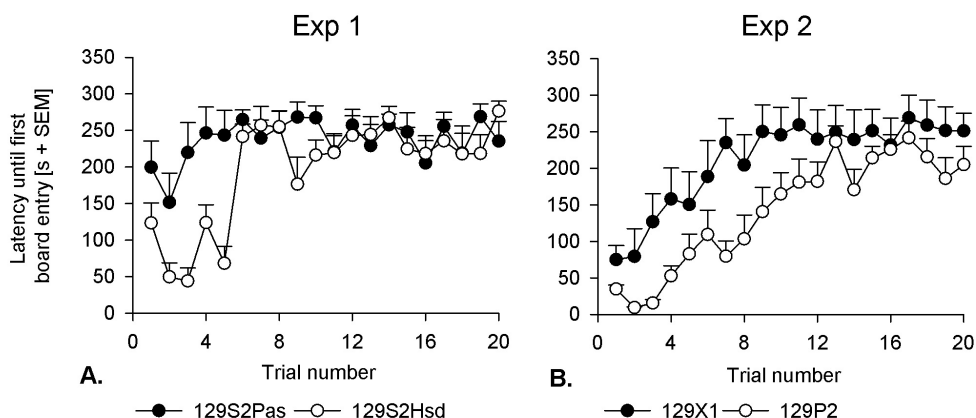

Fig. 1: Mean latency (seconds + SEM) until the first board entry in 129SPas, 129S2Hsd (1A) and in 129P2 and 129X1 (1B) mice.

EXPERIMENT 2: 129X1 VS. 129P2

Significant strain ($F_{1,16} = 5.21$, P<0.05) and trial effects ($F_{19,302.2} = 20.38$, P<0.01) were found for the latency until the first board entry. Both strains showed an increase in the latency until the first board entry across the experimental period (Fig 1B). The time spent on the board (Fig 2B) only showed a significant trial ($F_{19,302.2} = 10.98$, P<0.01) effect and both strains showed a decrease in time spent on the board across the experimental period. Significant strain ($F_{1,16} = 11.62$, P<0.05), trial ($F_{19,302.5} = 15.39$, P<0.01) and strain x trial interaction ($F_{19,302.5} = 3.35$, P<0.01) effects were found for the number of board entries. In both strains, the number of board entries decreased across the experimental period. In general, both strains thus showed increased avoidance behaviour over time.
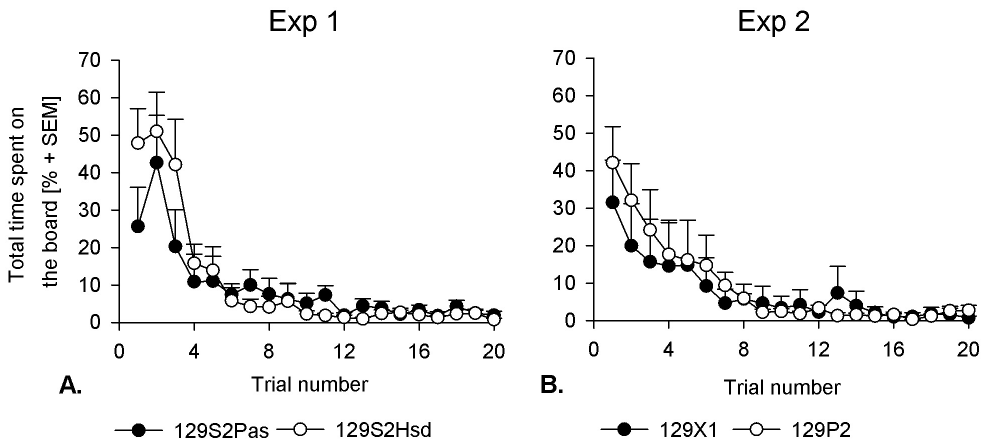

Fig. 2: Total time spent on the board (% + SEM) in 129S2Pas, 129S2Hsd (2A) and in 129P2 and 129X1 (2B) mice.

RISK ASSESSMENT

EXPERIMENT 1:129S2PAS VS. 129S2HSD

Significant trial effects were found for the total number of stretched attends ($F_{19,200} = 32.007$, P<0.001) and the latency until the first stretched attend ($F_{19,204} = 4.368$, P<0.001). Both strains showed an increase in latency until the first stretched attend (129S2Pas: 10.2 ± 5.8 in trial1, 212.6 ± 122.2 in trial 20; 129S2Hsd: 7.3 ± 3.2 in trial 1, 197.7 ± 89.5 in trial 20) and a general decrease in stretched attend postures (129S2Pas: 7 ± 12 in trial1, 4 ± 0.5 in trial 20; 129S2Hsd: 2 ± 17.5 in trial 1, 3 ± 1 in trial 20) across the experimental period.

EXPERIMENT 2:129X1 VS. 129P2

Significant trial effects were found for the total number of stretched attends ($F_{19,302.7} = 14.41$, P<0.01) and the latency until the first stretched attend ($F_{19,302.7} = 10.778$, P<0.001). Both strains showed an increase in latency until the first stretched attend (129X1: 52.3 ± 33.9 in trial1, 279.1 ± 19.0 in trial 20; 129P2: 42.3 ± 36.8 in trial 1, 300 ± 0 in trial 20) and a general decrease in stretched attends postures (129X1: 6 ± 10 in trial1, 0 ± 1 in trial 20; 129P2: 7 ± 12 in trial 1, 0 ± 0 in trial 20) across the experimental period.

LOCOMOTOR ACTIVITY
EXPERIMENT 1: 129S2PAS VS. 129S2HSD

Significant strain ($F_{1,23.6}$ = 4.363, P<0.05) and strain x trial effects ($F_{19,303.2}$ = 2.701, P<0.001) were found for the total number of line crossings (Fig 3A). 129S2Pas mice initially showed less line crossings compared to 129S2Hsd mice. Further, significant strain ($F_{1,19.4}$ = 11.896, P<0.001), trial ($F_{19,207.8}$ = 4.445, P<0.001) and strain x trial effects ($F_{19,207.8}$ = 3.587, P<0.001) were found for the latency until the first line crossing, which decreased across the experimental period in both strains. Locomotor activity was further analysed by immobility events. Significant strain and trial effects were found for the total time spent immobile (strain: $F_{1,21.8}$ = 12.163, P<0.001; trial: $F_{19,215.1}$ = 5.814, P<0.001) and the latency until the first immobility (strain: $F_{1,21.8}$ = 7.222, P<0.001; trial: $F_{19,215.1}$ = 10.278, P<0.001). Both strains showed increased immobility across the experimental period (129S2Pas: 3.6 ± 1.6 % in trial 1, 39.3 ± 6.7 % in trial 20; 129S2Hsd: 0.1 ± 0.1 % in trial 1, 17.9 ± 5.9 % in trial 20), whereas 129S2Pas mice overall spent more immobile than 129S2Hsd mice (table S1).
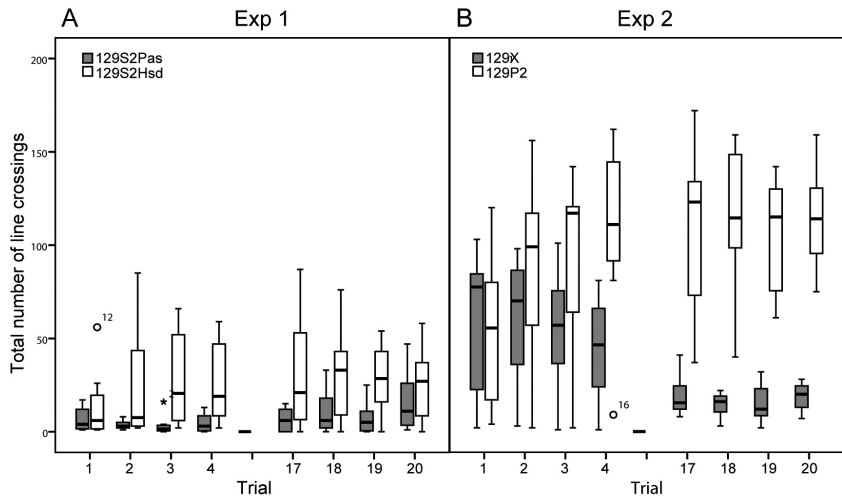

Fig. 3: Total number of line crosses (median ± IQR) on the first day and last day of testing (trials 1-4 and 17-20) in 129S2 mice (3A) and 129X1 and 129P2 mice (3B).

Table 1: Plasma corticosterone (CORT) levels before and after testing in both experiments. Data is represented in nmol/liter (± SEM) ns= non-significant effect

Strain	Statistics	pCORT	
		Before testing	After testing
Experiment 1			
129S2Pas	ns	371.06 ± 123.85	245.18 ± 60.95
129S2Hsd	ns	324.86 ± 92.99	144.41 ± 29.46
Experiment 2			
129X1	ns	79.74 ± 28.19	137.65 ± 48.67
129P2	ns	210.06 ± 74.27	103.51 ± 36.60

EXPERIMENT 2: 129X1 VS. 129P2

Significant strain, trial and strain x trial effects were found for the total number of line crossings (Fig 3B, strain: $F_{1,16} = 37.80$, P<0.001; trial: $F_{19,302.1} = 2.60$, P<0.001; strain x trial: $F_{19,302.0} = 7.67$, P<0.001) and the latency until the first line crossing (strain: $F_{1,16} = 25.58$, P<0.001; trial: $F_{19,302.0} = 6.56$, P<0.001; strain x trial: $F_{19,302.0} = 5.02$, P<0.001). Whereas 129X1 mice showed a decrease in line crossings, 129P2 mice showed a general increase in line crossings across the experimental period. Significant effects were further found for immobility duration (strain: $F_{1,16} = 40.76$, P<0.001; trial: $F_{19,302.0} = 19.12$, P<0.001; strain x trial: $F_{19,302.0} = 10.47$, P<0.001) and latency until the first immobility event (strain: $F_{1,16} = 12.88$, P<0.001; trial: $F_{19,302.0} = 19.52$, P<0.001; strain x trial: $F_{19,302.0} = 2.10$, P<0.01). Both strains showed increased immobility duration over time (129X1: 0 ± 0 % in trial 1, 56.5 ± 6.3 % in trial 20; 129P2: 0 ± 0 % in trial 1, 7.9 ± 3.7 % in trial 20), however 129X1 mice were more immobile than 129P2 mice (table S1).

GENERAL EXPLORATION
EXPERIMENT 1: 129S2PAS VS. 129S2HSD

Significant strain effects were found for the total number of rearings ($F_{1,20.8} = 5.900$, P<0.05) and latency until the first rearing in the box ($F_{1,20.8} = 11.762$, P<0.001). 129S2Hsd mice showed more rearings than 129S2Pas mice. More specifically, there was an increase in rearings over time in 129S2Hsd mice (trial 1: 3 ± 1.5, trial 20: 8 ± 5.5) and the amount of rearings in 129S2Pas mice stayed at the same level over time (trial 1: 3 ± 2, trial 20: 1 ± 1.5). The latency until the first rearing increased during the experimental period (129S2Pas: 98.6 ± 33.8 in trial1, 210.0 ± 60.7 in trial 20; 129 S2Hsd: 128.3 ± 13.9 in trial 1, 184.2 ± 16.5 in trial 20), indicated by a general trial effect ($F_{19,304} = 2.396$, P<0.001). No significant effects were found for the number of rearings on the board or latency until first rearing on the board, since both strains hardly displayed this behaviour. Significant trial effects were found for the number of hole explorations and ($F_{19,304} = 15.934$, P<0.001) and latency until the first hole exploration ($F_{19,304} = 6.746$, P<0.001). Both strains showed a general decrease in hole exploration (129S2Pas: 16 ± 9.5 in trial1, 4 ± 2 in trial 20; 129 S2Hsd: 9 ± 12 in trial 1, 2 ± 1 in trial 20) across the experimental period.

EXPERIMENT 2: 129X1 VS. 129P2

Significant strain x trial interaction ($F_{19,302.0} = 5.89$, P<0.01) effects were found for the number of rearings in the box. Whereas the number of rearings in 129X1 mice remained unchanged (trial 1: 3 ± 11, trial 20: 3 ± 5), 129P2 mice showed an increased number of rearings across the experimental period (trial 1: 0 ± 1, trial 20: 12 ± 9). Significant trial ($F_{19,302.0} = 2.164$, P<0.01) and strain x trial interaction ($F_{19,302.0} = 2.335$, P<0.01) effects were found for the latency until the first rearing in the box, both strains showed an increase in latency across the experimental period (129X1: 169.0 ± 35.3 in trial1, 137.3 ± 26.2 in trial 20; 129P2: 242.8 169.0 ± 32.0 in trial 1, 120.0 ± 31.8 in trial 20). Both 129P2 and 129X1 mice hardly displayed any rearings on the board, so no significant effects on numbers or latency regarding this behaviour were found. Significant trial effects for the number of hole explorations ($F_{19,302.0} = 16.37$, P<0.001) and the latency until the first hole exploration ($F_{19,302.0} = 23.15$, P<0.001) were found. Both strains showed a clear decrease in number of hole explorations (129X1: 13.5 ± 26 in trial1, 0 ± 1 in trial 20; 129P2: 15.5 ± 9 in trial 1, 1.5 ± 4 in trial 20) and increased latency to explore holes across the experimental period (table S1).

DIRECTED EXPLORATION
EXPERIMENT 1: 129S2PAS VS. 129S2HSD

No significant effects were found on the number of hole visits, since both strains hardly visited the holes. Nevertheless, a significant trial effect ($F_{19,304}$ = 2.303, P<0.01) was found for latency until the first hole visit, as both strains showed a small decrease in latency across the experimental period (table S1).

EXPERIMENT 2: 129X1 VS. 129P2

A significant strain effect ($F_{19,318.0}$ = 5.58, P<0.05) was found for the number of hole visits, although no significant effects were found for the latency until the first hole visit and both strains hardly visited the holes (table S1).

FOOD INTAKE INHIBITION
EXPERIMENT 1: 129S2PAS VS. 129S2HSD

Significant strain effects ($F_{1,16}$ = 5.533, P<0.001) were found for the latency until the first exploration of the unfamiliar food, as 129S2Hsd mice showed a lower latency (trial 1: 256.7 ± 22.1, trial 20: 273.0 ± 27.0) than 129S2Pas mice (trial 1: 300.0 ± 0, trial 20: 300.0 ± 0). Strain ($F_{1,17}$ = 9.371, P<0.01), trial ($F19,199.6$ = 2.447, P<0.01) and strain x trial effects ($F_{19;199.6}$ = 2.181, P<0.01) were found for the latency until the first exploration of the familiar food. Only the 129S2Hsd strain showed a decrease in latency to explore the familiar food (129S2Hsd: 205.6 ± 34.6 in trial 1, 155.0± 36.4 in trial 20; 129S2Pas: 276.6 ± 23.2 in trial 1, 244.4 ± 27.3 in trial 20). 129S2Hsd mice showed a lower latency compared to 129S2Pas.

EXPERIMENT 2: 129X1 VS. 129P2

A significant trial ($F_{19,302.0}$ = 3.79, P<0.01) and strain x trial interaction ($F_{19,302.0}$ = 3.67, P<0.01) effect was found for the latency until the first exploration of the familiar food. 129P2 mice showed a clear decrease in latency across the experimental time period (trial 1: 266.5 ± 23.5, trial 20: 152.5 ± 47.0), whereas this was not observed in 129X1 mice (trial 1: 186.6 ± 44.4, trial 20: 253.9 ± 30.2). This was also found for the latency until the first exploration of the unfamiliar food (trial: $F_{19,302.0}$ = 1.92, P<0.05; strain: $F_{1,16}$ = 5.062, P<0.05). 129P2 mice showed a decrease in latency across the experimental period in contrast to 129X1 mice that did not show this decrease (129P2: 193.6 ± 37.1 in trial 1, 60.9 ± 35.0 in trial 20; 129 X1: 183.7 ± 44.2 in trial 1, 181.4 ± 35.7 in trial20).

AROUSAL/DE-AROUSAL
EXPERIMENT 1: 129S2PAS VS. 129S2HSD

Significant trial and strain x trial effects were found for the total time spent grooming (trial: $F_{19,304}$ = 3.173, P<0.001; strain x trial: $F_{19,304}$ = 2.386, P<0.001), the latency until the first self-grooming event (trial: $F_{19,304}$ = 3.141, P<0.001; strain x trial: $F_{19,304}$ = 1.908, P<0.05) and the total number of self-grooming bouts (trial: $F_{19,304}$ = 2.861, P<0.001; strain x trial: $F_{19,304}$ = 1.784, P<0.05). Both strain showed an increase in grooming behaviour across the experimental period (129S2Pas: 0.0 ± 0 % in trial 1, 0.3 ± 0.2 % in trial 20; 129S2Hsd: 0.0 ± 0 % in trial 1, 2.1 ± 0.4% in trial 20). No significant effects were found for the number of defecations.

EXPERIMENT 2: 129X1 VS. 129P2

Significant trial, strain and strain x trial interaction effects were found for the total time spent grooming (trial: $F_{19,302.0} = 3.58$, P<0.01; strain: $F_{1,16} = 11.76$, P<0.05; strain x trial: $F_{19,302.0} = 2.17$, P<0.05), the latency until the first grooming event (trial: $F_{19,302.0} = 5.23$, P<0.01; strain: $F_{1,16} = 27.97$, P<0.01; strain x trial: $F_{19,302.0} = 2.45$, P<0.01) and the number of grooming events (strain: $F_{1,16} = 19.04$, P<0.01; trial: $F_{19,302.0} = 3.29$, P<0.01). Both strains showed an increase in grooming duration (129X1: 0.2 ± 0.2 % in trial 1, 5.0 ± 2.8 % in trial 20; 129P2: 0.4 ± 0.2 % in trial 1, 0.6 ± 0.3% in trial 20) and grooming events, although this was clearer for 129X1 mice (table S1).

Significant trial ($F19,302.0 = 3.06$, P<0.001) and strain x trial interaction ($F_{19,302.0} = 1.74$, P<0.05) effects were found for the number of defecations (table S1), whereas only 129X1 mice showed a decrease in number of produced boli during the experimental period (trial 1: 6 ± 3, trial 20: 3 ± 2).

CORTICOSTERONE

Corticosterone data is represented in table 1.

EXPERIMENT 1: 129S2PAS VS. 129S2HSD

No significant differences were found between basal/non-basal CORT values or between the two strains.

EXPERIMENT 2: 129X1 VS. 129P2

No significant differences were found between basal/non-basal CORT values within both strains nor were there significant strain differences. Non-basal values of 129P2 mice showed a trend to be higher than those of basal levels, although this difference did not reach significance (t=-2.602, p= 0.032, corrected p< 0.0167).

DISCUSSION

Like previously reported for 129P3/J mice (Salomons, *et al* 2010; Salomons, *et al* 2010a; Salomons, *et al* 2010b), the four substrains of the 129 family tested in the present study showed a lack of habituation of avoidance behaviour towards an initially novel area over time. Thus the 129 mouse strain seems to be characterized by a distinct profile that implies difficulties coping with environmental changes, although distinct substrain differences were found at the behavioural level as well. Notably, habituation reflects the adaptive process of integrating emotional and cognitive processes in order to enable an organism to adequately respond to changes in the environment (e.g. waning of an initial anxiety response after repeated exposure to the same stimulus (Bolivar 2009; Eisenstein and Eisenstein 2006)). A slow or impaired habituation can then be understood to indicate an inability to adapt and might therefore endanger an animals' welfare if it is exposed to (even mild) environmental challenges (Salomons, *et al* 2010a). Based on this hypothesis it has to be concluded that guidelines regarding husbandry and experimental procedures should account for strain- or even substrain-specific adaptive capacities in laboratory mice. In previous studies in which an identical test set-up and experimental procedure was used, C57BL6N male mice revealed a low level of avoidance behaviour during the initial exposure as well as over time (Ohl, *et al* 2001a; Ohl, *et al* 2003), while both DBA2 (Ohl, *et al* 2003) and BALB/c (Salomons, *et al* 2010a) mice displayed a high initial avoidance followed by a decrease in avoidance behaviour over time resulting in a stable baseline-level around the third day of testing. In contrast, all

129 substrains tested in previous (Salomons, *et al* 2010; Salomons, *et al* 2010a; Salomons, *et al* 2010b) and the present study fail to reveal a decrease in avoidance behaviour and even show an increase of this behaviour over time (Fig. 1 and 2). For a correct interpretation of such a behavioural phenomenon it is important to exclude potential confounding factors of the readout parameters. A lack of exploration of a distinct area for example might as well be the result of a low level of overall activity. The persisting level of high avoidance behaviour in 129 substrains therefore might have been a secondary effect to changes in other behavioural domains. Thus, in addition to different parameters indicating avoidance behaviour, we simultaneously investigated general exploratory and locomotor activity and found different behavioural profiles in the 129 mouse substrains. At first animals of the 129P2 substrain revealed only minor changes in locomotor activity over time (Fig 3, table S1), which is comparable to the previously tested 129P3 mice (Salomons, *et al* 2010; Salomons, *et al* 2010a). Additionally an increase in general exploratory activity (number of rearings) over time was found in this substrain (table S1), indicating that overall activity levels did not cause the increase in avoidance behaviour over time. Rather, this behavioural profile indicates that animals of the 129P2 substrain indeed are likely to be limited in their ability to adapt to novelty.

In contrast, 129S2 (129S2Hsd and 129S2Pas) mice were generally more inactive in comparison with the other 129 substrains. Especially 129S2Pas mice were immobile for about 50% of the total testing time. Further, they showed high latencies of the first line crossing and a low total number of line crossings already after the first couple of trials (Fig 3, table S1). In addition the rearing activity in this substrain was very low (table S1). Both the 129S2Hsd and the 129S2Pas substrain revealed, in accordance with the findings of others (Cook, *et al* 2002; De Visser, *et al* 2006; Pratte and Jamon 2009; Sik, *et al* 2003), low levels of locomotor and exploratory activity. A final interpretation of habituation profiles in these substrains thus remains difficult since either high avoidance behaviour may be caused by a low general activity, or this low general activity might be caused by high anxiety via behavioural inhibition (Gray 1982).

Conversely, the 129X1 substrain showed a more gradual increase in immobility and decrease in locomotor activity over time than the 129S2 substrains. Those animals initially displayed by equal amounts of line crossings as 129P2 mice, but locomotor activity decreased over time (Fig 3, table S1) as found before by Tang *et al*. (Tang and Sanford 2005). In consistency with findings on locomotor activity, the number of rearings decreased over time (table S1). This profile may suggest that the animals' during initial trials did gather sufficient information to conclude that there was no further need to explore the testing environment. However, the general decrease in activity makes it difficult to assess if the increase of avoidance behaviour seen in 129X1 mice can be considered adaptive (i.e. an adequate response to sufficient information gathering) or non-adaptive (i.e. an inability to overcome novelty-induced avoidance).

Based on the fact that habituation is a cognitive process (Bolivar 2009; Eisenstein and Eisenstein 2006; O'Keefe 1999) one may argue that a lack of habituation can be based on primary cognitive deficits. Indeed some 129 substrains have been described to be impaired in novel object learning when compared to other strains (Kim, *et al* 2005; Montkowski, *et al* 1997; Sik, *et al* 2003). However, in a previous experiment it was shown that 129P3 mice were able to discriminate between a novel and a familiar object in a 1-trial object recognition paradigm (Salomons, *et al* 2010a). A comparable indication of 1-trial recognition abilities is integrated

in the test procedure used in the present study by investigating the level of recognition of a familiar food object in comparison with an unfamiliar food object in the first trial (Ohl, *et al* 2001a). However, in none of the substrains tested here a significant discrimination between the two food objects could be observed during the first trial, which might be the result of the relatively high initial behavioural inhibition in all animals, resulting in exploration of both the familiar and the unfamiliar food only late or not at all (table S1). However, further investigation on cognitive abilities of the 129P2 strain (that showed a high discriminative learning over time in the present experiment) in the cognitive version of the modified hole board (Salomons, *et al* 2012 in press) indicates that at least spatial cognition is not impaired in this substrain, indicating that the increase in avoidance behaviour over time as seen here is probably not caused by cognitive dysfunctions.

The present study revealed similarities, but also considerable substrain specific differences in behaviour, comparable to other studies (Bothe, *et al* 2004; Bothe, *et al* 2005; Cook, *et al* 2002; Simpson, *et al* 1997; Tang and Sanford 2005). It is important to consider that the behavioural phenotype of animals is not only determined by genetic background but also by environment and gene-environment interactions (Crabbe, *et al* 1999; van der Staay, *et al* 2010). Notably, 129S2Hsd and 129S2Pas mice tested in the present study derive from the same 129S2/Sv strain, but are being kept as separate breeding colonies at different breeders. Thus, differences in behaviour as found here might be due to different environmental conditions at the breeding facilities as well as genetic drift (Casellas 2011).

However, we also found that the behavioural profile of 129P2 mice is comparable to that of the 129P3 strain tested in earlier studies (Salomons, *et al* 2010; Salomons, *et al* 2010a; Salomons, *et al* 2010b). Although these two strains are closely related (Simpson, *et al* 1997) 129P3 and 129P2 mice are derived from different breeders as well. The fact that two genetically similar substrains (129S2HSd and 129S2Pas) can considerably differ, while two genetically different substrains (129P3 and 129P2) show similar profiles may be the result of innumerable environmental and genetic factors which are difficult to pinpoint (Chesler, *et al* 2002).

In line of this reasoning and based on our substrain comparison, we conclude, however, that the genetic background of the 129 substrains is likely to cause an increased vulnerability for a quite limited ability to adapt to novel environments.

WELFARE IMPLICATIONS

Processes of adaptation are considered of potential relevance for our understanding of animal welfare: The concept of allostasis for example (Korte, *et al* 2007) states that an animal's welfare is not impaired as long as animals are able to adapt to (changing) environmental challenges such as exposure to novelty. Thus a high anxiety characteristic might not necessarily be detrimental to an animal's state of welfare as long as an individual is able to adapt, resulting in a decrease in anxiety towards a specific challenge over time (Ohl, *et al* 2008; Ohl and van der Staay 2012; Salomons, *et al* 2009). Given that the process of selection and inbreeding in laboratory mice has resulted in the (unintentional) co-selection of emotional traits (see for example (Belzung and Berton 1997; Clément, *et al* 2002; Jensen 2010; Laarakker, *et al* 2008; Ohl, *et al* 2003; Ohl, *et al* 2008)), we hypothesize that standard housing and treatment protocols for mice as a species may be insufficient to safeguard the welfare of different mouse (sub)strains equally. Compared to other strains, different 129 substrains reveal a reduced

ability to adapt to exposure to novelty. In addition others have found that several 129 substrains (including 129P3 and 129X1) also show impaired fear extinction (Camp, *et al* 2009; Hefner, *et al* 2008). Together with our results, this suggests that several 129 substrains have difficulties to cope with mild and more severe negative events. Although the question whether the welfare state of these mice might be generally compromised under standard laboratory housing conditions remains to be investigated, it is to be expected that environmental changes (such as transport between facilities or rooms, changes of light-regime, cage cleaning, or enrichment replacement) might be of higher impact on 129 substrains than in other strains. It might therefore be useful to consider guidelines and protocols that take into account the adaptive capabilities of mice at the strain- or even substrain-level.

ACKNOWLEDGMENTS

SUPPLEMENTARY INFORMATION

Table S1: Overview of the behavioural data in both experiments; data from the first (trial 1) and last (trial 20) trial are represented. Continuous data are represented as mean ± SEM and discrete data as median ± IQR. Mixed model analysis was used, the best fitting model was used per behavioural parameter (1 m = fixed model (fixed intercept/fixed slope), 2 m = random intercept (random intercept/fixed slope), 3 m = random intercept and slope (random intercept/random slope)). P<0.05 was considered significant. nr: number, s: seconds, T: trial, S: strain, S*T: strain x trial interaction and ns: non-significant.

Behavioural category and parameters	Experiment 1 Statistics	129S2Pas Trial 1	129S2Pas Trial 20	129S2Hsd Trial 1	129S2Hsd Trial 20	Experiment 2 Statistics	129X1 Trial 1	129X1 Trial 20	129P2 Trial 1	129P2 Trial 20
Avoidance										
Latency until first board entry [sec]	T, S*T (3m)	199.7±36.0	235.4±17.5	123.2±27.8	276.6±13.6	T, S (2m)	75.0±19.1	251.4±24.1	34.7±12.3	205.1±25.0
Total time spent on board [%]	T, S*T (3m)	25.6±10.5	1.9±1.1	47.9±9.1	0.8±0.4	T (2m)	31.5±11.3	0.7±0.5	42.1±9.7	2.7±1.3
Total number of board entries [nr.]	T, S*T (3m)	3±1.5	2±0.5	5±3.5	1±0	T, S*T, S (2m)	7.5±7	0±2	15.5±10	2±2
Risk assessment										
Total number of stretched attends [nr.]	T (3m)	7±12	4±0.5	2±17.5	3±1	T (2m)	6±10	0±1	7±12	0±0
Latency until first stretched attend [sec]	T (3m)	10.2±5.8	212.6±122.2	7.3±3.2	197.7±89.5	T (2m)	52.3±33.9	279.1±19.0	42.3±36.8	300.0±0
Locomotion										
Total number of line crossings [nr.]	S, S*T (3m)	13±4	28±11	22±6	35±27	T, S*T, S (2m)	77.5±65	20±12	55.5±70	114±42
Latency until first line crossing [sec]	S, T, S*T (3m)	104.4±30.0	143.7±30.4	87.0±25.7	116.1±42.3	T, S*T, S (2m)	7.6±1.2	124.8±27.2	13.8±4.9	9.1±4.1
Total time spent immobile [%]	S, T (3m)	3.6±1.6	39.3±6.7	0.1±0.1	17.9±5.9	T, S*T, S (2m)	0.0±0.0	56.5±6.3	0.0±0.0	7.9±3.7
Latency until first immobility [sec]	S, T (3m)	224.5±83.1	138.6±30.5	271.8±79.9	147.6±32.6	T, S*T, S (2m)	300.0±0.0	2.7±0.2	300.0±0.0	57.3±35.8

General exploration

Total number of rearings in the box [nr.]	S (3m)	3 ± 2	1 ± 1.5	3 ± 1.5	8 ± 5.5	S*T (2m)	3 ± 11	3 ± 5	0 ± 1	12 ± 9
Latency until first rearing in the box [sec]	S, T (2m)	98.6 ± 33.8	210.0 ± 60.7	128.3 ± 13.9	184.2 ± 16.5	T, S*T (2m)	169.0 ± 35.3	137.3 ± 26.2	242.8 ± 32.0	120.0 ± 31.8
Total number of rearings on the board [nr.]	ns (2m)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	ns (1m)	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Latency until first rearing board [sec]	ns (2m)	300.0 ± 0.0	300.0 ± 0.0	300.0 ± 0.0	300.0 ± 0.0	S (1m)	300.0 ± 0.0	300.0 ± 0.0	300.0 ± 0.0	300.0 ± 0.0
Total number of hole explorations [nr.]	T (2m)	16 ± 9.5	4 ± 2	9 ± 12	2 ± 1	T (2m)	13.5 ± 26	0 ± 1	15.5 ± 9	1.5 ± 4
Latency until first hole exploration [sec]	T, S*T (3m)	186.0 ± 112.0	147.5 ± 86.1	107.1 ± 47.4	176.2 ± 41.3	T (2m)	34.8 ± 15.7	267.9 ± 16.2	44.0 ± 8.0	226.7 ± 17.3

Directed exploration

Total number of hole visits [nr.]	ns (2m)	0 ± 0	0 ± 0.5	0 ± 0	0 ± 0	S (1m)	0 ± 1	0 ± 0	0 ± 1	0 ± 0
Latency until first hole visit [sec]	T (2m)	300.0 ± 0.0	274.5 ± 19.0	300.0 ± 0.0	281.5 ± 13.6	ns (1m)	300.0 ± 0.0	300.0 ± 0.0	256.2 ± 33.0	290.1 ± 9.9

Food intake inhibition

Latency first exploration unfamiliar food [sec]	S (2m)	300.0 ± 0.0	256.7 ± 22.1	273.0 ± 27.0	155.0 ± 36.4	T, S*T, S (2m)	183.7 ± 44.2	181.4 ± 35.7	193.6 ± 37.1	60.9 ± 35.0
Latency first exploration familiar food [sec]	S, T, S*T (3m)	276.8 ± 23.2	244.4 ± 27.3	205.6 ± 34.6	155.0 ± 36.4	T, S (2m)	186.6 ± 44.4	253.9 ± 30.2	266.5 ± 23.5	152.5 ± 47.0

Arousal

Total time spent grooming [%]	T, S*T (2m)	0.0 ± 0.0	0.3 ± 0.2	0.0 ± 0.0	2.1 ± 0.4	T, S*T, S (2m)	0.2 ± 0.2	5.0 ± 2.8	0.4 ± 0.2	0.6 ± 0.3
Latency until first self groom [sec]	T, S*T (2m)	300.0 ± 0.0	267.3 ± 23.2	300.0 ± 0.0	184.9 ± 29.1	T, S*T, S (2m)	282.4 ± 12.3	165.0 ± 37.9	270.4 ± 14.5	268.2 ± 17.1
Total number of self-groomings [nr.]	T, S*T (2m)	0 ± 0	0.5 ± 0.5	0 ± 0	1.5 ± 0.5	T, S (2m)	0 ± 1	1.5 ± 3	0 ± 1	0.5 ± 2
Total number of boli [nr.]	ns (2m)	3 ± 1	2 ± 1.5	2 ± 2.5	3 ± 3	T, S*T (2m)	6 ± 3	3 ± 2	5 ± 7	4 ± 7

Escape

Total number of jumps [nr.]	ns (2m)	0 ± 0	0 ± 0	0 ± 0	0 ± 0	ns (2m)	0 ± 0	0 ± 0	0 ± 0	0 ± 0
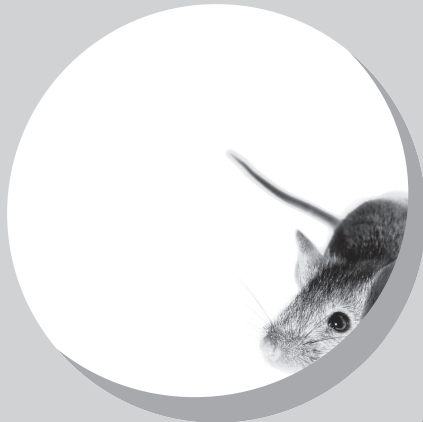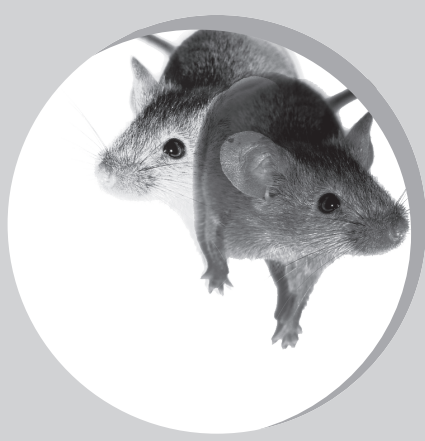
CHAPTER 3

EFFECT OF CHRONIC SOCIAL STRESS ON BEHAVIOURAL HABITUATION TOWARDS NOVELTY IN CD1 MICE

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ABSTRACT

Chronic stress is a known risk factor for the development of affective disorders in humans and animal models are used to better understand the underlying mechanisms of this effect. Recently, a novel chronic social stress (CSS) protocol has been developed for mice making use of social instability in adolescence and early adulthood. This protocol has been shown to cause an increase in HPA-axis activity and induce an increase in state anxiety. Still, these changes may as well represent adaptive responses instead of modelling pathological changes. Therefore the aim of the present experiment was to investigate the effect of the CSS protocol on adaptive capacities of CD1 mice via the measurement of behavioural habituation in a mildly aversive testing environment, the modified hole board (mHB). One group of mice, the CSS group, was exposed to the CSS protocol for 7 weeks and compared with mice housed in a stable social group, the SH group, in their behavioural and physiological responses. Our results confirm that application of the CSS protocol was indeed stressful for the mice, which is indicated by a decrease in body weight gain and fur condition, increased left adrenal weights and a decreased GR mRNA expression in the CA1 and the dentate gyrus. However, the effects of CSS did not appear to exceed adaptive capacities of the mice, i.e. after termination of the stress animals revealed normal habituation profiles. Further, a responder/non-responder effect in the CSS group indicated that some mice were more susceptible to the effects of stress than others.

INTRODUCTION

Chronic stress is known to be a risk factor for the development of affective disorders such as depression and anxiety related psychopathologies (Arborelius, *et al* 1999; Leonard 2007). In preclinical research, several paradigms have been developed to investigate the effects of chronic stress in laboratory rodents (Ducottet, *et al* 2003; Griebel, *et al* 2002; Mineur, *et al* 2003). For example the chronic mild stress (or CMS) paradigm, consisting of intermittent exposure to a range of unpredictable stressors, has been validated to cause depression like behaviour (Pothion, *et al* 2004; Strekalova, *et al* 2004; Willner, *et al* 1987) and increased anxiety-related behaviour in mice (Ducottet and Belzung 2004; Ducottet, *et al* 2004; Schweizer, *et al* 2009; Strekalova, *et al* 2004) .

However, depending on the type of protocol used, CMS has been reported not to affect anxiety related behaviour (Mineur, *et al* 2006) or even to cause anxiolytic effects (Mineur, *et al* 2006; Griebel, *et al* 2002; Schweizer, *et al* 2009) as well. It has therefore been proposed to use social factors as stressor that might have a higher biological relevance for a social species: For example mice (Bartolomucci, *et al* 2004) and rats (Blanchard, *et al* 1995) that have been exposed to repeated social defeat or that had been living in an unstable social environment (Baranyi, *et al* 2005; Haller, *et al* 1999; Maslova, *et al* 2010; Schmidt, *et al* 2007; Schmidt, *et al* 2010a) develop signs of chronic stress.

Recently, a chronic social stress (CSS) protocol for mice was developed by Schmidt *et al*. (Schmidt, *et al* 2007; Schmidt, et al 2008; Schmidt, *et al* 2010b) in which social instability is experimentally induced within a 7 weeks period during adolescence and early adulthood. This stage of development had been chosen because social factors appear to be especially important during the development of the brain in these periods of life and stressors are thought to have a high impact as it is a crucial period to develop social skills (Sachser, *et al* 1998). In short, the CSS protocol consisted of group composition changes of male CD1 mice in the home cage twice a week making it impossible for the mice to form a stable social hierarchy. This resulted in physiological and behavioural symptoms of chronic stress, for example, adrenal weights and morning corticosterone concentrations were increased and the mice showed indications of an increased state anxiety in the elevated plus maze and a suppression of familiar food intake in a novel environment (Schmidt, et al 2007). Further indications of chronic stress effects were found at the central nervous level, such as a decrease in expression of glucocorticoid receptors (GRs) and in mineralocorticoid receptors (MRs) in different brain areas (Schmidt, *et al* 2007; Sterlemann, *et al* 2008).

Although the CSS protocol in mice has proven to be effective in inducing for example anxiety and depression related behaviours, it is not known if such responses may be adaptive, i.e. if they represent a stress induced increase in anxiety that might actually help an animal to cope with and survive in a potential threatening environment (such as a testing environment) (Koolhaas, *et al* 1999). We have argued (Ohl, *et al* 2008; Salomons, *et al* 2009) and shown before that indeed in rodents high anxiety, for example in the BALB/c inbred mouse (Salomons, *et al* 2010; Salomons, *et al* 2010a) and in high anxiety (HAB) rats (Ohl, *et al* 2002), is not necessarily indicative of a pathological condition in that adaptive capacities are exceeded, but that acute high anxiety-responses still might be followed by rapid habituation. We therefore were interested to see whether the effects of the CSS protocol on acute behavioural responses in CD1 mice as found by Schmidt *et al*. would persist after termination of the stressful situation. In the present experiment, thus, the effects of the CCS protocol in CD1

mice on adaptive capacities was investigated by evaluating their behavioural habituation (behavioural change over time) to a mildly aversive testing environment, the mHB (Ohl, *et al* 2001; Ohl, *et al* 2001a; Salomons, *et al* 2010; Salomons, *et al* 2010a; Salomons, *et al* 2010b). In addition the effects of CSS on several physiological parameters (body weight, fur condition, plasma levels of CORT, adrenal weight and brain GR and MR mRNA expression) were investigated.

MATERIALS AND METHODS
ETHICAL NOTE

The protocol of the experiment (DEC-DGK number 2010.I.02.028) was peer reviewed by the scientific committee of the department of Animals in Science and Society, Utrecht University, The Netherlands, and approved by the Animal Experiments Committee of the Academic Biomedical Centre, Utrecht-The Netherlands. The Animal Experiments Committee based its decision on "De Wet op de Dierproeven" (The Dutch "Experiments on Animals Act" 1996) and on the "Dierproevenbesluit" (The Dutch "Experiments on Animals Decision", 1996). Both documents are available online at: http://wetten.overheid.nl. Further the animal experiments followed the "Principles of laboratory animal care" and refer to the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioural Research (National Research Council 2003).

ANIMALS AND GENERAL HOUSING CONDITIONS

All care taking and experiments were performed during the dark phase by well-trained members of the laboratory. A total of 48 male CD1 mice (26-28 days old at arrival) were ordered from Charles River Laboratories (Suzfeld, Germany) and housed socially in groups of 4 in Macrolon Eurostandard Type III cages (Technilab, Italy). The cages were enriched with a plastic shelter (Mouse House Techniplast®) and weekly provided with a tissue (Kleenex® Facial Tissue Kimberly-Clark). Mice were randomly assigned to either the Chronic Social Stress group (CSS) consisting of subgroups CSS1 (tested, n=10) and CSS2 (not tested, n=22) or to the Socially Housed control group (SH) consisting of subgroups SH1 (tested, n=10) and SH2 (not tested, n=6), see Fig. 1. These groups were housed separated from each other in individually ventilated animal rooms of the Netherlands Vaccine Institute (Bilthoven, The Netherlands), with temperature (± 22 °C) and humidity control (45-55%). Mice chow (CRM, Expanded, Special Diets Services Witham, England) and tap water were available *ad libitum*.
Mice were housed under a reversed 12/12h dark/light cycle (lights on 18.00 h and off 6.00 h, when the red light was turned on). One day after arrival the mice were chipped for individual identification. Eight weeks after arrival (after the CSS protocol in the CSS group) the mice were housed individually in cages of the same size and mice that were assigned to be tested in the mHB were moved to the room were the actual testing would take place. In this room the testing equipment was already installed and all other conditions were similar to those described above.

EXPERIMENTAL PROCEDURES
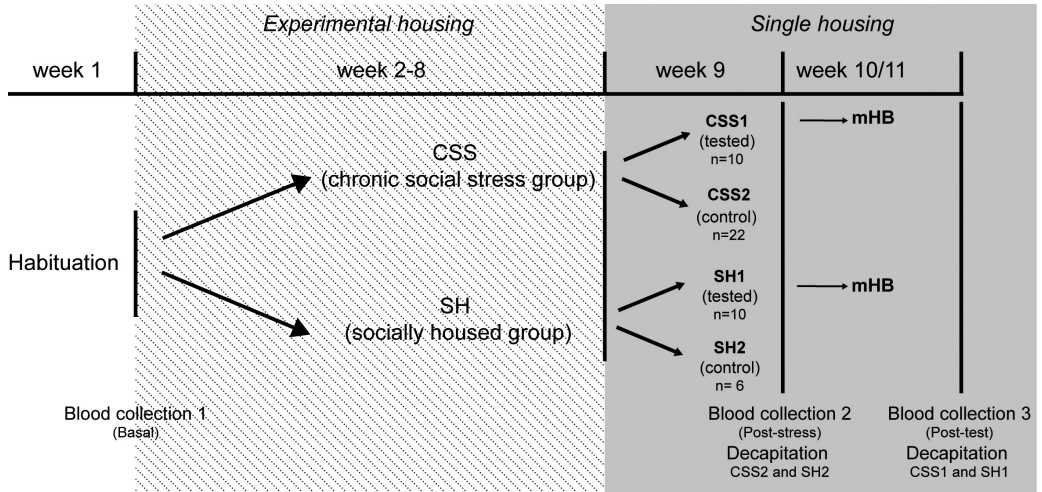An overview of the groups and experimental procedures over is shown in Fig. 1.

Fig 1. Overview of the experimental procedures over time: only mice from the CSS group underwent the chronic social stress procedure, mice from the SH group were housed in stable social groups from week 1 to 9. CSS2 and SH2 were decapitated after 1 week of single housing (week 9) and brain served as untested controls. Groups CSS1 and SH1 were tested in the mHB and decapitated after 5 days of testing in week 10 or 11.

CHRONIC SOCIAL STRESS (CSS) PROTOCOL
The CSS protocol was performed during adolescence and early adulthood of the mice; from 5 weeks of age until 12 weeks of age. After a habituation period of 1 week the CSS protocol started for mice in the CSS1 and CSS2 group (see Fig. 1). The group composition of the mice was changed twice a week for seven weeks in such a way that four mice from different cages were placed in a novel clean cage. The rotation schedule was based on the one previously used in the experiments from M. V. Schmidt (see for example (Schmidt, *et al* 2007)), which was kindly provided. This schedule was designed in such a way that the likelihood of a repeated encounter of the same mice during the course of the procedure was minimized. Every time the mixture procedure was performed in the CSS group, another person handled the mice in the SH group. This same person did the behavioural testing and never performed the mixing procedure to prevent association of this person with stress. Mice causing wounds in fights and mice too severely wounded because of fighting (only occurring in the CSS groups) were excluded from the experiment. In total 3 mice were excluded because of high aggression and 4 mice that had severe tail wounds.

BODY WEIGHT
Animals were weighed twice a week, in the groups CSS1 and CSS2 just before placing them in a novel cage with new cage mates and in the SH1 and SH2 groups at the same time as they were handled.

FUR CONDITION

Fur condition was scored on basis of a scale (1 to 4) developed by Ducottet et. al (Ducottet, *et al* 2003; Mineur, *et al* 2003). Fur scores of the groups CSS1 and CSS2 were obtained just before the mice were placed in a novel cage with new cage mates and in the SH1 and SH2 groups at the same time as they were handled. More specifically scores were defined as follows:

1. The mouse has a well groomed fur and body. The fur is smooth and shiny, with no tousled, spiky patches. Mice have long, normal whiskers and eyes have clear conjunctivae.
2. The mouse is slightly "fluffy" with some spiky patches. The rest of the appearance is similar to that described at number 1.
3. Mice are fluffy on most parts of the body and also may have slight staining of the fur. Whiskers may be abnormally trimmed and eye conjunctivae may be slightly red.
4. Mice are fluffy, stained, dirty and may have some bald patches or traces of wounds. Eye conjunctivae are red.

BLOOD SAMPLING

Blood samples were taken on three time points during the experiment always around 15.00 h (around the time the animals would be exposed to the last trial in the mHB). Basal samples (B1) were taken via tail vein incision with a sharp razor blade 7 days after arrival in week 1. After the 7 weeks of chronic stress and 5 days of individual housing another blood sample (B2) was collected via tail vein incision in the animals that were to be tested in the mHB (groups CSS1 and SH1). Blood collection from mice in groups CSS2 (n=15) and SH2 (n=6) was done via cheek puncture (9 gauge needle) at the same time point (B2) and these animals were decapitated immediately after. The third blood sample (B3) was taken from the animals that were tested in week 10 and 11 (SH1 and CSS1) via cheek puncture before decapitation. Since another investigation on the blood was planned as a separate experiment (looking at the effect of the procedure on immunological parameters) we decided to use a cheek puncture in the animals that were to be decapitated (and not just trunk blood). All blood sampling and decapitations took place in another room than the animals were housed in. The samples derived by tail vein incision were collected and stored in pre-chilled Microvette tubes (CB300, Sarstedt, Numbrecht, Germany) containing lithium heparin. Cheek puncture blood was collected in Minicollect tubes (1 ml EDTA, Greiner Bio-One GmbH, Kremsmünster, Austria). Blood samples were centrifuged (10 min at 10,000×g, 4 °C) and stored at−20 °C until measurement. pCORT levels were measured by radioimmunoassay (RIA) according to the protocol of the supplier with an ImmuChemTM Double Antibody Corticosterone kit for rats and mice (MPI Biochemicals, Amsterdam, The Netherlands). Due to technical problems not enough blood could be obtained from a substantial amount of animals on the different sampling moments (Sample 1: 12/48, sample 2: 20/48, sample 3: 3/20 missing), resulting in a data set with a lot of missing values, thus caution has to be taken on any conclusions drawn on the pCORT analysis.

BEHAVIOUR IN THE MODIFIED HOLE BOARD

The mHB consisted of an opaque grey PVC box (100 x 50 x 50 cm) with a separate board that was made of the same material (60 x 20 x 2 cm). The board had 20 cylinders (Ø 30 mm, height 30 mm) that were attached in three lines staggered

across the board. The board was placed in the middle of the PVC box and represented the unprotected area of the test arena. The area around the board (the box) was divided into 10 rectangles (20 x 15 cm) and 2 squares (20 x 20 cm). An additional stage light (white) was installed above the mHB that was directed at the board (± 120 lux) causing it to be more illuminated than the surrounding box (1-5 lux), which was under red light conditions. To investigate food intake inhibition in the mHB all animals that were to be tested (groups CSS1 and SH1) received a piece of almond (45 mg) in the home cage three days before testing, this food object was presented in the mHB as familiar food object. The unfamiliar food object was a Dustless precision pellet (45 mg, Bio-Serv, Frenchtown, USA). The food objects were placed opposite to the corner in which the mouse was placed in the mHB both on the same distance from the wall. Each mouse was always placed in the same corner of the mHB directly from the home cage and all trials lasted 5 minutes. After each trial the mHB was cleaned with tap water on a damp towel. A total of 20 trials was performed per animal, 4 trials per day over the course of 5 days. Testing order of the animals was random per day. Behaviour was scored directly by a trained observer with the help of the computer program "The observer 5.0" (Noldus Information Technology, Wageningen, The Netherlands). Behaviour was also recorded on a dvd-recorder (Panasonic) for data storage. The following behavioural parameters were measured and assigned to different behavioural categories according to previous studies (Ohl, *et al* 2001a); *avoidance behaviour*: the latency until the first board entry, the percentage of time spent on the board and the total number of board entries; *risk assessment*: the number of stretched attend postures and the latency until the first stretched attend; *locomotor activity*: the total number of line crossings, the latency until the first line crossing, the total time spent immobile and the latency until the first immobility event; *general exploration*: the total number of rearings in the box and on the board, the latency until the first rearing in the box and on the board, the total number of hole explorations and the latency until the first hole exploration (a hole was counted as explored when the animal's nose was directed to a hole; direct contact with the hole was not necessary); *directed exploration*: the total number of holes visited, (a hole was counted as visited when the mouse dipped the nose below the rim of the hole) and the latency until the first hole visit; f*ood intake inhibition*: the latency until the first exploration of the unfamiliar and familiar food object; *arousal or de-arousal*: the percentage of time spent self-grooming, the latency until the first self-grooming event, the total number of self-grooming events and the total number of faecal boli; *escape behaviour*: the total number of jumps.

BRAINS

After decapitation the brains of the mice were immediately removed and frozen in -60 °C 2-Methylbutane which was cooled on dry ice and subsequently stored in a -80 °C freezer.

Brains were sliced in coupes of 20μm and mounted on Menzel SuperFrost Plus slides (Menzel GmbH&Co, Braunsweg, Germany) and stored at –20 °C. Brains were sliced in 5 series. One series was used for the determination of the anatomic localization of the brain areas, these slides were stained with a Nissle staining and further examined together with the brain atlas (Franklin KGB and Paxinos G 1997). Two series were used *in situ* hybridizations..

For *in situ* hybridization [33P] UTP-labelled ribonucleotide probes were used that were complimentary to the cDNA sequence for mouse GR ad MR (as described in Hesen, *et al* 1996). The antisense GR probe was transcribed using T7 polymerase from a 520 bp mouse cDNA fragment (cloned by Uwe Strakle) that was amplified from plasmid DNA by PCR. The antisense MR probe was transcribed using T3 polymerase from a 1.2 kb mouse cDNA fragment from NcoI linearized plasmid DNA (pmg MR Eco5.0, cloned by Tim Cole). Corresponding sense probes for the GR and the MR were transcribed from the same cDNA fragments using T3 and T7 polymerase, respectively.

Tissue processing and hybridization was performed as described previously (see for example (Brinks, *et al* 2007)). Briefly, the sections through the hippocampus for *in situ* hybridization were allowed to acclimatize to room temperature, fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) (pH 7.4) and washed twice in PBS. Subsequently, the sections were acetylated in 0.25% acetic anhydride in triethanolamine (pH 8.0) for 10 minutes and rinsed in 2x sodium saline citrate buffer (SSC). Thereafter, the sections were dehydrated in graded alcohol series: 50%, 80%, 100% and 100% and left to air-dry at room temperature. The [33P] labelled probes were added to the hybridization mix in an amount of 25 x 10^6 counts per ml and denatured by heating the hybridization mix for 10 minutes at 85 °C. The hybridization mix contained 50% formamide, 5x SSC, 5x Denhards reagent, 250 μg/ml tRNA baker's yeast, 500 μg/ml Sonified Salmon Sperm DNA (ssDNA). The probe was added to the slides (3 x 10^6 counts/ slide), coverslips were applied and the sections were allowed to hybridize overnight at 55 °C.

The following day, the slides were washed in 2x SSC, treated with RNAse solution and rinsed in descending SSC concentrations (1x, 0.5x, 0.1x) and subsequently dehydrated in a graded alcohol series (50%, 80%, 100%, 100%) and air-dried. When dry, the slides were exposed to CL-Xposure film Thermo Fisher Scientific Inc. USA) for 2 weeks and developed using an automated film developer XR24nova (Dürr Dental, Germany). Autoradiographs were digitalized using a high resolution scanner and relative expression of GR and MR in CA1, CA2, CA3 and Dentate Gyrus of the hippocampus was determined by measuring optical densities using Image J analysis software (NIH, USA). After background subtraction, the mean of 4 measurements from both hemispheres and from 2 different slides for each animal was calculated.

ADRENALS

After decapitation also the adrenal glands were removed. The adrenals were carefully dissected from the abdominal area and dissected further from the surrounding fat. The adrenals were then weighed and immediately after that shock frozen in ice-cold liquid nitrogen. The adrenals were stored in a -80 °C freezer.

STATISTICAL ANALYSIS

All data was analysed with the help of the statistical program SPSS 16.0 for Windows (SPSS.Inc, IL, USA). Continuous data (latency and relative duration of behavioural parameters, GR and MR optical densities, body weight and relative adrenal weights (represented as mean ± standard error of the mean (SEM)), were first tested for Gaussanity using the Kolmogorov–Smirnov test. Homoscedasticity was tested by Levine's test. Group analyses using the Kolmogorov–Smirnov one sample test revealed a non-parametric distribution of several continuous

parameters. These parameters, as well as the total number of behavioural parameters, were either log transformed or rank transformed to achieve a normal distribution of the data (Conover and Iman 1982). The (transformed) data from the mHB experiments were subsequently analysed using repeated measures ANOVA (RM ANOVA) using Huyn–Feldt adjustment (trial number as within-subject factor and treatment as between-subject factor). Survival analysis was not needed because no censored data was included. The optical densities of the GR and MR situ's and pCORT were analysed by using a linear mixed models analysis (best fitting model with fixed slope and intercept), since for pCORT there were a lot of missing values we could still take the other measures of the animals into the analysis (which is not possible with a RM ANOVA). A backward strategy was adopted in which all non-significant interaction terms were removed.

If appropriate, post hoc analyses were done using an unpaired Student t-test for continuous data and the Mann–Whitney U-test for discrete data. For ANOVA analyses, a probability value less than 0.05 was considered to be statistically significant. To minimize the risk of a Type 1 error due to multiple comparisons, the level of significance was corrected for the post hoc analyses using Dunn Sidak correction (Ludbrook 1991).

RESULTS
BODY WEIGHT

Fig. 2 represents the progression of the body weight over the whole experimental period. The RM ANOVA revealed a significant time ($F_{17, 629} = 1180.9$, p< 0.001) group ($F_{1, 37} = 9.391$, p= 0.004) and time x group interaction effect ($F_{17, 629} = 7.503$, p< 0.001). The group difference is mainly attributable to the CSS period, after that the CSS group showed a recovery of body weight towards the average weights of the SH group.
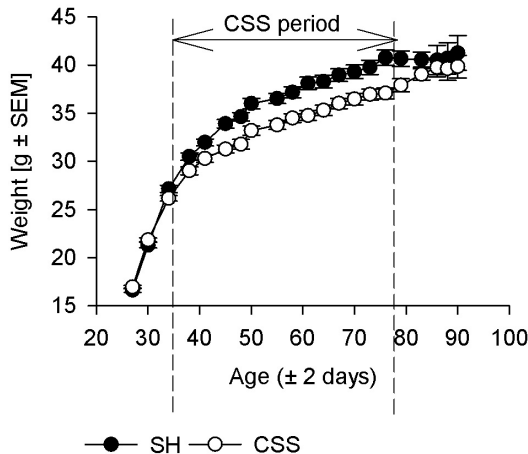

Fig 2. Body weight gain in chronically stressed (CSS) and Socially Housed mice (SH) represented as means ± SEM. A significant age, group x age interaction, and group effect was found.

FUR CONDITION

Fig. 3 shows the fur condition scores in percentages per scoring moment. Statistical analysis revealed a significant group difference ($F_{1,38} = 1827.7$, p<0.001) a significant age difference ($F_{14, 532} = 18.381$, p< 0.001) and a significant group x age interaction effect ($F_{14, 532} = 19.155$, p< 0.001). The SH group showed a good fur

condition in general, while the CSS group showed an increase in fur condition scores over time, indicating a decreased condition of the fur during progression of the CSS period in this group.
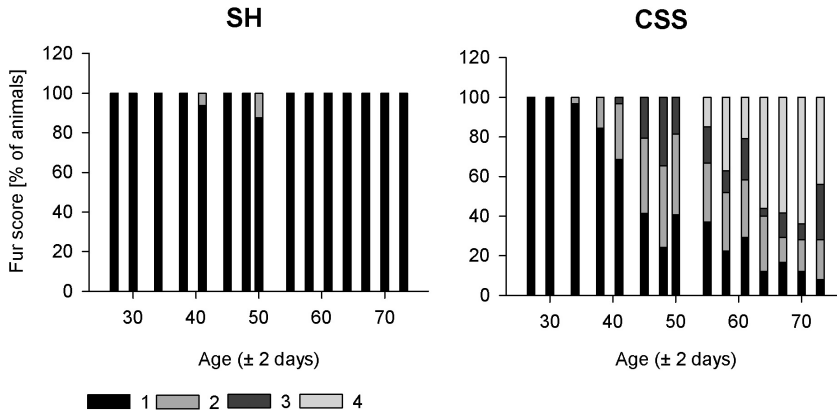

Fig 3. Body fur condition scores (represented as % of animals in a category) during the CSS period. Percentages differed significantly between groups and over time, group x time interaction was significant as well.
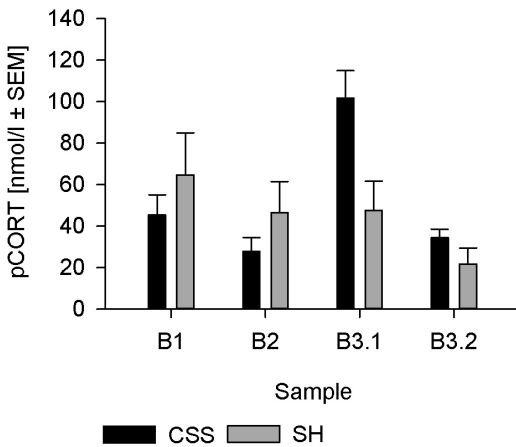

Fig 4. pCORT values represented as means (nmol/l ± SEM). No significant differences between the samples and groups were found. B1= blood collection 1 (basal), B2= blood collection 2 (Post-stress), B3.1= blood collection after testing in week 10 (post-test 1), B3.2= blood collection after testing in week 11 (post-test 2)

PCORT

Measured pCORT values are represented in Fig. 4. Mixed model analysis revealed a best fit of a model with fixed slope and intercept. Using this model for the analysis revealed no significant group ($F_{2, 19.9}= 0.287$, p= 0.753), group x time ($F_{2, 19.9}= 0.755$, p= 0.483) and time effects ($F_{1,22.2}= 0.021$, p= 0.886). Treatment as well as testing did not have any effect on the measured pCORT values.

ADRENAL WEIGHTS

Relative adrenal weight (mg/100g body weight) is represented separately for left and right and as a total of both in table 1. All adrenal data collected at the different time points was analysed together, since there were no differences within groups at the different time points. There were no significant differences in adrenal weights between the groups ($F_{1,34}$= 0.010, p= 0.920) and there was no significant side (left/right) x group interaction effect ($F_{1,34}$= 2.028, p= 0.163). Overall left adrenal weight was somewhat higher than right adrenal weight ($F_{1,34}$= 4.199, p= 0.048) and this difference seems to be caused by a difference in the CSS group, since the left-right difference is only significant in this group (paired t-test (corrected α p<0.025): t=-2.811, p= 0.011).

Table 1: Mean adrenal weights (mg ± SEM): both sides are represented separately as well as the total adrenal weights per group. * There was a significant difference in adrenal weights between sides (left and right adrenal) only in the CSS group.

	Statistics	Left adrenal (mg/100g BW)	Right adrenal (mg/100g BW)	Both adrenals (mg/100g BW)
CSS	side	10.8 ± 0.8	8.3 ± 0.6	19.2 ± 1.0
SH	ns	9.7 ± 0.7	9.6 ± 1.5	19.0 ± 1.7

BEHAVIOUR

A summary of all behavioural results (comparison 1st and 20th trial, to get an indication of the change over time) is listed in table S1.

AVOIDANCE BEHAVIOUR

A significant trial effect for the latency until the first board entry was found ($F_{19,323}$= 3.956, p= 0.000). Both groups showed a decrease in latency over time (see Fig. 5A) , however there were no significant group and group x trial interaction effects found ($F_{19,323}$= 0.710, p= 0.797 and $F_{1,17}$= 0.219, p= 0.645 respectively). Animals spent around 20% of their time on the board (see Fig. 5B) and the total time spent on the board did not significantly change over time ($F_{19,323}$= 1.302, p= 0.208), no significant trial x group interaction or group effects were found ($F_{19,323}$= 0.840, p= 0.624 and $F_{1,17}$= 0.052, p= 0.822 respectively). The total number of board entries decreased moderately over time ($F_{19,304}$= 2.746, p= 0.000), see table S1. Again, no group x trial or group effects were found ($F_{19,304}$= 0.631, p= 0.865 and $F_{1,16}$= 0.567, p= 0.462).

RISK ASSESSMENT

Risk assessment (measured by stretched attend postures) only took place on the first day of testing (see table S1) and did not occur in all animals, thus a significant decrease in latency was found for this parameter (trial effect: $F_{19,323}$= 39.691, p= 0.000). Also the latency until the first stretched attend shows a trend to be lower in the CSS group (group effect: $F_{1,17}$= 3.603, p= 0.075). The trial x group interaction effect was not significant ($F_{19,323}$= 0.950, p= 0.457). The total number of stretched attends decreased significantly over the 4 trials on the first day (trial effect: $F_{3,51}$= 60.400, p= 0.000), however no group ($F_{1,17}$= 0.918, p= 0.351) or group x trial

interaction effects ($F_{3,51}$=0.414, p= 0.695) were found on the number of stretched attends on the first day.
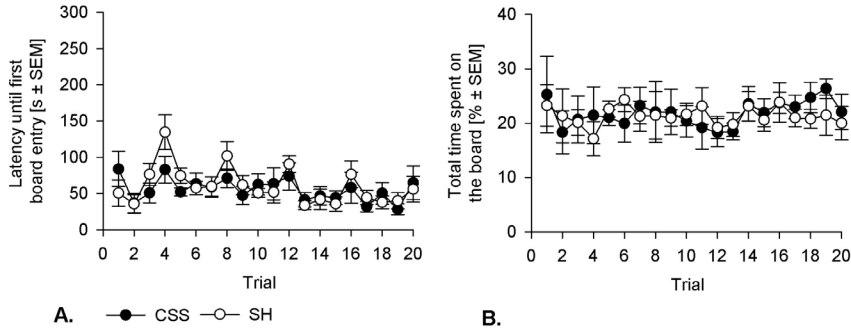

A. CSS — SH **B.**

Fig 5. Avoidance behavior: (A) Mean latency until the first board entry (in seconds ± SEM) and (B) the total time spent on the board (in percentage ± SEM). Latencies until the first board entry significantly decreased over time (trial effect).

CSS — SH
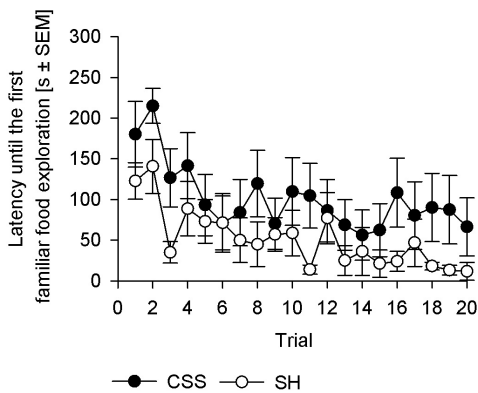
Fig 6. Food intake inhibition: Latency until the first familiar food object exploration (in seconds + SEM). Mice significantly decreased in latency to explore the familiar food object (trial effect).
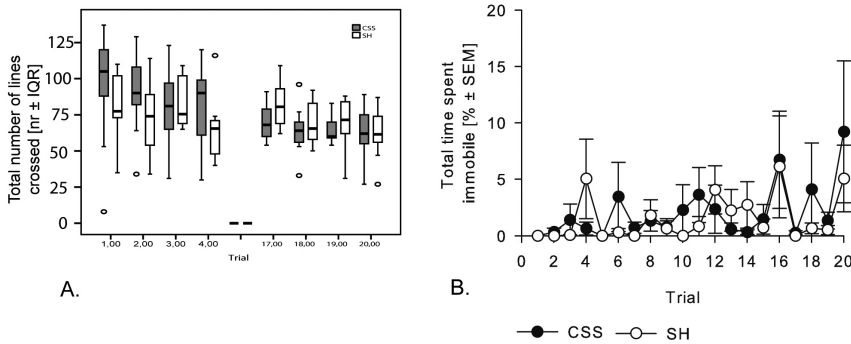

A. **B.**

CSS — SH

Fig 7. Locomotor activity: (A) Total number of line crossings (median ± IQR) and (B) total time spent immobile (in seconds + SEM). Mice significantly decreased their number of line crosses over time (trial effect).

FOOD INTAKE INHIBITION

Both groups showed a significant decrease in the latency to explore the familiar food object (trial effect: $F_{19, 304}$= 17.959, p= 0.000; see Fig. 6) and a significant decrease in the latency to eat the familiar food object (trial effect: $F_{19,304}$= 7.823, p= 0.000). The CSS group seems to show a consistent higher latency to explore and eat the familiar food object, however this difference is not statistically significant (exploration: $F_{1,16}$= 1.061, p= 0.318; intake: $F_{1,16}$= 0.731, p= 0.405) also for both exploration and intake of the familiar food object no significant trial x group interaction effects were found (exploration: $F_{19,304}$= 0.713, p= 0.797; intake: $F_{19,304}$= 0.528, p= 0.938).

The unfamiliar food object was hardly explored and eaten (see table S1), even though a trend for a group difference was found in the latency to unfamiliar food object intake ($F_{1,17}$= 3.778, p= 0.069).

LOCOMOTOR ACTIVITY

Both groups showed a significant decrease in the total number of line crosses over time (trial effect: $F_{19,323}$= 3.832, p= 0.000), see Fig. 7A. No significant trial x group interaction or group effects were found on this parameter (group effect: $F_{19,323}$= 1.481, p= 0.158; trial x group effect: $F_{19,323}$= 0.624, p= 0.778). Mice spent low amounts of time immobile (<10%), see Fig. 7B. On this parameter no significant effects were found (trial effect: $F_{19,323}$= 1.481, p= 0.158; trial x group effect: $F_{19,323}$= 0.624, p= 0.778; group effect: $F_{1,17}$= 0.242, p= 0.629). Immobility also seemed to be influenced by the time of testing, since animals seem to show more immobility on each 4th trial, which was performed in the afternoon.

EXPLORATION

Behavioural parameters indicative of general exploration include rearing (only in the box, since these hardly occurred on the board) and hole explorations (see table S1). The total number of rears in the box significantly decreased over time (trial effect: $F_{19,323}$= 2.586, p= 0.002), no significant trial x group interaction effect or group effects were found (trial x group: $F_{19,323}$= 1.251, p= 0.241; group: $F_{1,17}$= 1.532, p= 0.233). The latency until the first rear in the box also decreased over time in both groups (trial effect: $F_{19,323}$= 2.037, p= 0.010), latency trial x group interaction or group effects were not seen (trial x group effect: $F_{19,323}$= 0.909, p= 0.563; group: $F_{1,17}$= 1.289, p= 0.272). The total number of hole explorations decreased significantly over time in both groups (trial effect: $F_{19,323}$= 9.247, p= 0.000), but again no differences between groups were found over time and in general (group x trial effect: $F_{19,323}$= 0.775, p= 0.679; group effect: $F_{1,17}$= 0.465, p= 0.505). Also the latency to explore the first hole decreased over time in both groups ($F_{19,323}$= 2.864, p= 0.000) and no trial x group interaction or group effect was found (trial x group effect: $F_{19,323}$= 0.842, p= 0.647; group effect: $F_{1,17}$= 0.609, p= 0.446).

AROUSAL/DE-AROUSAL

Behavioural parameters indicative of arousal/de-arousal are grooming and defecation (table S1). The total time spent grooming increased over time in both groups (trial effect: $F_{19,323}$= 11.347, p= 0.000), but no significant trial x group interaction or group effects were found (trial x group effect: $F_{19,323}$= 0.713, p= 0.806; group effect: $F_{1,17}$= 0.732, p= 0.404). Grooming also seemed to be influenced by the time of the day, since animals groomed more on the third and fourth trial of each day. Also the latency until the first grooming event significantly decreased over time (trial effect: $F_{19,323}$= 10.591, p= 0.000), but did not differ between groups

(group effect: $F_{1,17}$= 0.474, p= 0.500) and no significant trial x group interaction effect was found (trial x group effect: $F_{19,323}$= 0.279, p= 0.999). The total number of defecations decreased over time (trial effect: $F_{19,323}$= 2.471, p= 0.004), but did not show a significant trial x group interaction effect or a group effect (trial x group: $F_{19,323}$= 0.652, p= 0.808; group: $F_{1,17}$= 0.151, p= 0.702). Both parameters together indicate a decrease in arousal over time.

Fig 8. MR mRNA expression levels indicated by dark intensity measures (arbitrary units + SEM) in the CA1 (A), CA2 (B), CA3 (C) and DG areas of the hippocampus. In the CA2 a significant group x tested interaction effect was found. t^1=0.039, t^2= 0.066

BRAIN IN SITU HYBRIDIZATION
MR MRNA EXPRESSION IN THE HIPPOCAMPUS

Data of the MR mRNA expression levels found in the separate subareas of the hippocampus are presented in Fig. 8. The linear mixed models analysis was performed using mouse as identifier and group (CSS or SH) and test (tested or not tested) as factors.

CA1

The interaction effect was not significant and removed from the model. In the CA1 area there appeared to be no differences in MR mRNA expression levels between groups and if the animals were tested or not (p>0.05).

CA2

In the CA2 area there was a significant group x tested interaction effect found ($F_{1,25}$= 4.530, p=0.043), but no separate effect of group or if the animals were tested or not (p>0.05) effects. Post-hoc testing revealed that the interaction effect can be explained by a trend for an increase in optical density in the CA2 of the CSS animals due to testing (see Fig. 8B; t= 2.178, p= 0.039, corrected α<0.025 is significant) and the trend for a higher optical density in the tested animals in the CSS group compared with the SH group (t= 1.919, p= 0.066, corrected α<0.025 is significant).

CA3

The interaction effect was not significant and removed from the model. In the CA3 area there appeared to be no differences in MR mRNA expression levels between groups and if the animals were tested or not (p>0.05)

DG

The interaction effect was not significant and removed from the model. In the CA3 area there appeared to be no differences in MR mRNA expression levels between groups and if the animals were tested or not (p>0.05).
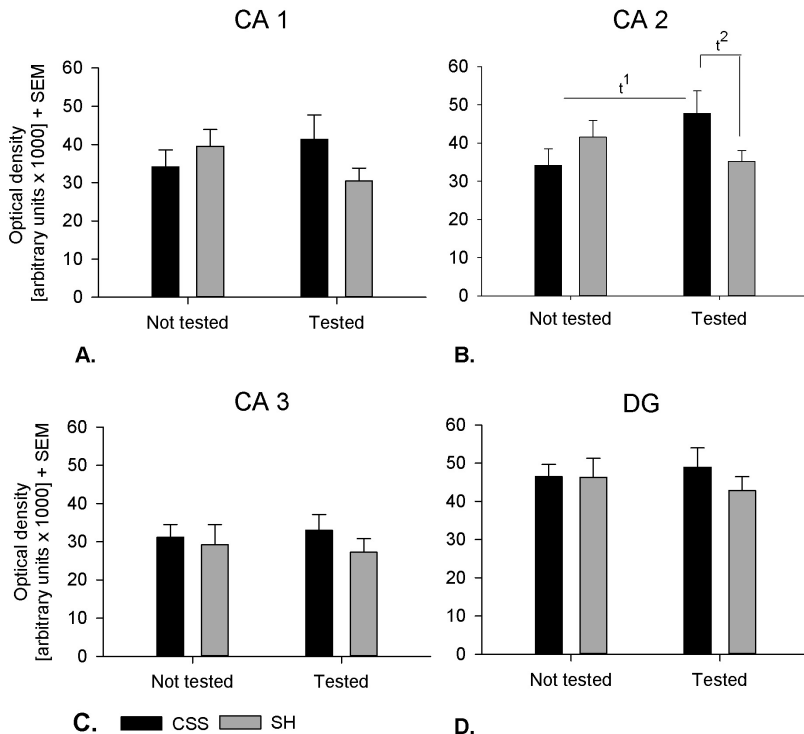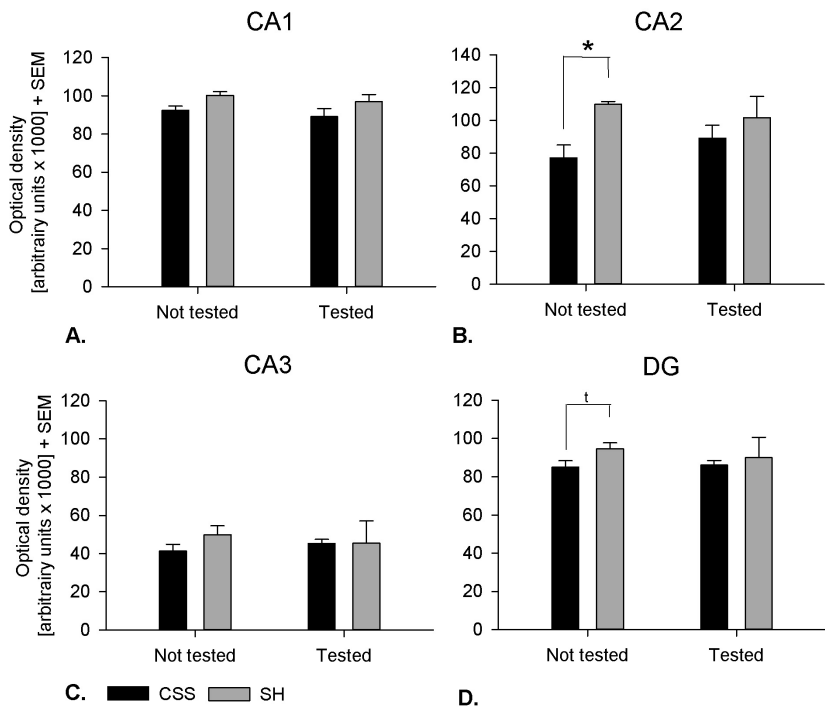

Fig 9: GR mRNA expression levels indicated by dark intensity measures (arbitrary units + SEM) in the CA1 (A), CA2 (B), CA3 (C) and DG areas of the hippocampus. General group differences were found in CA1, CA2 and DG areas. * p<0.025, t=trend p=0.038

GR MRNA EXPRESSION IN THE HIPPOCAMPUS
Data of the GR mRNA expression levels found in the separate subareas of the hippocampus are presented in Fig. 9. The linear mixed models analysis was performed using mouse as identifier and group (CSS or SH) and test (tested or not tested) as factors.

CA1
The group x tested interaction effect was not significant and removed from the model (p<0.05). There was a general group difference found; CSS mice had a lower GR expression level than SH mice ($F_{1,31}$= 4.721, p= 0.038), but there was no difference between mice that were tested and not tested ($F_{1,31}$= 0.108, p>0.05).

CA2
The group x tested interaction effect was not significant and removed from the model (p>0.05). There was a general group difference found; CSS mice had a lower GR expression level ($F_{1,31}$= 10.896, p=0.002) than SH mice but there was no difference between mice that were tested and not tested ($F_{1,31}$= 2.606, p= 0.117). Post-hoc testing revealed that the group difference was only significant within the non-tested animals (t=-3.486, p= 0.001).

CA3
The group x tested interaction effect was not significant and removed from the model (p>0.05). Also no significant effects were found between both the separate groups ($F_{1,31}$= 0.367, p>0.05) and if the animals were tested or not ($F_{1,31}$= 0.100, p>0.05).

DG
The group x tested interaction effect was not significant and removed from the model (p>0.05). However, there was a significant difference between the two groups ($F_{1,31}$= 4.560, p= 0.041); animals that underwent the CSS protocol had a lower GR mRNA expression level in the DG than mice that were not. This did not differ between tested and not tested animals ($F_{1,31}$= 0.234, p>0.05). Post-hoc testing revealed that the difference was most clear in non-tested animals (trend: t= -2.167, p= 0.038).

PVN
PVN mRNA expression levels are presented in Fig. 10. The group x tested interaction effect was not significant and removed from the model (p>0.05). There were no significant differences found between groups ($F_{1,30}$= 1.660, p>0.05) and between tested and non-tested animals ($F_{1,30}$= 0.028, p>0.05).
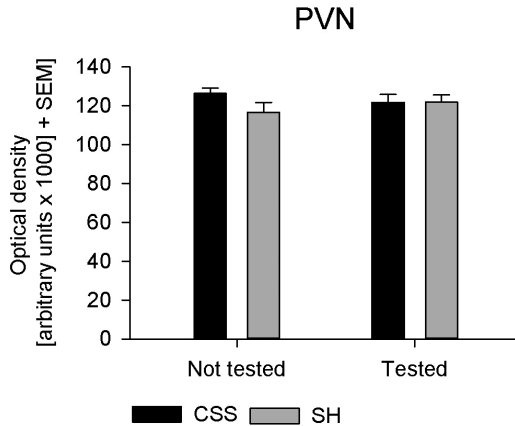
PVN

Fig 10: GR mRNA expression levels indicated by dark intensity measures (arbitrary units + SEM) in the PVN.

DISCUSSION

Male CD1 mice that were exposed to social instability (CSS protocol) during adolescence and early adulthood showed several symptoms that are indicative for chronic stress, i.e. delayed increase in bodyweight (Fig. 2) and a decrease of fur condition (Fig. 3), changes in HPA axis functioning (a decrease in GR mRNA expression in the CA1 and DG region of the hippocampus, Fig. 9), and a higher relative left adrenal weight (table 1). Interestingly, the exposure to the CSS protocol did not result in changes in behavioural habituation to a novel environment after termination of the CSS protocol. In contrast, it appeared that the CSS1 group showed little differences in habituation over time when compared with the SH1 group, suggesting that the stress induced by the CSS protocol did not exceed the adaptive capacities of the CD1 mice.

EFFECTIVENESS OF THE CSS PROTOCOL

A delay in body weight gain (Bartolomucci, *et al* 2004; Pothion, *et al* 2004; Schweizer, *et al* 2009; Singewald, *et al* 2009) and a deterioration of body fur condition (Mineur, *et al* 2006; Pothion, *et al* 2004) are commonly used parameters to show effectiveness of chronic (social) stress protocols, and the effects in the present study on these parameters support the idea that the CSS protocol was stressful for the mice. However, previous studies using the same CSS protocol (Schmidt, *et al* 2007; Schmidt, *et al* 2010a) were not able to find effects on body weight progression, even though similar procedures were followed. It is possible that the different lab environments and slight differences in procedures (reversed light/dark cycle changes) may contribute to variation in effects of CSS on body weight between different studies. Especially the group changes during the active phase that are likely to disrupt food intake in these animals, might explain the differences in findings.

The effectiveness of the CSS protocol might be further supported by an increase in HPA-axis activity, as for example being reflected by increased basal levels of corticosterone, increase in adrenal weights and decreased brain MR and GR expression (De Kloet, *et al* 1998; De Kloet, *et al* 2005). Although we did not find significant differences in pCORT levels between groups and samples over time, differences in GR mRNA expression levels in the brain do suggest that there is a persistently altered activity of the HPA-axis in CSS mice. GR mRNA expression

in the CA1, CA2 and DG regions of the hippocampus (Fig. 9) were decreased in CSS animals in comparison with SH animals one week after discontinuation of the CSS protocol (CSS2 and SH2) and also in the animals that were tested in week 10 or 11 (CSS1 and SH1). This is in concordance with other studies using the same (Schmidt, *et al* 2007; Sterlemann, *et al* 2008) and other chronic social stress protocols (Bartolomucci, *et al* 2003; Meyer, *et al* 2001). An decrease in GR mRNA expression is usually accompanied by a decrease in MR mRNA expression (Schmidt, *et al* 2007; Sterlemann, *et al* 2008), which could not be demonstrated here (Fig. 8). In contrast the significant interaction effect rather indicated that repeated behavioural testing increased MR mRNA expression in the CA2 region of the CSS animals. It seems likely that this effect may be due to the influence of behavioural testing, but no literature is available that investigates the effect of chronic stress and subsequent, additional behavioural testing on MR mRNA expression.

A heightened HPA-axis activity is usually accompanied by hypertrophy of the adrenal cortex in which corticosterone is produced (see for example Gamallo, *et al* 1986; Kuipers, *et al* 2003; Marti, *et al* 1994; Ulrich-Lai, *et al* 2006; Veenema, *et al* 2003). However, we only found an increase on relative left but not on right adrenal weights. A comparison of this finding with literature is difficult, since most studies do not differentiate between the left and right adrenal but report effects of chronic stress on average or total relative adrenal weights (Blanchard, *et al* 1995; Maslova, *et al* 2010; Razzoli, *et al* 2011; Schmidt, *et al* 2007; Schmidt, et al 2010b; Schwabe, *et al* 2008; Slattery, et al 2012; Sterlemann, *et al* 2008). Still, the increase in adrenal weight further supports that HPA-axis activity was heightened and subsequently the effectiveness of the CSS protocol.

CSS EFFECTS ON BEHAVIOUR
Together, the physiological effects suggest that the mice in the CSS group indeed were chronically stressed during the stress-period. However, when subsequently being tested behaviourally in a novel environment, behavioural parameters revealed little differences between both groups over time (see Figures 5, 6 and 7), suggesting that the CSS protocol did not exceed coping abilities in mice persistently, but that they were able to adapt to the subsequent testing situation. Chronic (social) stress has been reported to increase state anxiety in laboratory mice (Ducottet and Belzung 2004; Ducottet, *et al* 2004; Schmidt, *et al* 2007; Sterlemann, *et al* 2008; Strekalova, *et al* 2004) as indicated for example in CD1 mice by increased acute avoidance behaviour in the elevated plus maze and increased food intake inhibition (Schmidt, *et al* 2007; Sterlemann, *et al* 2008). In the mildly aversive modified hole board test used here, CD1 mice did not reveal increased avoidance behaviour, though a trend towards an increase in risk assessment behaviour in the CSS group (stretched attends, see table S1) was noted. Risk assessment behaviour has been demonstrated to represent a behavioural dimension that is independent of avoidance behaviour (Cruz, *et al* 1994; Ohl, *et al* 2001b; Rodgers and Johnson 1995) but that is indicative of anxiety since it is considered to be a defensive behaviour (Blanchard, *et al* 1993). However, overall behavioural parameters clearly show that the CD1 mice from the CSS group habituated as efficiently as the control animals to the novel environment. We therefore conclude that the previous stressful experiences did not exceed the adaptive capacities of the mice.

This contrasts earlier findings in several 129 substrains that show a lack of habituation in the same test setup (Boleij, *et al* 2012a; Salomons, *et al* 2010;

Salomons, *et al* 2010a). In one substrain, the 129P3 strain, animals were moreover found to be sensitive to the effects of chronic stress, in that the non-adaptive profile was further intensified by a chronic mild stress procedure (Salomons, *et al* 2010b). It has been suggested by others that environmental challenges such as chronic stress are more likely to result in a pathological state in individuals that have a susceptible genotype (De Kloet, *et al* 2005; see Schmidt, *et al* 2008 for reviews on this subject relating to chronic stress). Comparison of our result in CD1 mice with the previously tested 129 mice indicates that such a gene-environment interaction is likely to be involved for the development of a non-adaptive habituation profile as well.

It should be noted, however, that in contrast to homozygous inbred strains, the population tested in the present study consisted of individuals from an heterozygous outbred strain. Previous studies using the same stress protocol show that a sub-population of about 20% of the individuals are more susceptible to the effects of CSS (as reflected by a persistently increased basal corticosterone level several weeks after cessation of the stress protocol; (Schmidt, *et al* 2010b)). In support of these results we found that there was a higher variation in behaviours between animals from the CSS1 group than in individuals from the SH1 group (see for example Fig. 6), indicating that indeed a responder/non-responder effect might have occurred. It remains to be investigated if these responder animals show different habituation profiles than non-responder animals, therefore a higher number of animals needs to be tested in future experiments.

METHODOLOGICAL CONSIDERATIONS

Although similar procedures were followed as in Schmidt *et al.* (Schmidt, *et al* 2007; Schmidt, *et al* 2010a; Schmidt, *et al* 2010b; Sterlemann, *et al* 2008), several effects of the stress protocol could not be replicated here, i.e. no effects of the protocol on basal pCORT values were found, avoidance behaviour in the first trial did not differ between groups and we actually did find a deterioration in body weight progression. Some of these differences might be explained by the fact that we exposed the mice to the stress protocol during their activity phase and also tested the mice during this phase. Literature reports that application of stressors in the dark or in the light phase have different effects on behaviour and physiological parameters. In line with our findings and that of Schmidt *et al* (Schmidt, *et al* 2007; Schmidt, *et al* 2010b; Sterlemann, *et al* 2008), Bartlang *et al.* (2012) have shown that in C57Bl6 mice the application of repeated social defeat during the light phase of the animals had a more pronounced effect on the HPA-axis response. Moreover, it is known that pCORT levels are lower and less variable during the light phase and changes in basal levels are more easily measured (Akana, *et al* 1994; Retana-Márquez, *et al* 2003). In contrast, chronic stress applied during the dark phase has been reported to induce more profound effects on behaviour in mice and rats (Bartlang, *et al* 2012; Gorka, *et al* 1996). While therefore the comparably weak effects on HPA-axis activity found in the present study might be attributed to such methodological differences with other studies, they at the same time seem to emphasize our behavioural findings in stressed animals.

In conclusion, the CSS-protocol did not result in persistent effects on behaviour of CD1 mice and, thus, did not indicate any major effect on the ability of the mice to adapt to a mildly stressful testing environment after having been exposed to a period of chronic social instability. This lack of effect on adaptive capacities

cannot be attributed to a lack of effectiveness of the used stress protocol, since physiological and CNS-parameters indicated that the animals had been stressed during application of the CSS-protocol. Rather, the effects on GR mRNA expression in combination with normal habituation behaviour may indicate an allostatic process (Sterling and Eyer 1988), that is, the establishment of new internal set-points that still lie within the adaptive range of the animals (Koolhaas, *et al* 1999; Korte, *et al* 2007; McEwen and Wingfield 2003; McEwen 2008). The fact that the CSS protocol appeared to have affected only a subset of individuals in the stressed group however makes it difficult properly to analyse all parameters. Therefore, it is suggested further to explore inter-individual differences in male CD1 mice, and to differentiate between responders and non-responders in further stress experiments.
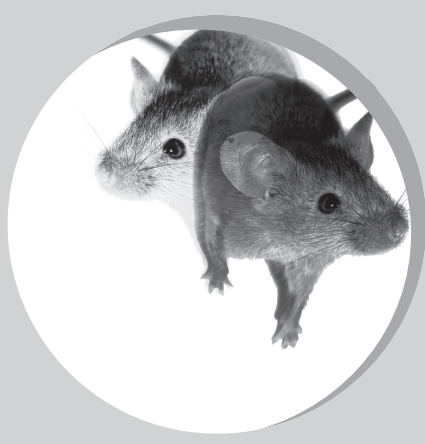
Table S1: Behaviours (ordered in categories) scored in the mHB. Continuous data is expressed in seconds ± SEM and discrete data in number ± IQR. T= Significant differences between trials (over time), G= significant differences between groups. * p< 0.05, **p<0.01

Behavioural category and parameters	Statistics	CSS		SH	
		Trial 1	Trial 20	Trial 1	Trial 20
Avoidance					
Latency until first board entry [sec]	T**	83.6 ± 24.1	64.8 ± 23.1	50.5 ± 18.1	55.7 ± 8.0
Total time spent on board [%]	ns	25.3 ± 7.0	22.1 ± 3.2	23.3 ± 3.8	20.0 ± 3.1
Total number of board entries [nr.]	T**	14 ± 14.5	14 ± 7	16.5 ± 6	10 ± 8.75
Risk assessment					
Total number of stretched attends [nr.]	T** , G (trend, p=0.075)	3 ± 12.5	0 ± 0	3.5 ± 9	0 ± 0
Latency until first stretched attend [sec]	ns	42.1 ± 32.2	300 ± 0	40.4 ± 29.2	300 ± 0
Locomotion					
Total number of line crossings [nr.]	T**	105 ± 50.5	62 ± 38	77.5 ± 34.5	61.5 ± 22.7
Latency until first line crossing [sec]	T**	8.6 ± 3.8	16.9 ± 11.8	9.4 ± 1.0	5.3 ± 0.9
Total time spent immobile [%]	ns	0 ± 0	9.2 ± 6.3	0 ± 0	5.1 ± 3
Latency until first immobility [sec]	ns	300 ± 0	234.9 ± 43.4	300 ± 0	243.3 ± 39.4
General exploration					
Total number of rearings in the box [nr.]	T**	24 ± 27.5	28 ± 24.5	26 ± 15.5	24.5 ± 18
Latency until first rearing in the box [sec]	T**	75.9 ± 33.3	90.8 ± 26.7	63.7 ± 13.9	42.7 ± 9.2
Total number of rearings on the board [nr.]	T**	0 ± 1	2 ± 5	0 ± 0	1 ± 4.25
Latency until first rearing board [sec]	T**	295.0 ± 3.5	201.6 ± 24.8	300 ± 0	215.4 ± 24.6
Total number of hole explorations [nr.]	T**	21 ± 24.5	6 ± 7.5	23.5 ± 11.5	7 ± 3.5
Latency until first hole exploration [sec]	T**	42.3 ± 20.7	101.6 ± 29.4	38.3 ± 13.4	64.8 ± 16.0

Directed exploration

Total number of hole visits [nr.]	T (trend, p=0.065)	2 ± 5.5	2 ± 2.5	3.5 ± 6.5	2 ± 4.25
Latency until first hole visit [sec]	T (trend, p=0.061)	155.6 ± 32.1	170.2 ± 36.9	99.2 ± 17.3	167.9 ± 32.9

Food intake inhibition

Latency first exploration unfamiliar food [sec]	T*	223.8 ± 39.0	300 ± 0	254.1 ± 30.8	269.9 ± 27.1
Latency first exploration familiar food [sec]	T**	180.3 ± 40.3	66.2 ± 35.7	122.8 ± 22.6	11.5 ± 11.0
Latency intake unfamiliar food [sec]	G (trend, p=0.069)	271.6 ± 28.4	300 ± 0	286.9 ± 11.0	300 ± 0
Latency intake familiar food [sec]	T**	180.3 ± 40.3	66.5 ± 35.6	122.8 ± 22.6	11.86 ± 10.95

Arousal

Total time spent grooming [%]	T**	0.3 ± 0.1	4.6 ± 2	0.4 ± 0.1	5.6 ± 1.5
Latency until first self groom [sec]	T**	270.9 ± 19.7	57.5 ± 11.2	235.6 ± 21.0	44.1 ± 2.3
Total number of self-groomings [nr.]	T**	0 ± 1.5	2 ± 2.5	1 ± 1	2.5 ± 1.25
Latency until the first defecation [s]	T**	239.1 ± 23.4	106.3 ± 37.8	244.5 ± 23.9	144.4 ± 36.4
Total number of boli [nr.]	T**	2 ± 4	3 ± 4	1.5 ± 4	2.5 ± 4

CHAPTER 4

A TEST TO IDENTIFY JUDGEMENT BIAS IN MICE

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ABSTRACT

Emotional states are known to affect cognitive processes. For example highly anxious individuals interpret ambiguous stimuli more negatively than low anxious people, an effect called negative judgement bias. Recently, the measurement of judgement bias has been used to try and indicate emotional states in animals. In the present experiment a potential test for judgement bias in mice was examined. Mice were trained with two distinct odour cues (vanilla or apple) predicting either a palatable or an unpalatable almond piece. Subsequently their reaction to mixtures of both odours, the ambiguous stimuli, was investigated. Mice of the BALB/cJ and 129P3/J inbred mouse strains (high initial anxiety and low initial anxiety phenotypes respectively) were tested. While BALB/cJ mice showed odour association learning and showed intermediate reactions to the ambiguous cues, 129P3/J mice did not discriminate between the cues. Additionally BALB/cJ mice that were tested under more aversive white light conditions revealed a higher latency to approach the almond piece than mice tested under less aversive red light conditions. The ambiguous stimulus however was interpreted as negative under both test conditions. Brain c-Fos expression levels (a marker for neuronal activity) differed between the BALB/c/J and 129P3/J in the lateral amygdala and the prelimbic cortex, indicating differences in ambiguous information processing between the strains. The behavioural results suggest that the present judgement bias test might be used to assess emotional states in at least BALB/c mice, however further research on both behaviour and on the involved brain mechanisms is necessary to confirm this idea.

INTRODUCTION

In humans it is well known that emotional states influence cognitive processes, an effect that is referred to as cognitive bias (Mathews and MacLeod 1994). People that are in a negative affective state reveal a better memory of negative events, tend to focus their attention on the occurrence of negative events, and interpret ambiguous stimuli more negatively (negative judgement or interpretation bias) (Cahill and McGaugh 1996; Chan and Lovibond 1996; Eysenck, *et al* 1991; Mogg and Bradley 1998; Telzer, *et al* 2008). People suffering from anxiety disorders and/or depression have a more negative judgement bias than healthy controls (MacLeod, *et al* 1997). Based on the knowledge mentioned above, a negative judgement bias is understood as an indicator of a negative affective state (Eysenck, *et al* 1991; Mathews, *et al* 1989; Mathews, *et al* 1997; Richards and French 1992).

In animals a measurement of judgement bias is of additional value next to existing behavioural and physiological indicators of emotions, since the measurement of judgement biases includes the cognitive component of emotions and could be used as indicator of emotional valence (Mendl, *et al* 2009). Recently, the phenomenon of judgement bias has been investigated in several animal species, some being aimed at welfare assessment while others are more interested in judgement bias in animal models of human affective disorders (Bateson and Matheson 2007; Bateson, *et al* 2011; Brydges, *et al* 2011; Burman, *et al* 2008; Doyle, *et al* 2011; Enkel, *et al* 2010; Harding, *et al* 2004; Matheson, *et al* 2008; Mendl, *et al* 2010a; Salmeto, *et al* 2011; Tsetsenis, *et al* 2007). Judgement biases in animals are measured by testing their behavioural response to an ambiguous stimulus after performing a conditioning procedure in which two different stimuli (of the same sensory modality) are associated with either reward or lower-value reward/ punishment. For example, a tone of 2 kHz predicts a food reward and a tone of 4 kHz predicts an aversive white noise, in a test session the reaction of the animals to tones of 2, 3.5 and 4 kHz is investigated (Enkel, *et al* 2010; Harding, *et al* 2004) by comparing this with the reaction to the positive and negative associated cues. Anxiety seems to influence judgement biases in animals like it does in humans (Bateson, *et al* 2011), causing a more negative interpretation of ambiguous stimuli (Burman, *et al* 2009; Mendl, *et al* 2010a; Tsetsenis, *et al* 2007). One way to manipulate state anxiety in laboratory rats is to alter light conditions during testing: Rats are nocturnal and testing under bright light conditions increases state anxiety (=anxiety at a specific moment in time) (Cosquer, *et al* 2005; Garcia, *et al* 2005; Valle 1970). Notably, rats that are trained under low light conditions and tested under high light conditions show a more negative judgement bias than rats trained under high light conditions and tested under low light conditions, implying that state anxiety can alter judgement biases in rats like in humans (Burman, *et al* 2009). Interestingly, recent results also demonstrate that dogs suffering from separation anxiety and stereotyping starlings have a more negative bias (Brilot, *et al* 2010; Mendl, *et al* 2010a), suggesting that high trait anxiety (=general anxiety trait) may affect judgement bias in animals. This notion elicits the question whether judgement bias may in turn represent a potential read-out parameter for affective states in animals.

The aim of the present study was, firstly, to investigate if judgement bias can be measured in mice and, secondly, if judgement bias would be affected by state or trait anxiety respectively. As different strains of mice are frequently used as animal models of (pathological) anxiety and are often subject of transgenic studies, it seems of high interest to investigate judgement bias in this species.

Recently anxiety-related behaviour in two inbred mouse strains, BALB/cJ (BALB/c) and 129P3/J (129P3) was evaluated in our lab and it appeared that BALB/c mice behave highly anxious when initially exposed to a test environment, but show a rapid habituation over time, while 129P3 mice are initially less anxious but do not habituate to the testing environment (Salomons, *et al* 2010; Salomons, *et al* 2010a). Previously, BALB/c mice have been suggested to represent a phenotype of trait anxiety because they show high state anxiety in multiple testing situations (Belzung and Berton 1997; Belzung and Griebel 2001; Griebel, *et al* 1993; Salomons, *et al* 2010a). Thus to our first aim we performed the test in these previously characterized 129P3 and BALB/c mice (experiment 1) expecting a more negative judgement of the initially highly anxious BALB/c mice.

To elucidate effects of state anxiety, BALB/c mice in addition were tested under different test conditions (red or white light, experiment 2), expecting a more negative judgement of the mice tested under white light conditions. An additional third experiment evaluated the odour perception abilities of 129P3 mice.

Next to the behavioural tests on judgement bias, brain area's known to be relevant for emotional processes involved in judgement bias, i.e. the prelimbic cortex (Marquis, *et al* 2007), lateral septum (Sheehan, *et al* 2004; von Cramon, *et al* 1993) and amygdala (Blasi, *et al* 2009; Davis and Whalen 2001) were analysed for c-Fos expression, a marker for neuronal activity.

In mice, no procedure has been performed yet that focuses on the effects of anxiety on judgement bias. Thus, in the present study a conditioning procedure was used in which the animals were trained to associate odours with either a positive or a negative experience and their reaction to an ambiguous stimulus (mixture of both odours) was subsequently investigated.

MATERIALS AND METHODS
ETHICAL NOTE

The protocols of the experiments were peer reviewed by the scientific committee of our department and approved by the local Animal Experiments Committee. Further the animal experiments followed the "Principles of laboratory animal care" and refer to the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioural Research (National Research Council 2003). For more details see supplementary material.

ANIMALS AND GENERAL HOUSING CONDITIONS

Husbandry procedures and animal experiments were performed by well-trained members of the laboratory. The experiment on judgement bias (experiment 1) was performed with 50 naive male BALB/cJ (BALB/c) mice and 50 male 129P3/J (129P3) mice. The light effect experiment (experiment 2) was performed with 84 naive male BALB/c mice. An additional odour perception experiment (experiment 3) was performed with 6 naive male 129P3 mice. All mice were obtained from The Jackson Laboratory (Bar Harbour, Maine, USA) and were 6-8 weeks old at arrival. The animals were housed individually at the animal laboratory of the Netherlands Vaccine Institute (Bilthoven, The Netherlands), in a temperature (22±2 °C) and humidity (45%-50%) controlled room under a 12/12h reversed light/dark cycle (lights on at 18.00 h. and off at 6.00 h.). Training and behavioural testing was performed in the same room. Mice chow (CRM, Expanded, Special Diets Services Witham, England) and tap water were available *ad libitum*.

During the two-week pre-experimental period the person that performed the

actual experiment handled and weighed the mice regularly. All mice were kept in Eurostandard type 3 macrolon cages (40 cm x 26 cm x 20 cm) with standard cage bedding (Aspen chips), a plastic shelter (Mouse House Techniplast®) and tissue (Kleenex® Facial Tissue Kimberly-Clark) as enrichment. The testing equipment had already been installed in the room before the animals arrived.

BEHAVIOURAL TESTING

All testing was performed with odours as conditioned stimuli, considering the ability of mice to discriminate even slight differences between odours (Bodyak and Slotnick 1999); this ability is also found in individuals of the BALB/c and the 129S1/SvImj sub-strain (Brown and Wong 2007). Odour mixtures have been used before in a judgement bias experiment with honeybees (Bateson, *et al* 2011). Both visual and auditory stimuli were excluded, since specific inbred strains (including the 129P3 strain) have been shown to possess a mutation (*Cdh23[ahl]*) that causes hearing loss within three months of age (Zheng, *et al* 1999), moreover the albino BALB/c mice tend to be visually impaired, which makes visual stimuli less suitable. Testing was performed in the home cage of the animals to avoid unwanted environmental stress, potentially induced by testing in a novel environment (Misslin, *et al* 1982; Misslin and Cigrang 1986; Misslin and Cigrang 1986).

Pieces of almond (approximately 0.05 g) were used as rewards; mice eat these readily even if they are fed *ad libitum* (see for example (Ohl, *et al* 2003)).

The odour stimuli were vanilla and apple (Micro-Plus, Stadtoldendorf, Germany), dissolved in distilled water (0.05%), since mice are attracted by those (e.g. (Ohl, *et al* 2003)). Both odours were dissolved in a low concentration because the stock solution is highly concentrated and similar concentrations were used before. Odour mixtures for the test sessions were made with the 0.05% solutions, mixing them in the required proportions (see below and table 1).

EXPERIMENTS 1 AND 2: JUDGEMENT BIAS TEST

APPARATUS

Experiment 1: During training and test trials almond pieces were presented on a small Petri dish (ø 5.5 cm). The odours were spread on a filter paper (ø 5.5 cm) in an amount of 0.1 ml per odour that was positioned in the Petri dish (Schellinck, *et al* 2001). The Petri dish with the filter paper was covered by a lid with several holes to let the odours diffuse through the top (see Fig. 1A). From now on this dish will be called the odour cup.

Experiment 2: During training and testing almond pieces were presented in an odour apparatus that consisted of a grey PVC cylinder (ø 3.0 cm, height 3.0 cm) that could be fastened on a transparent Perspex plate (20.0 cm x 9.5 cm), see Fig. 1B. From now on this apparatus will be called the odour cylinder. The odour cylinders are similar to those used in the modified hole board and suitable for mice (see for example (Salomons, *et al* 2010a)). Odours (0.05 ml) were spread on a filter paper (ø 3.2 cm) that fitted underneath the cylinder.
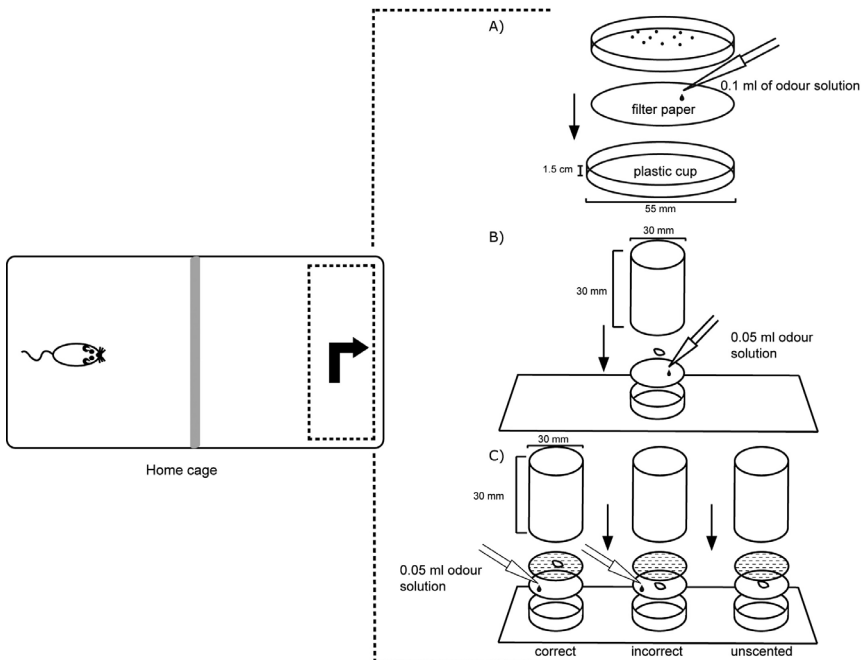
Fig. 1: A diagram of the different odour apparatuses that were used to present the odours in the home cage during training and testing. (A) Odour cup that was used in experiment 1 (adapted figure from Schellinck et al. (2001)) (B) Odour cylinder that was used in experiment 2 and (C) odour apparatus that was used for the odour discrimination test in experiment 3. One cylinder is marked with a filter paper with vanilla odour, one with apple odour and one is unscented.

TRAINING AND TESTING

Animals were trained and tested when being most active between 9.00 and 13.00. Mice were habituated to eating the piece of almond (30 mg), by offering it with tweezers in the home cage on days 14, 15 and 16 after arrival. On day 19 after arrival the training procedure started. During all trials the home-cage was placed on a table in front of a video camera (placed on the side of the odour cup) that was connected to a dvd-recorder (Panasonic). First of all, the enrichment and water bottle were removed from the home cage. A trial started with placing the odour cup in the home cage (see Fig. 1). The training trials were terminated when the almond piece was eaten. Test trials lasted 5 minutes.

During training in a positive (POS) trial the odour cup or cylinder was presented with a normal tasting almond piece and in a negative (NEG) trial the odour cup or cylinder was presented with a bitter tasting almond piece. Almond pieces were made bitter by dipping them in a 180 mmol odourless quinine solution (Sigma-Aldrich) and drying them overnight. Half of the mice from one group received the normal tasting almond piece paired with vanilla and the bitter almond piece paired with apple, and the other half the other way around. In the test trials all odours were presented with a normal tasting almond piece. Learning effects were investigated by statistically comparing latencies to eat the almond piece in the POS trials with that in the NEG trials, a statistical significant difference indicated

that the animals had learned the association (on the group level).

Experiment 1: BALB/cJ and 129P3/J mice were trained similarly. For testing, animals of both strains were randomly assigned to five groups (n=10 per group) and the separate groups were tested on their reaction to their group-specific odour concentration. In total all mice received 4 training trials (3 POS trials and 1 NEG trial) over 4 days, one trial per day. The separate groups were either tested (1 trial) on the POS, MIX 1 (85% POS-15% NEG), MIX 2 (50% POS-50% NEG), MIX 3 (15% POS-85% NEG) or the NEG stimulus on the 5th day depending on their experimental group (see table 1 for an overview of the groups). All mice in this experiment were trained and tested in the dark (red light). Animals eating the whole almond piece in the NEG sessions were removed from the analysis (in total 2 129P3 and 6 BALB/c mice), assuming that the bitter taste of the almond was not experienced as being negative by these animals. We therefore assumed that the NEG stimulus could not be considered being 'negative' in these cases.

Experiment 2: BALB/c mice were trained similarly, but different groups (6 groups, n=14 per group) were tested on their reaction to different odour concentrations either in the dark (red light) or in the light (white light, provided by a desk lamp of approximately 120 lx, directed on the animal to be tested). All training was performed in the dark. In total the mice received 8 training trials (5 POS and 3 NEG trials) over 4 consecutive days (two trials per day). The first training day consisted of two POS trials, the other training days of one POS and one NEG trial in a random order. The inter trail interval was approximately 2 hours. Animals were either tested (1 trial) on the POS, MIX (50% POS-50% NEG) or NEG stimulus on day 5 depending on their experimental group (more details on the treatments per group can be found in table 1).

Table 1: experimental groups (experiment 1 and 2), tested with different odour concentrations. In the POS (= positive conditioned stimulus) sessions the almond pieces were presented with one odour (either apple or vanilla, odour 1) and in the NEG (= negative conditioned stimulus) sessions bitter tasting almond pieces presented with the other odour (odour 2).

Groups	1	2	Training Odour POS, almond piece Odour NEG, bitter tasting almond piece				Test Presentation POS, NEG and MIX
Experiment 1: strains							
	BALB/c	**129P3**	Day 1	Day 2	Day 3	Day 4	Day 5
1	POS	POS	POS 1	POS 2	POS 3	NEG 1	POS
2	MIX 1	MIX 1					MIX 1 (85% POS, 15% NEG)
3	MIX 2	MIX 2					MIX 2 (50% POS, 50% NEG)
4	MIX 3	MIX 3					MIX 3 (15% POS, 85% NEG)
5	NEG	NEG					NEG
Experiment 2: light conditions							
	Dark (red light)	**Light** (white light)	Day 1	Day 2	Day 3	Day 4	Day 5
1	POS	POS	POS 1 POS 2	POS 3 NEG 1	POS 4 NEG 2	POS 5 NEG 3	POS
2	MIX	MIX					MIX (50% POS, 50% NEG)
3	NEG	NEG					NEG

Initially in our first experiment a one-trial learning procedure was applied (one NEG trial) in order to minimize the number of aversive trials, since we have previously found that mice from the 129P3/J strain have difficulties to habituate to a mildly aversive environment (Salomons, *et al* 2010a; Salomons, *et al* 2010a; Salomons, *et al* 2010b). As we continued with BALB/c in experiment 2, some extra trials were added to insure that the animals learned the odour associations. From literature it is known that mice are able to learn odour associations relatively quick (Schellinck, *et al* 2001), which was the reason to choose for the present design. A disadvantage of the one-trial learning procedure (experiment 1) is that it is not possible to make a learning curve for individual mice. However, a comparison between the POS and NEG groups in the test session will reveal whether there is a learning effect on the group level. Since inbred strains of mice were used we did not expect major differences.

In contrast to other studies on cognitive bias, we were interested in investigating neuronal activation in the brain by looking at c-Fos expression. This was only possible if separate groups of mice were exposed to the positive, ambiguous and negative stimulus in the test trial (between-animal design).

EXPERIMENT 3: ODOUR PERCEPTION IN 129P3/J MICE

Due to the results of experiment 1 an additional experiment was designed to investigate whether the lack of discrimination between the different odours in the test session of experiment 1 in 129P3/J mice (no differences in latencies to eat the almond piece between the groups) was due to a deficiency in odour perception or discrimination. 129P3 mice showed a rapidly decreasing latency to eat the almond piece over trials in experiment 1 indicating that they learned to make the positive association between the odour cup and the almond rapidly. Therefore in this third experiment again a conditioning paradigm was used, but now in such a way that we could draw conclusions on the olfactory capabilities of 129P3 mice.

ODOUR APPARATUS AND ALMOND PRESENTATION

The odour apparatus consisted of three grey PVC cylinders (ø 3.0 cm, height 3.0 cm) that could be fastened equispaced from each other on a transparent Perspex plate (20.0 cm x 9.5 cm), see Fig. 1C. A trial was initiated by putting the odour apparatus (see experiment 1 & 2) in the home cage. Behaviour during testing was recorded via a camera that was placed above the test set-up. Again odours (0.05 ml) were spread on a filter paper (ø 3.2 cm) that fitted underneath the open cylinders of the apparatus. One of the cylinders was marked with 0.05% apple odour, another with 0.05% vanilla odour and the remaining cylinder was not marked. The almond was coupled to one of the odours and presented in the corresponding cylinder: half of the mice could obtain the piece of almond in the vanilla scented cylinder and the other half in the apple scented cylinder. The correct cylinder (the one containing the almond piece) was presented randomly at one of the three locations over trials. To make sure the mice could not identify the correct odour cup by the scent of the almond itself also the other cylinders contained an almond that the mice were unable to obtain (it was contained under a round piece of wire mesh). A total of 30 trials per mouse was performed, 6 trials per day during 5 consecutive days. A trial started with placing the odour apparatus in the home cage and ended after the almond piece was eaten.

BEHAVIOURS SCORED

Behaviour during the training and the test trials of experiment 1 was scored afterwards from the video material using the computer program "The Observer" version 5.0 (Noldus b.v., Wageningen, The Netherlands). Behaviour in experiments 2 and 3 was scored live with the same computer programme. Behaviours were scored in a continuous way, i.e. all-occurrence recording of the behaviours of interest. The following behavioural parameters were measured:

Experiments 1 and 2: Latency until eating the almond piece was used as indicator of odour cues judgement (i.e. low latency with a positive interpretation and a higher latency with a negative interpretation). Other measures included exploration (sniffing) of odour cup/cylinder (latency and duration), picking up almond piece (latency), locomotor activity: line crossings between front and back (latency and total number), general exploration: rearing (latency and total number), grooming (latency, total duration and total number).

Experiment 3: Head dipping in the correct cylinder was recorded as a correct response, head dipping in the incorrect and unscented cylinder as an incorrect response. Other behaviours that were recorded were exploration (sniffing) of odour cup, head dip (latency and total number), general exploration: rearing (latency, total duration and total number).

EUTHANASIA, BRAIN REMOVAL AND C-FOS ANALYSIS

All mice were decapitated two-and-a-half hours after the test session, in a separate room adjacent to the experimental room. Immediately after decapitation the brains of the mice (experiments 1 and 2) were removed and frozen in liquid (-80 °C) 2-methylbutane which was cooled with dry ice and stored at –80 °C. A c-Fos immunohistochemistry was performed only on the brains of experiment 1 to get a general impression of the emotion related brain areas involved in the present test. Brains of experiment 2 are stored and might be further analysed in the future.

Experiment 1: Coronal sections were cut (20 μm) and mounted on Menzel SuperFrost Plus slides (Menzel GmbH & Co, Braunschweig, Germany) and stored at –20 °C. For the immunohistochemical detection of c-Fos, rabbit anti-c-Fos (SC-52 Santa Cruz Biotechnology) was used. During the staining procedure the sections were rinsed several times after every step in 0.01 M phosphate-buffered saline (PBS) (pH 7.4).

First, the sections were dehydrated. Endogenous peroxidase was blocked by treatment with H_2O_2 (0.1%) for 30 min. Sections were pre-incubated with 5% normal donkey serum (NDS) and 1% bovine serum albumin (BSA) in PBS (PBS-BSA 1% + NDS 5%) for 30 min before the rabbit anti-c-Fos incubation (1:1500 in PBS-BSA 1% + NDS 5%, 4 °C, 24 h). Negative controls, used to control for aspecific binding of the Biotin SP conjugate (Jackson ImmunoResearch Laboratories, Inc., PA, USA), were incubated with the PBS-BSA 1% + NDS 5% solution. Next, the sections were incubated with the donkey–anti-rabbit IgG Biotin SP conjugate (1:400 in PBS-BSA 1% + NDS5%) for 45 min. Subsequently, the sections were incubated with avidin horseradish peroxidase solution (1:400 in PBS-BSA 1%+ NDS 5% VECTASTAIN® ELITE ABC, Brunswich Chemie, Amsterdam) for 60 min and pre-incubated with diaminobenzidine tetrahydrochloride (DAB) solution containing nickel sulphate. For visualization of bound peroxidase complexes, the substrate H_2O_2 (30%, 1:2000) was added to the DAB solution and incubated for 5 min. Afterwards the sections were dehydrated in alcohol and cover slipped.

IMAGE QUANTIFICATION

The images of brain sections were projected (10× magnification) and digitalized using an Olympus BX 51 microscope (Olympus, Tokyo, Japan) with a high-resolution digital camera interfaced with a computer. The following brain regions that have been implicated to be involved in anxiety (Arzt and Holsboer 2006; Muigg, *et al* 2007; Nguyen, *et al* 2006) (numbers correspond with the Bregma location) were investigated: prelimbic cortex (1.78), lateral septum (0.86) and the amygdala (basolateral nucleus and central nucleus, -1.58). The anatomical localization was aided by use of adjacent Nissl stained sections and the illustrations in a stereotaxic atlas (Franklin KGB and Paxinos G 1997). For each region at least two overt landmarks were used. For quantitative analysis of c-Fos positive cells, the program LEICA QWIN (image processing and analysis software, Cambridge, UK) was used. Left and right hemispheres were analysed in one section separately and averaged for each animal and calculated for stained neurons per square millimetre.

CORTICOSTERONE

In experiment 2 blood samples were collected via tail vein incision to determine the influence of testing on plasma corticosterone (pCORT) levels of the animals in the different groups, i.e. if indeed testing under white light was more stressful for the animals. Only pCORT from experiment 2 was analysed because no differences in stress levels were expected in experiment 1. Basal blood samples were taken 5 days before testing (BASAL) and another sample half an hour after testing (POST-TEST). All blood sampling took place in a separate room adjacent to the experimental room under red light conditions to not disturb the other animals. To prevent any influence of handling and blood sampling on pCORT, the procedures were done as fast as possible with a maximum of 3 minutes.
A small blood sample was collected (±50 μl) and stored in pre-chilled Microvette tubes (CB300, Sarstedt, Numbrecht, Germany) containing lithium heparin. Blood samples were centrifuged (10 min at 20,000×g, 4 °C) and stored at –20 °C until measurement. pCORT levels were measured by radioimmunoassay (RIA) according to the protocol of the supplier with an ImmuChemTM Double Antibody Corticosterone kit for rats and mice (MPI Biochemicals, Amsterdam, The Netherlands).

STATISTICS

Statistical analyses were performed with the statistical program SPSS for Windows (version 16.0, SPSS.Inc., IL, USA). Continuous data (latencies, durations and number of c-Fos positive cells per square millimetre) were presented as means with a standard error of the mean (SEM) as index of variance. Discrete data (numbers of occurrence) were presented as Median with the Inter Quartile Range (IQR) as index of variance. The Kolmogorov–Smirnov one-sample test was used to check Gaussianity of the continuous data. Group analyses using the Kolmogorov–Smirnov one-sample test showed a non-parametric distribution of several continuous parameters. These parameters, as well as the total numerical parameters, were either rank transformed (Conover and Iman 1982) or log transformed (continuous data). The (transformed) data from the experiment were subsequently analysed with a 2-way ANOVA with group and strain as factors. Another possibility is to perform a multiple regression analysis and using the odour concentration as a continuous variable. This analysis was performed and confirmed the significant effects found by applying the 2-way ANOVA (results

not shown). Comparisons within and between the groups in the acquisition phase were done with a repeated measurements ANOVA using group and strain as between subject factors (experiment 1) and trial as within subject factor, differences between positive and negative trials (experiment 2) were assessed with a paired t-test. Post-hoc testing was done using a Dunn-Ŝidák correction. pCORT data was represented as delta scores (POST test values-BASAL values) + SEM as we were interested in the change of the pCORT levels caused by testing to get an indication of state-anxiety induced pCORT. A one-way ANOVA was performed to investigate condition effects. Between condition effects were further investigated by performing a t-test on the separate groups (POS, MIX, NEG) using condition as an independent variable (α was corrected with Dunn- Ŝidák). The choice data in experiment 3 were analyzed with a one sample t-test on the percentage of correct choices for each day against performance on chance level (33.33%). The other data (latencies, duration and numbers over trials) in this experiment was analysed with a repeated measurement ANOVA, number data were ranked transformed prior to analysis.

RESULTS
BEHAVIOUR
A summary of the behavioural data of all experiments can be found in the supplementary material. Behaviour related to the measurement of judgement bias and odour discrimination will be described in more detail in this section.

EXPERIMENT 1
TRAINING
Mice from both strains became significantly faster in picking up the almond piece over training trials (trial effect: $F_{1,89}= 10.089$, $p=0.000$) and an overall strain difference was found in the latency to eat the almond piece in the training trials (129P3 mice were faster than BALB/c mice; strain effect: $F_{1,89}= 7.373$, $p=0.008$). No strain x trial interaction effect in the training was found ($F_{3, 89}=109.720$, $p=0.561$), data not shown. In total 2 129P3 and 6 BALB/c mice ate the whole bitter almond piece in the NEG trial and were excluded from the test session data.
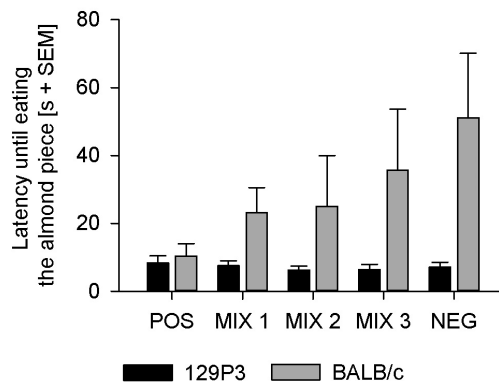

Fig. 2: Time in seconds (+ SEM) from the start of the trial until the almond piece is eaten in the 3rd POS trial and the test session (all mice tested in the dark) of experiment 1. In the test trial a significant strain difference was found, as well as a significant increase in latency between the start of the trial and picking up the food for the BALB/c strain (p<0.005) when compared with the 3rd POS session. A significant difference between the 129P3 and BALB/c strain was found in the test session (p<0.05).

TEST

In the test session the different groups of 129P3 mice showed comparable latencies to eat the almond piece (POS: 8.75 ± 2.1, MIX 1: 7.6 ± 1.4, MIX 2: 6.3 ± 1.1 MIX 3: 6.3 ± 1.6 and NEG: 7.1 ± 1.4 seconds respectively), whereas this latency increased in BALB/c mice when the concentration of the negative odour in the odour mix increased (POS: 10.3 ±3.7, MIX 1: 23.2 ± 7.4, MIX 2: 25.0 ± 14.9, MIX 3: 35.7 ± 18, NEG: 51.1 ± 19 seconds respectively), see Fig. 2. The 2-way ANOVA did not reveal a group difference ($F_{4,90}$= 0.585, p>0.05) , but did reveal a strain difference ($F_{1,90}$= 4.552, p= 0.036). No group x strain interaction effect ($F_{4,90}$= 0.369, p>0.05) was found. Latencies in the third POS session were significantly lower compared with latencies in the test session (data not shown) only as a main effect in the BALB/c group (t=-3.109, p<0.005), post hoc testing revealed no separate group effects (all p>0.025).
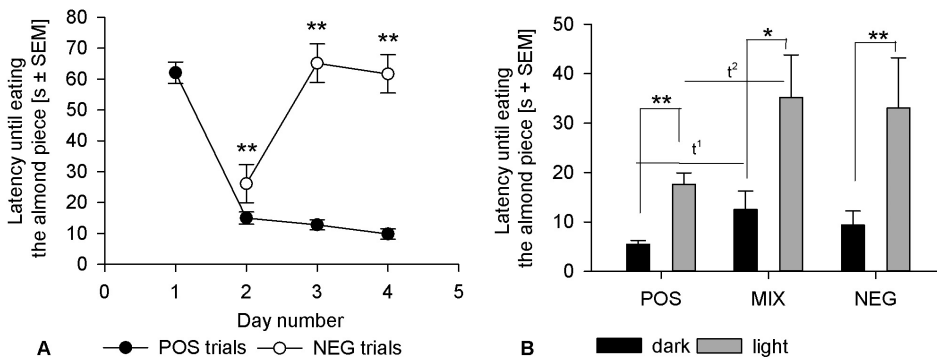
A —●— POS trials —○— NEG trials

B ■ dark ■ light

Fig. 3: Behaviour experiment 2. (A) Latencies to eat the almond piece of BALB/c mice (presented as mean ± SEM) on training days. Significant differences were found between POS and NEG trials, **p<0.001. (B) Latencies of BALB/c mice (presented as means + SEM) to eat the almond piece in the test session. A significant effect was found for light conditions (p<0.001), the group effect for both conditions failed to be significant (p=0.09). *p<0.01, **p≤0.001, t^1= 0.086, t^2= 0.075

EXPERIMENT 2

TRAINING

The mice showed a decrease in latency to start eating the almond pieces in the positive training trials (F_4= 173.419, p<0.001), and an increase in latencies to start eating the bitter tasting almond pieces in the negative training trials (F_2= 17.882, p<0.001), see Fig. 3A. In addition, there were significant differences in picking up the almond piece between positive and negative trials on days 2, 3 and 4 (t=-3.900, p<0.001; t= -10.218, p<0.001 and t= -9.686, p<0.001, respectively).

TEST

The latency to eat the almond piece in the test session is presented in Fig. 3B. Mice tested under white light conditions showed a higher latency to eat the almond piece than mice tested in the dark (condition effect $F_{2,78}$ = 47.293, p< 0.001). Post hoc testing revealed a significant condition effect when the POS (t= -5.865, p< 0.001) MIX (t=-3.324, p= 0.003) and NEG groups (t= -3.811, p= 0.001) were compared between light conditions, (adjusted α=0.017, Dunn-Ŝidák

correction). The two-way ANOVA a showed a trend for differences in latencies to eat the almond piece between the groups ($F_{2,77}= 2.482$, p= 0.09), no group x condition interaction effect was found ($F_{2,77}= 0.015$, p= 0.985). Mice from the MIX groups showed a similar latency to eat the almond piece when compared with the NEG group from the same condition (dark: t= 0.646, p=0.524; light: t= 0.104, p= 0.918). When the MIX group and POS group within the same condition (dark or light) were compared the latencies to eat the almond piece show a trend to be higher in the MIX groups in both conditions (dark: t= -1.840, p= 0.087; light: t= -1.919, p= 0.075).

EXPERIMENT 3

CHOICE

The percentage of trials in which the correct choice was made is presented in figure 4. During the first four days of testing the mice made no difference between the three cylinders; choice for the correct cylinder was not significantly different from chance level (day 1: t= 1.085, p= 0.328; day 2: t= -1.168, p= 0.296; day 3: t= 1.746, p= 0.141; day 4: t= 1.936, p= 0.111). On the last day of testing (day 5) the mice chose on average 58.33% ± 5.69 of the time the correct odour cylinder which was significantly different from chance level (t= 4.392, p= 0.007).
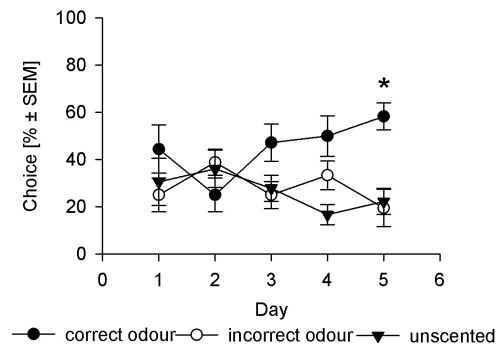

Fig. 4: Mean percentage of correct hole visited during testing on day 1 till 5 in experiment 3. Choice for the correct odour cylinder was compared with performance on chance level (33%). On day 5 the mice chose the correct odour cylinder more than was expected on chance level, *p=0.007.

C-FOS EXPRESSION EXPERIMENT 1

Data are presented in Fig. 5.

PRELIMBIC CORTEX

For the c-Fos expression in the prelimbic cortex no general strain ($F_{1,37}=1.538$, p=0.223) or group ($F_{2,37}=0.359$, p=0.7) effect was found, however the strain x group interaction approached significance ($F_{2,37}=2.945$, p=0.065), this was due to the trend for a difference in positive cells between strains in the group exposed to the ambiguous stimulus (t11=-2.091, p=0.061).

LATERAL AMYGDALA

In the lateral amygdala a significant difference was found between strains ($F_{1,40}=12.631$, p=0.001) and groups ($F_{2,40}=4.010$, p=0.026) the strain x group interaction ($F_{2,40}= 2.028$, p=0.145) was not significant. There were no differences in c-Fos expression levels in BALB/c mice of the different groups (POS: 10.2 ± 1.5, MIX 3: 10.3 ± 1.2 and NEG: 9.1 ± 0.9 cells/mm^2). There were differences between the groups of 129P3 mice (POS: 5.3 ± 1.5, MIX 3: 9.7± 1.4 and NEG: 5.0 ± 0.8

cells/mm^2). Post hoc testing revealed a significant difference between strains in the POS and NEG groups (t_9=3.323, p= 0.009 and t_{15}=3.408, p=0.004 respectively) and a significant difference between the MIX 3 and NEG group (p=0.006) and a trend for a difference between the POS and MIX 3 group (p=0.021) in the 129P3 strain (corrected α p<0.0085).

CENTRAL AMYGDALA
In the central amygdala no differences were found between strains ($F_{1,40}$=0.396, p=0.533) and groups ($F_{2,40}$= 0.016, p=0.984), also the strain x group interaction effect was not significant ($F_{2,40}$=1.986, p=0.150) (Fig. 5).

LATERAL SEPTUM
The expression of c-Fos in the lateral septum was not different between strains ($F_{1,37}$=0.377, p=0.543) and groups ($F_{2,37}$=0.996, p=0.379) and no significant group*strain interaction effect was found ($F_{2,37}$=1.322, p=0.279). A difference between groups could be seen in BALB/c mice (POS: 24.9 ±3.5, MIX 3: 14.7 ± 2.0 and NEG: 24.4 ± 2.7 cells/mm^2). When tested separately on a group effect these differences indeed appeared to be significant (ANOVA $F_{2,22}$= 4.234, p= 0.029).

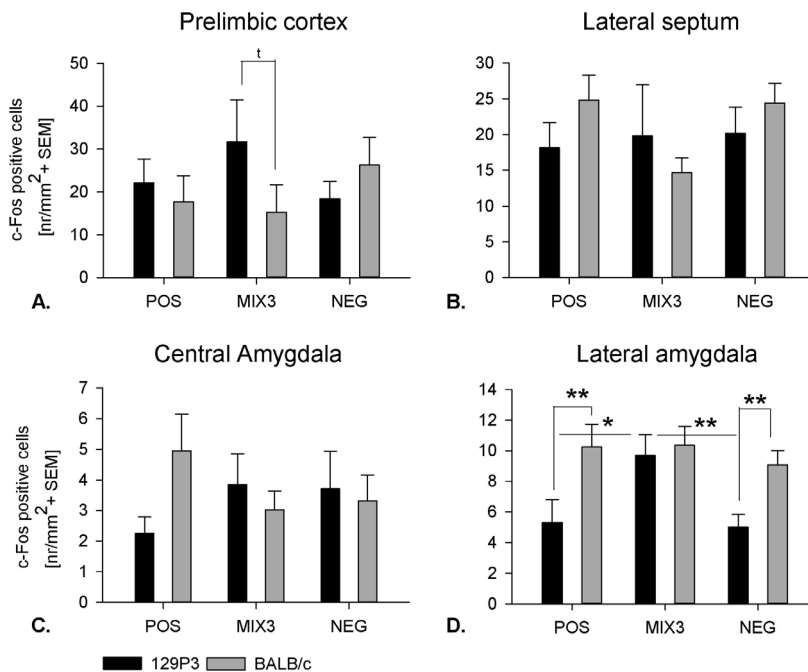Fig. 5: c-Fos expression levels in experiment 1 (expressed as the number of positive cells per mm^2 + SEM) in (a) the prelimbic cortex, (b) the lateral septum the central (c) and lateral (d) amygdala. A trend was found for a group strain interaction for the prelimbic cortex (p=0.065). In the lateral amygdala a significant strain and group effect was found (p= 0.001 and p=0.026). t=trend p=0.061, ** p<0.01, *p<0.05

PCORT EXPERIMENT 2

Delta values between BASAL and POST test samples are represented in Fig. 6. There was a significant difference in delta pCORT values between testing conditions ($F_{5,47}$= 1.266, p= 0.046), mice tested under white light had higher delta values than mice tested under red light. No group ($F_{2,47}$=0.002, p=0.998) or group x condition ($F_{2,47}$= 1.118, p= 0.336) interaction effect was found. Post hoc testing (corrected α= 0.025) revealed only a trend for a difference between conditions in the MIX group (t= 2.327, p= 0.033) and not between conditions in the POS and NEG groups (POS: t= -0.429, p=0.674; NEG: t= -0.728, p= 0.477). Actual and delta pCORT values of BASAL and POST TEST blood plasma samples can be found in the supplementary material.
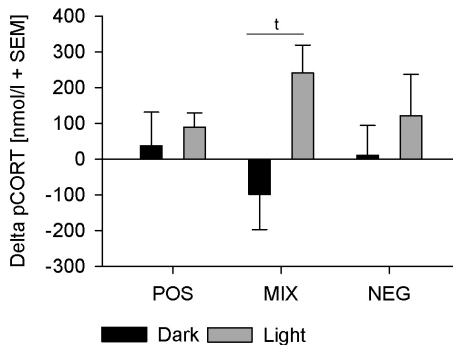

Fig. 6: Delta pCORT (nmol/l + SEM) levels between BASAL and POST testing plasma samples of experiment 2. t= trend p= 0.034

DISCUSSION

BALB/c mice showed a differentiation between positive and negative stimuli in both judgement bias experiments (experiments 1 and 2), i.e. already after exposure to one negative trial BALB/c mice show increased latencies to eat the almond piece (Fig. 3A) and responded with an increased latency to both mixed and negative odour cues in the test trial comparison with the third positive trial. Moreover their response latency seemed to be gradually increased by mixing the positive odour with increasing amounts of the negatively associated odour (Fig. 2) in the test, although this effect did not reach statistical significance. Further, when the light conditions were changed during testing towards more aversive white-light conditions (experiment 2), BALB/c mice revealed an increase in response times to all odour cues next to elevated pCORT levels after testing (Fig. 6), together indicating an increase in state anxiety. Notably, response latencies towards the mixed and negative cues were similar under both testing conditions and differed from the positive cue, suggesting that testing under red and white light conditions induced a negative judgement bias in BALB/c mice. We therefore conclude that the present test set-up provides a basis for the investigation of judgement bias effects in mice.

However, in contrast to BALB/c individuals, 129P3 mice did not respond differently to the different odour mixtures. Other studies have shown that BALB/c mice are relatively fast learners in paradigms using odours as conditioned stimuli (Restivo, *et al* 2006; Roman, *et al* 2002) and have a high odour sensitivity (Lee, *et al* 2003) in comparison to other strains. Restivo *et al*. (2006) hypothesized that this difference in learning capacity could be related to eyesight; in general albino

mice (CD1 and BALB/c) had a better ability to learn odour associations than non albino mice (129S2/SvPasCrl, C57/Bl6 and DBA2).

To our knowledge, no data on the olfactory learning capacities of 129P3 mice are available. Yet, the results of our third experiment confirmed that 129P3 mice are able to discriminate between both odours (Fig. 4). 129P3 mice revealed rapidly decreasing latencies to approach all odour cups after a few training trials in both odour conditioning tasks, showing that 129P3 mice are able to learn the spatial location of a reward, a finding that confirms earlier results of our lab showing that these mice are relatively fast in learning the location of reward in the modified hole board test (Salomons, *et al* 2010a; Salomons, *et al* 2010b). However, 129P3 mice did not seem to build any negative association with the odour predictive for the bitter-tasting almond piece readily as indicated by comparable response times to different odours in the test session. It might be hypothesized that 129P3 mice need more trials than BALB/c animals to establish the association with positive and negative cues, respectively. This hypothesis has to be explored in further experiments.

An alternative explanation for the lack of discrimination between the negative and positive odour, respectively, in 129P3 mice may be that 129P3 mice experience the bitter taste of the almond as less aversive than BALB/c mice. However, this explanation seems unlikely because almost all of the mice rejected the bitter tasting almond in the negative trial.

EFFECTS OF TEST CONDITIONS (WHITE LIGHT VS. RED LIGHT) ON JUDGEMENT BIAS

To evaluate whether the test set-up allows for assessing the effects of a more negative emotional state on judgement bias in mice, BALB/c mice were tested under white light in experiment 2, a condition that has previously been shown to increase avoidance behaviour in the same strain (Salomons, *et al* 2010a). It is remarkable that the latencies to eat the almond piece under dark testing conditions were shorter than the latencies found in experiment 1, which might be explained by the different test set-ups used and the familiarity with the test. Regardless of this it was hypothesized that testing under more aversive bright light conditions would cause a more negative judgement bias than testing under dark (red light) conditions. Yet, it was found that mice under both dark and light testing conditions showed indications of a negative judgement bias, i.e. the response latency in BALB/c mice towards the ambiguous and the negative stimulus was identical under both light conditions, while it tended to differ between the ambiguous and the positive stimulus (Fig. 3). A judgement bias by definition is a relative reaction (or "interpretation") to an ambiguous stimulus: if the reaction to the ambiguous stimulus is similar to the negative stimulus, a negative bias is to be concluded while a positive bias is indicated by a comparable reaction to the positive stimulus (Mathews and Mackintosh 1998). The response profile in BALB/c mice to the different ambiguous stimuli in experiments 1 and 2 was similar to that of previous studies on cognitive bias in rats and sheep, in which the response time to the ambiguous stimulus was higher when the presented ambiguous cue was more similar to the negative cue (Burman, *et al* 2008; Doyle, *et al* 2011; Harding, *et al* 2004).

Some concerns regarding this apparent negative judgement bias under both testing conditions might be raised. Firstly, most cognitive bias experiments in animals describe a relative negative bias when comparing a negatively manipulated group with an appropriate control group that shows a more positive

bias and show no differences in reaction to the positive and negative cues (Burman, *et al* 2008; Burman, *et al* 2011; Doyle, *et al* 2011). Here all groups tested under bright light conditions, irrespective of whether they were tested on either a negative or a positive odour, revealed an increase in latency to explore and pick up the almond, indicating a general anxiety-induced behavioural inhibition. In addition, post-testing stress hormone levels (pCORT) were increased in mice that were tested under white light, confirming that testing under these conditions indeed was more stressful for the animals. Although this is in accordance with previous results showing that an aversive environment (such as exposure to novelty or predator odour) causes an inhibition of familiar food intake in mice (Merali, *et al* 2003; Sterlemann, *et al* 2008), it is difficult to compare the groups tested under the different light conditions regarding their relative judgement bias. Further, it might be discussed whether results were confounded in that the presentation of a negative associated odour cue itself induced a more negative affective state and whether, thus, the mere presence of this odour in the mixture inhibited the mice from eating the almond piece. Here, latencies to explore the odour cups and cylinders did not differ between the groups in both experiments 1 and 2 (see supplemental material), indicating that the motivation to search for food at least did not differ between the groups. Others have resolved this problem by using a conditioning paradigm based on expectancy of reward size or value as indication of reward (e.g. (Brydges, *et al* 2011)). However, for the measurement of anxiety such an approach might be less suitable, since high anxiety is hypothesized to cause an increase in the expectancy of negative events and not a decrease in the expectancy of positive events (MacLeod, *et al* 1997).

Rats show a difference in judgement bias between dim and bright light testing (Burman, *et al* 2009). In contrast with our study these rats were trained under dim light conditions, but tested under bright light conditions or vice versa. It appeared that a shift towards a more aversive test condition induced a negative judgement bias, while shifting towards less aversive conditions resulted in a positive judgement bias. In our experiment, all animals were trained under dim (red) light conditions and tested either under the same or more aversive white light conditions which could explain the difference with the mentioned rat study. A more negative interpretation of ambiguous cues is thought to be related to a more negative affective state, which again can be influenced by current environmental conditions, trait affect and previous experiences (Mendl, *et al* 2010b). Notably, it has been argued that the BALB/c inbred strain represents a high trait anxiety phenotype (Belzung and Berton 1997; Belzung and Griebel 2001), which would be in line with a given sensitivity to establish a negative bias under less-aversive and aversive conditions.

C-FOS EXPRESSION

Despite the apparent lack of discrimination between the different odour stimuli in 129P3 mice (experiment 1), a higher c-Fos expression was found in the lateral nucleus of the amygdala in the group that had been exposed to the ambiguous stimulus in comparison with the groups exposed to the positive or negative stimulus, respectively. In addition, and similar to the lateral nucleus of the amygdala, a trend for an increase in c-Fos expression was found in the prelimbic cortex in the 129P3 group that was exposed to the ambiguous cue, while no differences were found in BALB/c mice. The connection of this region with the amygdala might explain the similarity in the c-Fos responses in both regions, i.e. the prelimbic cortex projects to the basal part of the lateral amygdaloid nucleus

and neurons from this same part also send projections back (Groenewegen, *et al* 1997; McDonald, *et al* 1996).

Lesion experiments suggest that the amygdaloid nuclei involved in appetitive and aversive learning are functionally similar (Everitt, *et al* 2003), which may explain why in the present experiment no differences in c-Fos expression were found between the groups exposed to either the positive or the negative cue. However, in combined action with higher order regions such as the prefrontal cortex, the basolateral amygdala is indicated to be involved in this evaluation of ambiguous and uncertain situations (Davis and Whalen 2001). In humans there is some evidence that exposure to uncertainty and ambiguous cues results in an higher amygdala activation (Blasi, *et al* 2009; Herry, *et al* 2007; Hess, *et al* 1997; Hsu, *et al* 2005; Whalen 1998). In addition some authors have suggested that uncertainty is processed similar to ambiguity since the chance of a forthcoming event in both situations cannot be foreseen (Herry, *et al* 2007; Hsu, *et al* 2005). Experimental work has indicated that unpredictability increases c-Fos expression in the mouse lateral amygdala (Herry, *et al* 2007) and might thus also be implicated in response to ambiguous cues. Thus, while the increased amygdala and prelimbic activity that was seen in 129P3 mice in response to exposure to the ambiguous cue might indeed seem to indicate that the ambiguity of the cue is processed at the brain level, it remains unclear why these mice were unable to translate process into an appropriate behavioural response.

While in BALB/c mice no differences were found in both the lateral nucleus of the amygdala and the prelimbic cortex, in the lateral septum there appeared to be a decrease in c-Fos expression in response to the ambiguous cue. The lateral septum is an essential node in integrating cognitive information with emotional information (Sheehan, *et al* 2004). This area acts as a system that compares known information with actually presented information, which is especially important for the identification of ambiguous cues. A human patient for example with lesions in this region has been reported to reveal problems with judging the valence of novel environmental information (von Cramon, *et al* 1993). c-Fos expression in the the lateral septum revealed a trend towards reduction in response to ambiguous cue exposure in the BALB/c strain (when the statistical analysis was done separately from 129P3, the difference reached significance), but not in 129P3 animals. It may be hypothesized that this difference in the processing of ambiguous and predictable information between 129P3 and BALB/c mice in the lateral septum may be related to differences in behaviour in the test session. The nature of the difference found on the brain level remains to be investigated, as c-Fos expression as a quantitative measure only can offer a first indication.

CONCLUSIONS

In summary, the behavioural data reveal that there is a strain difference in performance in the odour discrimination task intended to measure judgement bias in mice: BALB/c mice discriminate between an odour predicting an almond piece and an odour predicting a bitter tasting almond piece, while 129P3 mice respond very fast to all odour cues presented. BALB/c mice also are more reluctant to eat almond pieces that are presented together with the ambiguous odours and reveal a negative judgement bias both under red and white light conditions. Therefore we conclude that the present test provides a basis for evaluating judgement bias in BALB/c mice. At the brain level, c-Fos expression in the amygdala, prelimbic cortex and lateral septum indicated that there may

be strain differences in information processing: while c-Fos expression levels did not differ between positive and negative cue exposure in both strains, exposure to the ambiguous cue increased c-Fos activity in the lateral nucleus of the amygdala and the prelimbic cortex in 129P3 mice and seemed to decrease c-Fos activity in the lateral septum in BALB/c mice. These results suggest that 129P3 mice may perceive the ambiguous cue as different from the positive and negative cue at the level of the brain, only this perception is not translated into a behavioural response. Notably, exposure to an ambiguous cue affected c-Fos activity in the lateral septum in BALB/c, but not in 129P3 mice. This area is important for linking emotional with cognitive information and it has been shown in other experiments that neuronal activation of this specific area differs between the two strains. Thus the lateral septum might be an important target to investigate in future experiments.

ACKNOWLEDGEMENTS

SUPPLEMENTARY INFORMATION

Table S1: Ethogram of the behaviours scored in all three experiments. From all behavioural parameters the latency until first expression of the behaviour and numbers were recorded, as well as the duration for state categories (sniffing and grooming).

Behavioural category	Behaviours included
Experiment 1 & 2	
Behaviour directed at odour cup/cylinder	Exploration odour cup Directing nose at a distance < 2cm at the odour cup and/or touching it with nose
Behaviour directed at almond	Pick-up almond piece Taking food into the mouth
	eating the almond piece Chewing and ingesting the almond piece
Exploration	Rearing Upright posture with forelegs moving into the air (above 45º) or leaning against the wall of the enclosure
	Line crossings mouse crosses line between front and back of the cage with all 4 paws
Arousal	Grooming Mouse scratches or licks fur, washes face or licks genitalia
Experiment 3	
Choice	Head dip correct cylinder Head dip incorrect cylinder Head dip unscented cylinder Head is dipped in the odour cup, eyes are under the upper brim of the odour cup
Directed exploration	Sniffing correct cylinder Sniffing incorrect cylinder Sniffing unscented cylinder Nose directed at <2 cm from the odour cup and/or touching it with nose
General exploration	Rearing Upright posture with forelegs moving into the air (above 45º) or leaning against the wall of the enclosure

Table S2: Overview of behavioural data of experiment 1 and the significant effects. Latency and duration data is presented as mean ± SEM (seconds) and number data as median ± IQR . s= strain effect, g= group effect, s*g= interaction effect. * p< 0.05, **p<0.01, ***p<0.001, t^1=0.065, t^2=0.090.

Behaviour	Statistics s = strain g = group	BALB/c					129P3				
		POS	MIX 1	MIX 2	MIX 3	NEG	POS	MIX 1	MIX 2	MIX 3	NEG
Exploration odour cup											
Latency	ns	9.14±3.89	8.88±3.01	2.83±0.96	4.53±1.15	7.4±2.00	5.65±1.99	5.02±1.25	3.33±0.91	3.77±1.48	3.77±1.24
Duration	ns	22.87±3.99	28.64±3.81	23.33±3.36	27.71±4.08	31.01±4.11	26.12±3.36	23.84±2.27	27.66±3.36	27.18±3.23	32.55±4.07
Number	ns	15.5±6.75	15±4	14±4.5	15±9	17±6	13±6	16±7	17±5.5	15.5±10	14.5±12.75
Pick up almond piece											
Latency	s t1	8.68±3.68	22.29±7.39	52.92±32.82	27.28±17.28	33.36±14.65	7.04±2.06	6.66±1.32	5.15±1.07	5.22±1.56	6.06±1.36
Eating											
Latency	s*	10.35±3.68	23.16±7.38	25.01±14.94	35.70±18.02	51.11±19.00	8.35±2.08	7.58±1.42	6.26±1.12	6.36±1.56	7.12±1.42
Rears											
Latency	s*	30.07±10.35	12.76±2.74	16.1±3.94	10.54±3.62	10.98±3.14	26.50±4.49	18.84±4.62	18.89±3.82	17.86±4.26	21.77±5.57
Number	s***	68±14.5	71±22.5	71±22	65.5±16.25	61±22.75	52±11.5	53±18	57±15	53.5±7.5	53.5±7.25
Line crosses											
Latency	gt2	10.44±3.19	8.93±2.51	13.72±3.34	4.73±1.21	6.62±2.03	15.34±3.57	6.94±2.68	12.35±3.16	7.79±2.51	9.73±2.85
Number	s***	35.5±10.25	32±7.5	32±12	31.5±9.25	28±13	22±10.5	27±13	23±6	30.5±8.25	25.5±6
Grooming											
Latency	s** , s*g***	231.47±28.26	123.82±23.96	169.88±31.17	118.94±23.74	207.49±28.07	204.58±31.69	237.57±31.80	253.30±32.10	256.55±16.51	150.27±24.53
Duration	ns	2.35±0.82	3.26±0.27	2.60±0.53	2.92±0.42	1.54±0.52	2.02±0.67	1.62±0.78	1.76±0.88	2.58±0.78	3.34±0.64
Number	s* , s*g***	0.5±1.75	1±1	1±0.5	2±1	1±1.75	1±1	0±1	0±1.5	1±1	1±1.25

Table S3: Overview of behavioural data in BALB/c mice of experiment 2 and the significant effects. Latency and duration data is presented as mean ± SEM (seconds) and number data as median ± IQR. g= group effect, c= condition effect, g*c= interaction effect. * p< 0.05, **p<0.001

Behaviour	Statistics c= chance t= trial	Day 1	Day 2	Day 3	Day 4	Day 5
Choice						
Head dip						
Correct	c* day 5	44.44 ± 10.24	25 ± 7.14	47.22 ± 7.95	50 ± 8.61	58.33 ± 5.69
Incorrect		25 ± 7.14	38.89 ± 5.56	25 ± 5.69	33.33 ± 6.09	19.44 ± 7.95
Unscented		30.56 ± 10.02	36.11 ± 7.95	27.78 ± 5.56	16.67 ± 4.30	22.22 ± 5.56
Other behaviour						
		Trial 1	Trial 7	Trial 13	Trial 19	Trial 25
Sniffing						
Duration correct	ns	9.89 ± 2.09	10.59 ± 2.98	16.86 4.47	13.32 ± 5.36	16.56 ± 14.08
Duration incorrect	ns	9.60 ± 2.14	11.09 ± 5.62	4.25 ± 2.65	11.13 ± 3.21	6.39 ± 3.89
Duration Unscented	ns	9.03 ± 1.49	12.26 ± 3.14	5.60 ± 5.35	11.76 ± 3.66	5.56 ± 3.48
Total number	t**	31 ± 18.75	4.5 ± 5.5	1.5 ± 2.25	2.0 ± 3.25	2.5 ± 3.25
Hole visits						
Latency	t**	331.21 ± 108.79	26.94 ± 6.92	32.03 ± 21.99	24.14 ± 8.82	55.12 ± 28.49
Latency correct	t**	462.06 ± 84.04	55.30 ± 18.04	84.71 ± 66.66	47.36 ± 8.8	64.20 ± 27.30
Number	t*	2 ± 2	3 ± 2.5	1.5 ± 1.25	3 ± 4	2 ± 2.5
Rears						
Number	t**	19 ± 9.5	0 ± 0	1 ± 1.75	0.5 ± 2	0 ± 5

Table S4: Overview of behavioural data of experiment 3 and the significant effects. Latency and duration data is presented as mean ± SEM (seconds) and number data as median ± IQR. c= choice effect (significant difference from chance performance), t = trial effect. *p<0.01, **p< 0.001.

Behaviour	Statistics s = strain c = condition	Dark			Light		
		POS	MIX	NEG	POS	MIX	NEG
Eating							
Latency	c**, g[t3]	5.74±0.78	12.81±3.74	9.78±2.83	18.58±2.23	35.62±8.60	34.25±10.07
Sniffing							
Latency	c**	1.79±0.21	3.13±0.56	2.92±0.6	9.24±1.60	9.16±1.79	10.82±2.27
Duration	c**	5.48±1.99	5.17±1.47	4.09±0.73	5.28±0.46	7.45±1.33	6.16±0.71
Number	c[t1]	1±0.25	1±0.5	1±1.25	1±1.25	1.5±2.5	2±1.25
Rear							
Latency	ns	140.22±9.8	121.17±15.35	119.20±16.38	100.45±18.46	84.34±18.28	110.20±17.46
Number	c*	0±0	0±0.25	0±0.25	0±1	0.5±1.25	0±1
Pick up food							
Latency	c**, g[t2]	5.46±0.78	12.51±3.74	9.39±2.83	17.62±2.30	35.21±8.59	33.07+10.18
Head dip							
Latency	c**, g[t1]	3.93±0.52	9.99±2.97	8.30±2.72	15.75±2.29	31.62±8.56	30.85±10.06
Number	ns	1±0	1±1	1±0	1±0	1±0	1±0.25
Grooming low occurence							

Table S5: Overview of the actual pCORT values (nmol/l) and their delta values in the plasma samples of experiment 2. Data is presented as mean ± SEM. t= trend p= 0.066, * p<0.05

| Sample	Statistics c = strain g = group s = sample	Dark			Light		
		POS	MIX	NEG	POS	MIX	NEG
pCORT (nmol/ml)							
BASAL		354.93± 87.81	404.58± 60.23	224.44±35.11	223.92± 37.15	330.62± 39.37	296.57± 35.09
POST TEST	s^t	374.79± 70.29	359.49± 62.10	335.61± 59.05	419.93± 134.41	464.13± 95.89	424.70± 138.72
TRUNK		312.20± 69.07	342.46± 55.28	240.68± 33.27	311.66± 52.74	454.33± 91.44	347.95± 47.72
Delta pCORT (nmol/ml difference)							
POST TEST - BASAL	c*	37.62± 94.14	-99.12± 98.37	11.40± 83.22	90.46± 39.06	87.81± 163.26	121.75± 115.95
TRUNK - POST TEST	ns	-157.84± 75.46	-67.92± 54.98	-81.44± 66.62	-11.13± 42.91	67.64± 157.36	-185.32± 90.36
TRUNK - BASAL	ns	-124.50± 107.01	-59.93± 95.38	-13.69± 59.75	97.22± 62.85	55.74± 246.53	37.93±55.27

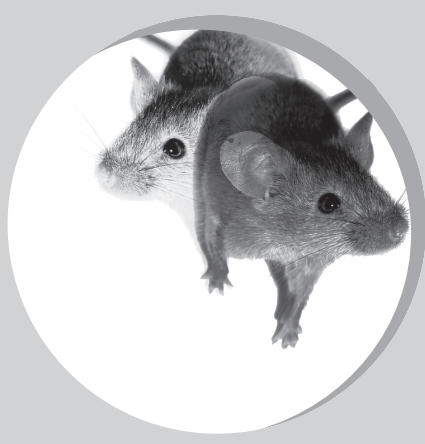## BOX 1: LIGHT AND TESTING INDUCED C-FOS EXPRESSION PATTERNS IN BALB/C MICE

In extension to the findings described in chapter 4 (experiment 1), showing strain differences in neuronal activation of distinct brain areas after performance of the judgement bias test under red-light conditions (Fig. 5; page 72), we investigated the influence of different testing conditions (experiment 2; chapter 4) on c-Fos expression patterns in BALB/c mice. Further, c-Fos immunohistochemistry results were analysed separately for both the right and left hemisphere.

The results showed that differences between light and dark testing conditions and between stimuli were most apparent in the subnuclei of the amygdala and (dorso) lateral septum (Fig. 1). More specifically, c-Fos expression in the left basolateral amygdala (BLA) was higher for mice tested in the light when compared to those animals tested in the dark ($F_{1,46}$= 4.189, p= 0.046). Post hoc analysis showed that this light/dark difference was only tending to be different in response to the positive (t= -2.495, p= 0.024) and the negative stimulus (t= -2.194, p= 0.041) while in the right BLA c-Fos expression was higher in response to the negative stimulus only in mice tested in the dark (condition x stimulus interaction: $F_{2,43}$= 4.632, p= 0.015, post hoc difference between POS-NEG: p= 0.083). In addition, c-Fos expression in the left central amygdala (CeA) was higher in mice exposed to the positive cue than in mice exposed to the ambiguous or negative cue (stimulus effect: $F_{2,44}$= 3.628, p=0.035, post hoc p< 0.011). A similar effect was found in the right dorsolateral part of the lateral septum (LSD): mice tested in the light and exposed to an ambiguous cue showed a lower c-Fos expression than mice exposed to a positive or negative cue, whereas mice tested in the dark showed a different response (condition x stimulus interaction: $F_{2,35}$= 4.279, p= 0.022). Further, post hoc testing showed that mice tested in the dark showed a higher c-Fos expression in response to the ambiguous cue (t=3.021, p= 0.012) and c-Fos expression for mice exposed to an ambiguous cue in the light was lower than that for mice exposed to a positive cue in the light (p=0.014). Thus, in summary more or less aversive test conditions result in differential effects on neuronal activation especially in the amygdala and the lateral septum, brain areas involved in the regulation of emotions (Davis and Whalen 2001; Kirk 1998; LeDoux 2003; see for example McNaughton and Gray 2000; Millan 2003; Pratt 1992; Sheehan, *et al* 2004), which might be a reflection of differences in state anxiety under these test conditions, since mice showed more behavioural inhibition (figure 3; page 70) and a higher testing induced pCORT response (figure 6; page 73) under white light conditions. Interestingly, human literature also indicates that the amygdala is important for the processing of uncertain and ambiguous information (Blasi, *et al* 2009; Herry, *et al* 2007; Hess, *et al* 1997; Hsu, *et al* 2005; Whalen 1998), while the lateral septum is more involved in integration between emotional and cognitive information (Sheehan, *et al* 2004), a process that is important for judgement bias processes. Notably, different patterns of c-Fos expression occurred in right and left

hemispheres, respectively and significant effects only were observed when hemispheres were analysed separately. This is not surprising since lateralization effects are well known in humans; for example the right amygdala has been shown to be more involved in the processing of ambiguous information than the left amygdala (Blasi, *et al* 2009). However, in most experimental research in animals c-Fos data of left and right hemispheres are being pooled and analysed together, as we had done in chapter 4 as well. Thus, it is possible that the data presented in chapter 4 contains some more subtle lateralized effects, which still needs to be analysed. Anyway, for future experiments we suggest to investigate right and left brain areas separately in order to avoid missing potential lateralization effects.

Fig. 1: Testing-induced c-Fos expression levels (number of cells/mm^2 ± SEM) in brains of BALB/c mice exposed to positive (POS), ambigous (MIX) and negative (NEG) stimuli under dark or white light testing conditions, respectively (total brains analysed: n=52). Statistical analysis was done on left and right hemispheres separately using a two-way ANOVA with 'stimulus' and 'light condition' as main factors. BLA= basolateral amygdala, CeA= central amygdala, MeA= medial amygdala, LSD= dorsolateral septum. t^1= 0.027, t^2= 0.040, t^3= 0.083, *p< 0.05, ** p <0.01 (Post-hoc testing was done with an adjusted α p<0.017, Dunn-Sidâk correction)
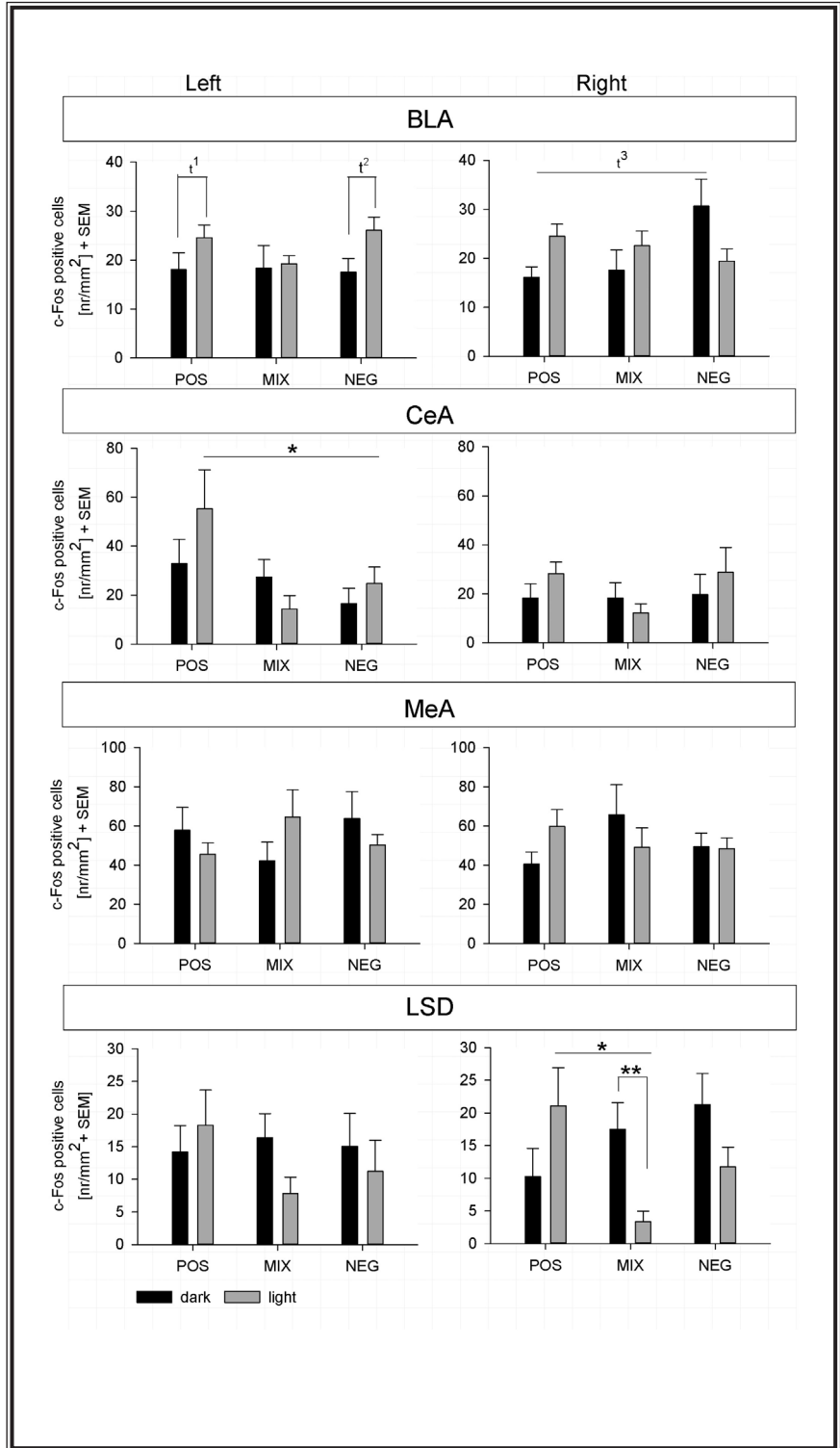
CHAPTER 5

BEHAVIOURAL EFFECTS OF DIAZEPAM IN A JUDGEMENT BIAS TEST FOR MICE

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ABSTRACT

The interpretation of ambiguous cues is influenced by affective states, an effect called judgement bias. In reverse logic, judgement bias tests can be used to assess an individual's perception of its own affective state, a method that has recently been established in different animal species. Male BALB/c mice have been shown to reveal a negative judgement bias under white light test-conditions after having been trained to associate distinct odour cues with either a palatable or an unpalatable food reward. Here, the response of BALB/c mice towards an ambiguous odour (50/50 odour mixture) was investigated after pre-treatment with the anxiolyticum diazepam, in order to validate the causal relationship between affective state and judgement bias in mice. While only a subgroup of animals revealed a negative bias (responders), it was these responders that showed a reduced latency to explore the ambiguous odour cue after treatment with (1 and 3 mg/kg) diazepam. Although statistically weak, these results support the idea that a causal relationship exists between high state anxiety and negative judgment bias in mice. The present study provides a basis for future experiments that should evaluate responder/non-responder effects and group differences in more detail.

INTRODUCTION

The interpretation of ambiguous information is influenced by emotional states and traits, a process called judgement- or interpretation bias (Mathews, *et al* 1997). In effect, people that are characterized by a high trait anxiety show a more negative interpretation of ambiguous information than people with a low trait anxiety (Chan and Lovibond 1996; Mathews, *et al* 1989; Mathews and MacLeod 1994). Correspondingly, it has been shown that people with anxiety disorders show negative judgement biases as well (Eysenck, *et al* 1991). In general, this interaction between anxiety and cognitive processing of information might be considered adaptive, since in potentially dangerous situations it may be advantageous rather to avoid an ambiguous stimulus than to approach it (Bateson, *et al* 2011). However, when anxiety is persistently or inadequately increased anxious reactions and cognitive bias can develop into a pathological and, thus, maladaptive variant (Ohl, *et al* 2008) in that a too negative bias can result in the avoidance of non-dangerous and even useful resources (Berger-Tal and Avgar 2012).

Recent research shows that conditioning procedures using positive and negative association learning and subsequent exposure to ambiguous cues might be used to measure judgement biases in animals as well (Bateson and Matheson 2007; Bateson, *et al* 2011; Brilot, *et al* 2010; Brydges, *et al* 2011; Burman, *et al* 2008; Burman, *et al* 2009; Burman, *et al* 2011; Douglas, *et al* 2012; Doyle, *et al* 2011; Enkel, *et al* 2010; Matheson, *et al* 2008; Mendl, *et al* 2010a; Pomerantz, *et al* 2012; Richter, *et al* 2012; Salmeto, *et al* 2011; Sanger, *et al* 2011). It has been proposed that the assessment of judgement bias in animals may be used to get an indication of the animal's internal emotional state (Paul, *et al* 2005). Reliable indications of the animal's own perception of its emotional state are of relevance both for the understanding and assessment of animal welfare (Boissy, *et al* 2007a; Dawkins 2006; Mendl, *et al* 2009; Ohl and van der Staay 2012) and the validation of animal models for human affective disorders (Anderson, *et al* 2012a; Blanchard, *et al* 2001; Enkel, *et al* 2010; Richter, *et al* 2012; Richter, *et al* 2012). In previous studies, we have made use of a potential judgement bias test for mice to investigate the interpretation of ambiguous information in BALB/c mice under more or less aversive testing conditions, respectively (Boleij, *et al* 2012b): it appeared that mice that were tested under more aversive bright light showed similar reactions towards the ambiguous and negative cues indicating a negative judgement bias when compared to animals that were tested under less aversive dim light conditions. Although it is known that bright light induces an increase in anxiety related behaviour in BALB/c mice (Salomons, *et al* 2010a), a causal relationship between the "negative judgement bias" that was found and an increased state anxiety in BALB/c mice cannot be concluded from the previous experiments. Therefore the aim of the present study was to investigate if a pharmacological modulation of state anxiety would shift the negative judgement bias towards a more positive bias in BALB/c mice.

Benzodiazepines (BZs) have been widely used in the treatment of anxiety disorders (Nash and Nutt 2005; Shader and Greenblatt 1993). In animal research benzodiazepines have been shown to decrease anxiety as well: For example, BALB/c and C3H mice treated with chlordiazepoxide spent more time in the lit compartment of the light/dark box and in a novel compartment in the free exploratory paradigm (Kopp, *et al* 1999) and diazepam has been found to decrease avoidance behaviour in the modified hole board in BALB/c mice (Ohl, *et al* 2001a). Anxiolytic effects in rodents however appear to be strain- and/or

behavioural-test-dependent, (Clément, *et al* 2009; Kopp, *et al* 1999; Ohl, *et al* 2001a) and especially BALB/c mice, that are characterized by a high state anxiety when exposed to a novel (test) situation (Salomons, *et al* 2010; Salomons, *et al* 2010a), seem to be sensitive to the anxiolytic effects of BZs (Belzung and Berton 1997; Belzung and Griebel 2001; Griebel, *et al* 1993). Therefore diazepam was selected in the present experiment to modulate state anxiety in BALB/c mice. The judgment bias test used in this study consists of a conditioning procedure coupling one odour with a palatable almond piece and another odour with an unpalatable (bitter tasting) almond piece. In the test animals are exposed to a mixture of both odours and the response (latency to explore/eat the almond piece) towards this stimulus is compared with the response towards the positive and negative stimuli, respectively. Again, bright (white) light was used to increase state anxiety during the test session as was done in our previous experiment (Boleij, *et al* 2012b). The effect of a reduction of state-anxiety via pre-test treatment with two doses of diazepam on judgement bias as well as several behavioural parameters and plasma corticosterone (pCORT) levels in the mice was investigated.

MATERIALS AND METHODS
ETHICAL NOTE
The protocol of the experiment (DEC-DGK number 2011.I.08.80) was peer reviewed by the scientific committee of our department and approved by the local Animal Experiments Committee. The Animal Experiments Committee based its decision on "De Wet op de Dierproeven" (The Dutch "Experiments on Animals Act" 1996) and on the "Dierproevenbesluit" (The Dutch "Experiments on Animals Decision", 1996). Both documents are available online at: http://wetten.overheid.nl. Further the animal experiments followed the "Principles of laboratory animal care" and refer to the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioural Research (National Research Council 2010).

ANIMALS AND GENERAL HOUSING CONDITIONS
Husbandry and experimental procedures were performed by well-trained members of the laboratory. 42 male BALB/cJ (BALB/c) mice were obtained from Charles River laboratories (Margate, UK) and were 6-8 weeks of age at arrival. The experiment took place at the animal laboratory of the Netherlands Vaccine Institute (Bilthoven, The Netherlands) in a humidity (45%-50%) and temperature ($22 \pm 2°C$) controlled room. The animals were housed individually in Eurostandard type 3 macrolon cages (40 cm x 26 cm x 20 cm) with standard cage bedding (Aspen chips) a plastic shelter (Mouse House Techniplast®) and facial tissue (Kleenex® Facial Tissue, Kimberly-Clark) as standard enrichment. Animals were housed under a reversed light/dark cycle (the dark period started at 6:00 and lasted until 18:00 h, with red lights on) and radio music was turned on as background noise during the dark phase in the whole experimental period. During the first two weeks after arrival (habituation period) the animals were handled for ~3 minutes per mouse three times a week (between 9:00 and 11:00 h) by the experimenter who also did behavioural testing and were weighed twice a week. All testing took place in the animals housing room and equipment was installed before the animals arrived.

DIAZEPAM

Diazepam (Centrafarm Services, Etten-Leur, The Netherlands) was dissolved in saline under sterile working conditions and injected in a volume of 10 ml/kg; 1 mg diazepam was dissolved in 10 ml for the 1 mg/kg dose (1 DZ), 3 mg diazepam was dissolved in 10 ml for the 3 mg/kg dose (3 DZ) and saline (0 DZ) was used in the vehicle control group. The diazepam doses were chosen based on previous experiments using male BALB/c mice showing clear anxiolytic effects on behaviour in the mHB and the open field test (see Ohl, *et al* 2001a; Salomons, *et al* 2012). Drugs were injected i.p. 30 minutes before testing in the experimental room.

EXPERIMENTAL PROCEDURES

The paradigm used was similar to that used before (see (Boleij, *et al* 2012b)), although the test set-up was somewhat different. In the present experiment a within subject comparison instead of a between subject comparison was used and more training trails were performed (see also Training and Testing).

ALMOND PIECES, BITTER STIMULI AND ODOUR STIMULI

Pieces of almond were used as rewards, mice eat these readily even if they are fed *ad libitum* (see for example (Ohl, *et al* 2003)). For the negative trials (NEG) almonds were soaked for 5 minutes in 180 mM quinine hydrochloride (Sigma-Aldrich) dissolved in demineralised water, and dried overnight. Odour stimuli that were used were vanilla and apple odour (Micro-Plus, Stadtoldendorf, Germany), dissolved in distilled water (0.05%), since mice are attracted by those (e.g. (Ohl, *et al* 2003)). Both odours were dissolved in a low concentration because the stock solution is highly concentrated and similar concentrations were used before (Boleij, *et al* 2012b).

APPARATUS

During training and test trials almond pieces were presented on a small petri dish (ø 5.5 cm). The odours were spread on a filter paper (ø 5.5 cm) in an amount of 0.1 ml per odour that was positioned in the petri dish (see (Boleij, *et al* 2012b)). The petri dish with the filter paper was covered by a lid with several holes to let the odours diffuse through the top. From now on this dish will be called the odour cup.

TRAINING

Animals were tested during the dark (active) phase between 9:00 h and 16:00 h. Mice were habituated to eating the piece of almond (30 mg), by offering it with tweezers in the home cage on days 14, 15 and 16 after arrival. On day 19-23 after arrival the training and testing procedure took place, consisting of 6 trials per mouse per day (session). Briefly, a trial started with the placement of the home-cage on a table in front of a video camera (placed on the side of the odour cup) that was connected to a dvd-recorder (Panasonic). During preparation of the home cage for the trials the mouse was placed in a small empty cage next to the home cage. Before each training or test session enrichment and the water bottle were removed from the home cage and the correct odour cup with the almond piece was placed in the middle of one of the short cage sides (see (Boleij, *et al* 2012b)), the trial was started with gently placing the mouse on the opposite end of the cage. Trials were ended when either the whole almond piece was eaten or when the cut-off time of 2.5 minutes was reached. The next trial started

after an inter-trial interval of 1 minute.

During training in a positive (POS) trial the odour cup or cylinder was presented with a normal tasting almond piece and in a negative (NEG) trial the odour cup or cylinder was presented with a bitter tasting almond piece, and within groups half of the mice received apple as a POS conditioned stimulus and vanilla as a NEG conditioned stimulus (counterbalanced design). On the first day of training (day 19 after arrival) the mice received 4 POS trials and 2 NEG trials, on the second, third and fourth day mice received 3 POS and 3 NEG trials. All trials were presented in a random order and mice were tested randomly each day.

TEST SESSION AND EXPERIMENTAL GROUPS

In the test session (day 23 after arrival), in addition to 2 POS and 2 NEG trials, 2 ambiguous (MIX) trials were performed, in which a 50/50 mixture of both odours was presented with a palatable almond piece. All trials were presented in a random order and mice were tested in a random order. 30 minutes before the test session mice were i.p. injected with either 0 mg/kg DZ (0 DZ, n=14), 1 mg/kg DZ (1 DZ, n=13) or 3 mg/kg DZ (3 DZ, n=14).

A further subdivision of the groups was made, based on the responses of the animals in the test session, i.e. a clear distinction could be made between animals that decided to pick up and eat the bitter almond piece and animals that were avoiding the bitter almond piece. Animals were defined as non-responder if they started eating the bitter tasting almond piece in both negative trials of the test session and as a responder if they did not. This resulted in the following subdivision within groups (responders/non-responders respectively): 0 DZ (12/2), 1 DZ (10/4) and 3 DZ (7/7). Since only the responders showed a sufficient avoidance in the negative trials, conclusions on judgement bias are based on responder data. We recognize that the subdivision causes limitations for the statistics on especially the non-responders. Therefore the presented data on the non-responders and differences between responders and non-responders are cannot be reliably interpreted at present.

BEHAVIOURS SCORED

Behaviour during testing was scored live using the computer program "The Observer" version 5.0 (Noldus b.v. Wageningen, the Netherlands). Behaviours were scored in a continuous way, i.e. all-occurrence recording of the behaviours of interest. The following behavioural parameters were measured: Latency until picking up and eating the almond piece, exploration (sniffing) of odour cup (latency and duration), locomotor activity: line crossings between front and back (latency and total number), general exploration: rearing (latency and total number), grooming (latency, total duration and total number).

CORTICOSTERONE

Blood samples were collected via tail vein incision to determine the influence of diazepam and testing on plasma corticosterone (pCORT) levels of the animals in the different groups, i.e. if treatment with diazepam had an effect on testing induced stress in the animals. To this aim basal blood samples were taken 5 days before testing (BASAL) and another sample half an hour after testing (POST-TEST). A third blood sample was obtained from the trunk blood (TRUNK) after decapitation, which was performed two hours after testing. All blood sampling took place in a separate room adjacent to the experimental room not to disturb the other animals under red light conditions. To prevent any influence of

handling and blood sampling on pCORT, the procedures were done as fast as possible with a maximum of 3 minutes. A small blood sample was collected (±50 µl) and stored in pre-chilled Microvette tubes (CB300, Sarstedt, Numbrecht, Germany) containing lithium heparin. Trunk blood after decapitation was collected in Minicollect tubes (1 ml Lithium Heparin, Greiner Bio-One GmbH, Kremsmünster, Austria). Blood samples were centrifuged (10 min at 20,000×g, 4 °C) and stored at–20 °C until measurement. pCORT levels were measured by radioimmunoassay (RIA) according to the protocol of the supplier with an ImmuChemTM Double Antibody Corticosterone kit for rats and mice (MPI Biochemicals, Amsterdam, The Netherlands).

STATISTICS

Statistics were performed with the computer programme SPSS 16.0.1 for Windows (SPSS Inc. IL, USA). Continuous data (plasma CORT, latency and duration of behavioural parameters) are represented as mean ± standard error of the mean (SEM), and were first investigated for gaussianity using the Kolmogorov-Smirnov test. Homoscedasticity was tested by Levine's test. Discrete data on the ordinal scale (total number of behavioural parameters) are represented as median with interquartile range. Some of the parameters revealed a non-parametric distribution and were either log-transformed (continuous data) or rank transformed (number data) to get a normal distribution. All data were subsequently analysed using linear mixed model analysis. If a certain behaviour did not occur during the trial latencies were set at 150 s (maximum trial length). Before analyses, the most appropriate test for each parameter was defined by varying the linear mixed model test with or without random intercept and slope. Based on the value of the 2-log likelihood of the Chi-square distribution, the significantly best test on most parameters appeared to be a model with a random intercept and a fixed slope so this model was chosen to be used on all parameters. Furthermore, a backward strategy was adopted in which all non-significant interaction terms were removed. Fixed factors in the model were trial, diazepam dose, stimulus, if the animal was a responder or a non-responder and their interactions. For linear mixed model analyses a probability value less than 0.05 (two-tailed) was considered as statistically significant. Post hoc testing was performed using a Bonferroni corrected test using a nested model.

RESULTS
EATING

Training revealed a decrease in latency to eat the palatable almond piece over time in the positive trials and an increase in latency to start eating the unpalatable almond piece in the negative trials (see Fig. 1); a significant trial ($F_{12,937}$= 11.004, p= 0.000), stimulus ($F_{1,937}$= 1937.104, p= 0.000) and trial x stimulus interaction effect was found ($F_{10,937}$= 31.281, p= 0.000). In addition, non-responders (independent of treatment) show a lower latency to eat the almond pieces in some trials on day 2 and 4, reflected by a significant responder ($F_{1,44}$= 6.509, p= 0.014) and responder x trial interaction effect ($F_{12,937}$= 2.334, p= 0.006). On the last training day 39 out of 42 animals (93%) did not start eating the unpalatable almond piece in all negative trails, while in the test session there seems to be an effect of DZ treatment on the decision to start eating the unpalatable almond piece; i.e. in the 0 DZ group 12 out of 14 animals (86%), in the 1 DZ group 10 out of 14 animals (71%) and in the 3 DZ group 7 out of 14 animals (50%) did not

start eating the almond piece. Animals that started eating the bitter piece in both negative trials in the test session were defined as non-responders, this was taken as an extra factor (responder effect) in the statistical analysis.

Linear mixed model analysis revealed a significant effect of responder ($F_{1,40}$= 81.206, p= 0.000), trialnumber ($F_{1,201}$= 6.066, p= 0.015), stimulus ($F_{2,200}$= 172.308, p= 0.000) and significant responder x stimulus ($F_{2,200}$= 84.471, p= 0.000) and trialnumber x stimulus ($F_{2,201}$= 3.048, p= 0.05) effects in the latency time to start eating the almond piece in the test session (Fig. 2A). Within both the responder and non-responder groups the latency until eating the almond piece was significantly higher in response to the NEG stimulus than in response to the POS and MIX stimulus (POS-NEG and MIX-NEG comparison, p<0.01, corrected α p<0.017 is significant). Although the latency until eating the almond piece seems to be lower in the group treated with 3 DZ in the NEG trials, no significant group and group interaction effects were found (p>0.05).

LOCOMOTOR ACTIVITY (LINE CROSSES)

There were no differences between responders and non-responders in the latency until the first line cross (table 1), as well as between the different doses of DZ and the stimulus that was presented (p>0.05). During the negative test trials responders crossed more lines than non-responders ($F_{1,41}$= 6.342, p= 0.016) also the total number of line crosses differed between the first and the second negative trial ($F_{1,41}$= 6.323, p= 0.016), see table 1. The group x responder x trial effect also appeared significant ($F_{2,41}$= 3.911, p= 0.028). Within the responders, animals treated with 1 DZ tended towards a higher number of line crosses than animals treated with 3 DZ in both the first and the second NEG trial (trial 1: t= 2.277, p=0.025; trial 2: t= 2.138, p= 0.036, corrected α p<0.017 is significant), 1 DZ treated animals also crossed more lines than 0 DZ treated only in the second NEG trial (t= 3.520, p= 0.001). The total number of line crosses within the responders did not differ between 0- and 3 DZ treatment (p>0.05). Within non-responders no differences in the total number of line crosses were found between the groups (p>0.05).
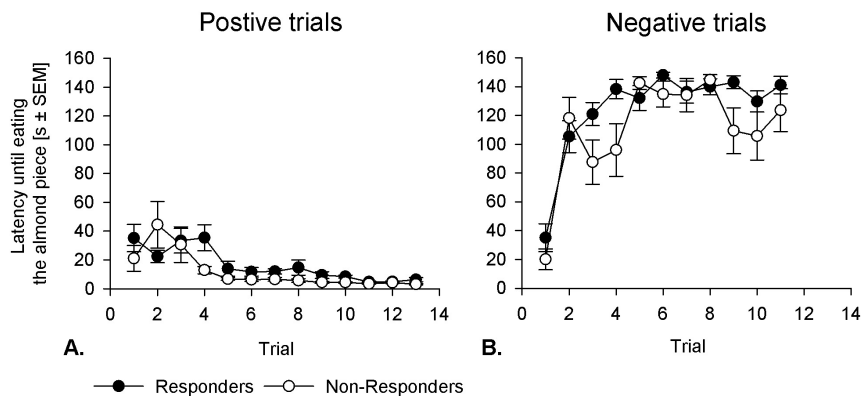

Fig 1: Latency to eat the almond piece(in seconds ± SEM) in positive (A) and negative (B) training trials: Latencies significantly decreased in the positive trials (p<0.01) and increased in the negative trials (p<0.01). Further, there was a significant responder x trial and responder effect (p<0.05).
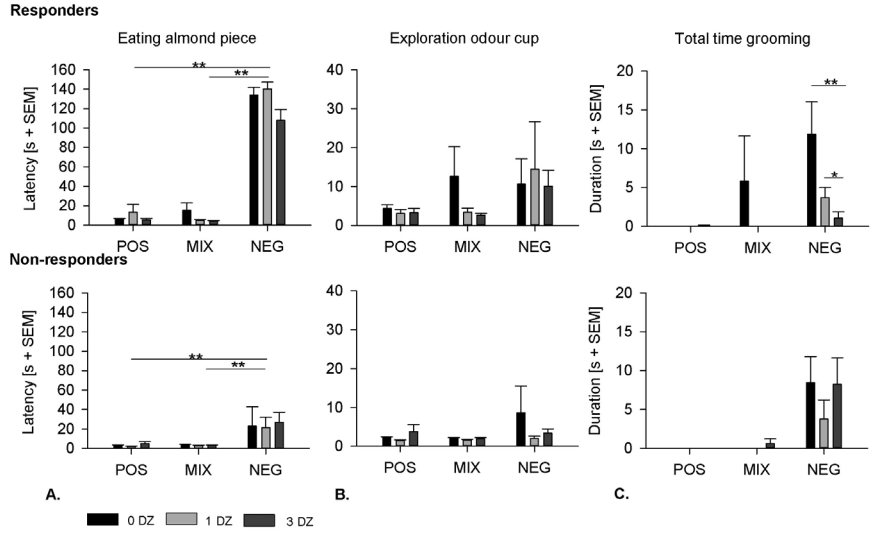
Fig 2: Behaviour in the test session as presented in separate plots for responders and non-responders:

A. Average latency to start eating the almond piece (in seconds + SEM),

B. Average latency to start exploring the odour cup (in seconds+ SEM) and

C. Total time spent grooming (in seconds+ SEM). ** p< 0.01, * p< 0.05

EXPLORATION

As an index of general exploration rearing of the mice was measured (see for example Ohl, *et al* 2001a). The latency until the first rear differed between stimuli ($F_{2,205}$=43.452, p= 0.000) , groups ($F_{2,41}$= 9.433, p= 0.000) and responder and non-responders ($F_{1,41}$= 4.532, p= 0.039), see table 1. Post hoc testing revealed that the group treated with 3 DZ showed a higher latency to rear than the other two groups (0-3 DZ: t=-2.935, p=0.005; 1-3 DZ: t= 4.254, p=0.000, corrected α p<0.0085) and that the latency to rear was higher in response to the POS and MIX stimulus than the NEG stimulus (POS-NEG: t=6.968, p= 0.000; POS-MIX: t= 8.847, p= 0.000, corrected α p<0.0085). Since POS and MIX trials had a shorter duration as the NEG trials, rearing in these trials was scarce and only the total number of rears in the negative trials is shown and analysed here. The total number of rears in the negative trial (see table 1) showed effects of group ($F_{2,41}$= 4.379, p= 0.019), responder ($F_{1,41}$= 8.034, p= 0.007) and a significant group x responder interaction effect ($F_{2,41}$= 5.370, p= 0.008). More specifically, within the responders animals treated with 1 DZ showed a higher number of rears than animals treated with either 0 DZ (t=2.811, p= 0.008) and 3 DZ (t=4.260, p= 0.000). In addition, 3 DZ animals showed a trend for a lower number of rears than animals treated with 0 DZ (t=-1.907, p= 0.064, corrected α p<0.0017).

As an index of odour exploration also the latency until the first odour cup exploration (Fig. 2B) and the total duration of odour cup exploration (table 1) was measured. In the latency until odour cup exploration, all interaction effects were non-significant and removed from the model. Only a general effect of stimulus (independent if animals were responders or non-responders) showed a trend to be significant ($F_{2,205}$= 2.907, p= 0.057) all other effects (group, responder

and trial) were not significantly different (p>0.05). The duration of odour cup exploration differed between stimuli ($F_{2,205}$= 84.672, p= 0.000) and almost differed significantly between the first and the second trial ($F_{1,205}$= 3.602, p= 0.059). It appeared that a difference between the first and second trial differs for the separate stimuli, since a stimulus x trial effect was found ($F_{2,205}$= 3.128, p= 0.046). Post hoc testing revealed that the animals explored the negative odour cup longer than the positive as well as the ambiguous odour cup , in both the first (POS-NEG: t=-5.285, p= 0.000; MIX-NEG: t=-5.275, p= 0.000) and second trial (POS-NEG: t=-5.093, t= 0.000; MIX-NEG: t=-5.278, p= 0.000, corrected α p=<0.017 is significant). The responder x stimulus x trial interaction was almost significant ($F_{=2.732}$, p= 0.067) and seems to be caused by a decrease in odour cup exploration in the second trial only within the responder group.

GROOMING

Grooming hardly occurred in the POS and MIX trials, thus only NEG trials were analysed on this behaviour. No significant interaction effects were found (P>0.05) so these were removed from the model. Further analysis revealed that there was a significant difference between groups in grooming duration ($F_{2,41}$= 4.773, p= 0.014), see Fig. 2C. Post hoc testing revealed that both doses of diazepam significantly decreased grooming behaviour in comparison with the 0 DZ control group (0 DZ-1 DZ: t=-2.784, p= 0.008; 0 DZ-3 DZ: t=2.567, p= 0.014, corrected α p=<0.017 is significant).
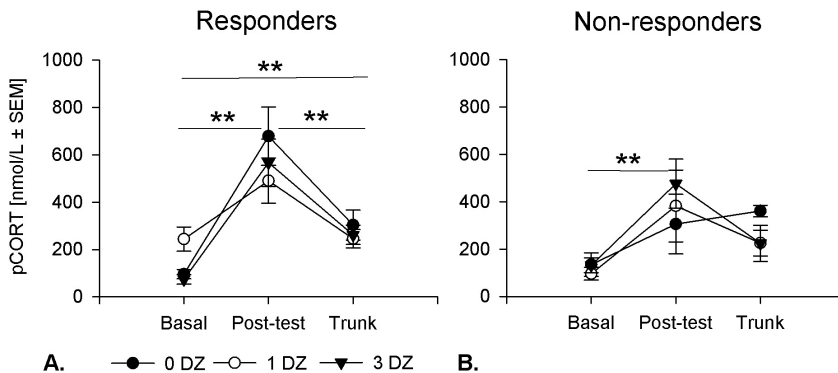
Fig 3: pCORT levels (in nmol/l ± SEM) from samples collected before (Basal), 30 minutes after (Post-test) and 2 hours after testing (Trunk). Behavioural testing resulted in increased pCORT levels (**p<0.01) in both responders and non-responders, which decreased again after testing (**p< 0.01) only in responders.

PCORT

pCORT levels are presented in Fig. 3. pCORT levels significantly differed between samples ($F_{3,104}$= 22.392, p= 0.000), and trends were found for a responder ($F_{1,104}$= 3.569, p= 0.062) and a responder x sample ($F_{2,104}$= 2.389, p= 0.097) effect. No significant differences between the diazepam treated and saline treated groups were found ($F_{2,104}$= 0.277, p= 0.759). Post hoc testing (adjusted α: p< 0.017 is significant) revealed that pCORT levels increased after the animals were tested in both responders (Basal-Post-Test: t= 8.876, p= 0.000) and non-responders (Basal-Post-Test: 3.671, p= 0.000). Trunk blood pCORT levels were still higher

than basal levels (Basal) within the responders (Basal-Trunk: t=3.223, p= 0.002) while this effect was not significant non-responders (Basal-Trunk: t=1.845, p= 0.068, corrected α, p<0.016 is significant), in addition within the responders pCORT levels significantly decreased from 30 minutes (Post-Test) to 2 hours after testing (Trunk) (t= 6.213, p= 0.000) while this effect was not significant in the non-responders (t=2.048, p= 0.043, corrected α, p<0.016 is significant).

DISCUSSION

The interpretation of the respective judgement bias is depending on the behavioural response towards the ambiguous stimulus: a comparable response towards both the positive and the ambiguous stimulus would indicate a positive bias, while a comparable response towards the negative and the ambiguous stimulus would indicate a negative bias (Mathews and Mackintosh 1998).
The latency to explore the odour cup in the present experiment seemed to indicate that the saline treated BALB/c mice interpreted the ambiguous cue negatively, similar to what had been found before (Boleij, *et al* 2012b). Moreover, mice treated with 1 and 3 mg/kg diazepam, respectively, did not reveal this indication of a negative judgement bias, suggesting that indeed there might be a causal relationship between state anxiety and a more negative judgement bias in these mice.
During training all animals showed a decrease in latency to start eating the almond piece in the positive trials and an increase in latency to start eating the bitter tasting almond piece in the negative trials (Fig. 1). These results are in concordance with our previous study (Boleij, *et al* 2012b) and indicate that the mice learned to associate the respective odour with the taste of the presented almond, resulting in a strong avoidance response towards the bitter tasting almond piece.
Based on the previous study (Boleij, *et al* 2012b) it was expected that BALB/c mice treated with saline would show a negative judgement bias in the test sessions of the current experiment. However, in contrast with our previous study, no negative judgement bias could be concluded from the latency to *eat* the almond piece. This difference with our previous study can be attributed to the differences in test set-up between the present and previous experiment: the avoidance of the bitter piece in the test session of the present study was much higher (on average between 100 and 130 seconds) than in the previous study (on average between 30 and 50 seconds) which we consider a result of using bitter tasting almond pieces in the negative trials of the present experiment in order to prevent animals from learning that the NEG cue was no longer combined with a bitter almond piece. This change in test setup was necessary to allow for a within-animal comparison in contrast to the previously used between-animal comparison. If indeed the presentation of a bitter almond did cause the increased eating latency in the negative trials, it would have to be concluded that BALB/c mice were able to "sense" the bitter taste additive irrespective of the conditioned odour, which would exclude the food intake response from providing a reliable indication of a judgement bias.
Still, a negative judgement bias indeed seemed to be indicated by the latencies to *explore* the odour cup, since the response towards the ambiguous stimulus was more similar to the negative than to the positive stimulus (Fig. 2B) in saline treated mice. In addition, this negative judgement bias was not visible in animals treated with 1mg and 3mg diazepam, respectively (though statistical

analysis only revealed a significant substance effect) indicating that treatment with this anxiolytic might have shifted the negative towards a more positive judgement bias. A similar effect has been reported in lambs (latency to approach a feeding bucket presented on an ambiguous location) that were treated with diazepam (Destrez, *et al* 2012). Since similar doses of diazepam have been shown to decrease anxiety related behaviour of BALB/c mice in the open field test (Salomons, *et al* 2012), free exploration test (Griebel, *et al* 1993) and modified hole board (Ohl, *et al* 2001a), it seems reasonable to conclude that a causal relation exists between state anxiety and a negative judgement bias in BALB/c mice. This conclusion is further supported by the notice that other behavioural parameters measured in the present test indicated that state anxiety was decreased by treatment with diazepam. For example grooming behaviour, (Fig. 2C) which is thought to be indicative of arousal/dearousal (see Kalueff and Tuohimaa 2004) was dose dependently decreased by diazepam, while rearing (table 1) was increased only in the group treated with 1mg diazepam, but not in the group that received 3mg diazepam. These results suggest that the increased exploratory drive due to the anxiolytic effect of the lower dose may be masked by a sedative effect induced by the higher dosage, an effect that has been demonstrated before (see (Ohl, *et al* 2001a)).

A decrease in arousal after treatment with diazepam may be expected to reduce testing induced plasma corticosterone (pCORT) levels as well, as has been demonstrated in earlier studies (see for example De Souza 1990). Still, although diazepam treated animals seem to have lower pCORT levels (Fig. 3A), the variation between animals was too high to detect any significant differences. A previous study in our lab also revealed a high inter-individual variance in pCORT levels under influence of 1 and 3 mg/kg diazepam (Salomons, *et al* 2012). Further, it appeared that a high inter-individual variation did not only occur in plasma corticosterone levels, but in behavioural parameters as well. Closer analysis revealed for example that only part of the animals treated with diazepam avoided the bitter almond piece and, in parallel, anxiolytic responses to diazepam appeared only in the sub-group of animals that was consistently avoiding the almond piece. We therefore analysed the results based on a differentiation between those animals that avoided the almond piece in the negative trials (responders) and those that did not (non-responders). Non-responders seemed to be less responsive towards the anxiolytic effects of diazepam than responders (see Fig. 2 and table 1), although the differentiation between responders and non-responders is statistically weak due to a now relatively low number of animals per sub-group. It is however an interesting finding that apparently this (genetically homogenous) population of mice differs with respect to individual trait and state anxiety levels, which might be reflected by their differing response to diazepam. In support of this hypothesis we found that non-responders had lower pCORT levels than responders after testing (Fig. 3). It is of note that for example in humans, treatment with anxiolytics is only found to be effective in reducing state anxiety if healthy subjects show a sufficiently increased state anxiety in the first place; indeed it has been reported that only people with high trait anxiety or clinically anxious people show effective reductions in state anxiety when treated with an anxiolytic (Debus and Janke 1980; Parrott and Kentridge 1982; diazepam: Wilkinson 1985), while low anxiety trait individuals reveal an diazepam induced increase of aggression, but not a reduction in state anxiety (Wilkinson 1985). Further, a low anxiety trait and state recently was suggested to be causally related to the lack of effect of diazepam in

Lister-Hooded rats that were tested on judgement bias (Anderson, *et al* 2012b). It seems thus reasonable to hypothesize that the responder/non-responder effect found in the present study might indeed relate to individual trait anxiety levels, which would as well explain why some individuals showed an anxiolytic effect of diazepam on judgement bias and others did not.

Individual differences in responsiveness towards pharmacological treatments have been found before. For example one study shows that 20% of the mice (strain unknown) were found to be responders towards Baclofen (GABA-B agonist), while 65% was defined as mediate responder and 15% as non-responder (Rago, *et al* 1986). In addition, male DBA inbred mice were divided in 55% responders and 45% non-responders towards R121919 treatment (CRH antagonist)(Erhardt, *et al* 2009). This is comparable to the amount of responders and non-responders towards 3 mg/kg diazepam found in the present study in the BALB/c inbred strain. Another study shows that responder/non-responder differences in Sprague-Dawley rats treated with the benzodiazepine chlordiazepoxide (Patel, *et al* 1984) could be pointed towards a higher affinity of BZ binding sites in the hippocampus of non-responder animals. Similar mechanisms could be involved here and would be interesting to investigate in future experiments, since BZ receptor binding has been shown to not only differ between the BALB/c and C57/BL6 inbred strains, but to also be influenced by maternal care (Caldji, *et al* 2004), indicating that other factors than genetic background (environment, gene x environment interactions) are important for the development of cognitive-emotional traits.

Diazepam however is known to have different side-effects, which might influence the intake of bitter tasting almond pieces as well. For example, Berridge *et al.* (1995) have shown that effects on food intake under influence of diazepam are modulated by an increase in food palatability, because positive hedonic reactions towards a bittersweet solution are increased while aversive reactions are hardly affected under influence of diazepam in rats (Berridge and Peciña 1995; Richardson, *et al* 2005). In fact, in the present test diazepam might have increased the taste palatability of the almond piece itself, masking the bitter taste for the diazepam treated non-responder mice and increasing their motivation to start eating the bitter piece. With increasing dose of diazepam more mice tended to start eating the bitter tasting almond piece, which is in agreement with the hypothesis that the threshold for palatability modulation of BZ is probably higher than that modulating anxiety (possibly regulated by different neural systems) (Berridge and Peciña 1995), and based on the present experiment this threshold might be suggested to differ between (high and low trait-anxious) individuals. Anterograde amnesia (influences on acquisition of memory after drug administration (in humans: Lister 1985; in animals: Thiebot 1985)), another side effect of diazepam, is unlikely to account for the effects found in the present study because the mice were not treated with diazepam during the training, but only before the test session.

CONCLUSION

While we acknowledge that some methodological aspects need to be carefully considered when interpreting the results of the presented judgement bias test, we feel that there is some support for the idea of a causal relationship between state anxiety and a negative judgement bias in BALB/c mice. At present, we chose not to add more animals to the experiment in order to increase the likelihood of reaching significant effects, considering that, to reduce animal use, a modification of the test set-up might be more appropriate to investigate the hypothesized differences and draw more reliable conclusions on group differences. Thus this study provides a basis for further research on the relation between a negative judgement bias and state anxiety, implicating that future experiments should account for a differentiation between responders and non-responders within the tested population of mice. In addition, further experiments to improve the test set-up are necessary to filter out the possible effect of the taste additive used on detectability of the cued almond piece. It might for example be suggested to change the negative consequence (different bitter substance that is less volatile) and implement operant responses in the test.

Table 1: Overview of the behavioural responses of the experimental groups (0 DZ, 1 DZ and 3 DZ) towards the different stimuli in the test session, data are averaged for the first and second trial. In the POS (= positive conditioned stimulus) trials the almond pieces were presented with one odour (either apple or vanilla) and in the NEG trials bitter tasting almond pieces presented with the other odour and in the MIX trials (ambiguous stimulus) a 50/50 mixture of both odours was presented with normal almond pieces. Continuous data is presented as mean ± SEM and ordinal data as median ± IQR.

Behavioural category and parameters	Statistics		0 DZ POS	0 DZ MIX	0 DZ NEG	1 DZ POS	1 DZ MIX	1 DZ NEG	3 DZ POS	3 DZ MIX	3 DZ NEG
Eating											
Latency until eating the almond piece [sec]	r, t, s, r*s, t*s	R	6.0 ± 1.0	15.1 ± 7.7	13.4 ± 7.9	13.4 ± 8.0	5.0 ± 0.9	140.3 ± 7.0	5.4 ± 1.5	4.2 ± 0.5	107.9 ± 11.2
		NR	3.1 ± 0.3	3.5 ± 0.5	22.8 ± 19.8	2.0 ± 0.2	2.7 ± 0.5	21.6 ± 10.6	4.9 ± 2.0	3.2 ± 0.2	26.9 ± 10.2
Exploration											
Latency until the first cup exploration [sec]	s (p=0.057)	R	4.3 ± 0.9	12.6 ± 7.6	10.6 ± 6.5	3.1 ± 1.0	3.4 ± 1.0	14.5 ± 12.2	3.3 ± 1.1	2.6 ± 0.5	10.1 ± 4.1
		NR	2.2 ± 0.3	2.0 ± 0.3	8.6 ± 6.9	1.4 ± 0.2	1.6 ± 0.2	2.1 ± 0.6	3.8 ± 1.8	1.9 ± 0.3	3.4 ± 1.1
Total duration of cup exploration [sec]	s, s*t	R	7.7 ± 1.5	7.1 ± 1.5	50.9 ± 13.1	12.6 ± 6.7	10.5 ± 2.2	42.1 ± 10.4	16.4 ± 2.5	13.4 ± 2.5	52.7 ± 12.2
		NR	10.3 ± 2.8	15.5 ± 3.0	35.7 ± 14.1	10.5 ± 1.6	15.9 ± 3.6	34.4 ± 12.4	12.0 ± 2.6	9.5 ± 2.2	61.4 ± 13.0
Latency until the first rear [sec]	s, g, r	R	69.7 ± 20.1	104.4 ± 18.5	31.5 ± 13.6	61.5 ± 23.9	79.0 ± 22.6	11.9 ± 2.2	124.3 ± 12.3	122.6 ± 20.7	51.8 ± 15.6
		NR	118.6 ± 31.4	86.1 ± 0.5	41.7 ± 23.7	99.1 ± 36.1	99.4 ± 36.1	40.0 ± 18.8	132.4 ± 11.4	150 ± 0	66.7 ± 23.0
Total number of rears [nr]	g, r, g*r	R	0.5 ± 1.5	1 ± 1.5	17 ± 15	1 ± 2	1 ± 1.75	24 ± 11	0 ± 1	0 ± 0.5	5 ± 17.5
		NR	1#	0.25#	14.75#	0.8 ± 1.9	0.5 ± 1.1	4 ± 5.8	0 ± 0	0 ± 0.5	5 ± 9
Locomotion											
Latency until the first linecross [sec]	ns	R	3.0 ± 0.7	10.2 ± 7.3	3.9 ± 1.7	1.5 ± 0.3	2.8 ± 1.1	2.1 ± 0.6	2.0 ± 1.2	1.7 ± 0.5	1.7 ± 0.5
		NR	1.2 ± 0.3	1.1 ± 0.1	1.4 ± 0.4	0.8 ± 0.2	0.9 ± 0.2	0.9 ± 0.3	1.8 ± 0.6	1.1 ± 0.3	2.3 ± 0.8
Total number of line crosses [nr]	t, r, t*r	R	1 ± 1	2 ± 1	8.5 ± 9.25	2 ± 2	2 ± 2	18.5 ± 11.5	1 ± 1	1 ± 2	9.5 ± 16.5
		NR	1 ± 1.5	2.5 ± 1.75	13.3 ± 6.75	2 ± 1.75	1 ± 1.5	7 ± 5.25	1 ± 1.25	1 ± 1	4.5 ± 12.5
Arousal											
Total time spent grooming [sec]	g	R	0 ± 0	5.8 ± 5.8	11.9 ± 4.2	0 ± 0	0 ± 0	3.7 ± 1.3	0.1 ± 0.1	0 ± 0	1.1 ± 0.8
		NR	0 ± 0	0 ± 0	8.4 ± 3.37	0 ± 0	0 ± 0	3.8 ± 2,5	0 ± 0	0.6 ± 0.6	8.2 ± 3.4

n=2, no IQR, s= stimulus effect, g= group effect, t= trialnumber effect, r= responder effect, interaction effects are indicated with a *

CHAPTER 6

FOOD AVOIDANCE IN MICE: EFFECTS OF QUININE AND DENATONIUM BENZOATE

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ABSTRACT

In a previous experiment we used quinine as a bitter taste additive to almond pieces in a home cage odour conditioning test for mice. BALB/c mice appeared to avoid these almond pieces, but some of the mice seemed to detect the quinine prior to intake, which interfered with the test set-up. Therefore the aim of the present experiment was to investigate if another bitter substance, denatonium benzoate (denatonium), was suitable as an alternative bitter taste additive in this test. To this aim a quinine soaked almond piece was paired with one odour (apple or vanilla) in the negative trials in one group of mice, while in another group the same procedure was performed with denatonium benzoate. In the positive trials both groups received a palatable almond piece coupled to the other odour.
In total 10 positive and 8 negative trials were performed over 3 days. Mice showed an increase in latencies to eat the bitter almond piece in the negative trials and this increase was higher for mice in the quinine group. In addition, a higher percentage of mice ate the whole bitter almond piece by the end of the training period in the denatonium group, which indicates that these mice might have habituated to the denatonium taste. Also other behavioural parameters, such as rearing (exploration) and behaviour in the positive trials indicated that quinine may be more aversive than denatonium. We conclude that denatonium benzoate cannot be used as an alternative to quinine to cause persistent food avoidance in BALB/c mice.

INTRODUCTION

Most mammals react aversively towards bitter tastes. This innate aversive response is an evolutionary adaptation since bitter taste often is indicative of toxic substances (Glendinning 1994). Typical responses, such as tongue retraction and mouth gaping that are elicited by a bitter taste are reflexes and comparable across a variety of species (including humans) (Erickson and Schulkin 2003; Grill and Norgren 1978; Steiner 1979). Further, bitter-tasting substances are learned very quickly and induce food avoidance behaviour (Glendinning 1994; Scott and Mark 1987). In mice aversive responses towards bitter tastes have been found as well (Kiefer, *et al* 1998). However, differences between rodent species (Kleinkauf, *et al* 1999; Willoughby, *et al* 2011; Wong 1994) and even between rodent strains and individuals exist (Lush 1984; Tordoff 2007; Tordoff, *et al* 2008; Wong and Brown 2007) in terms of sensitivity to bitter stimuli.

Bitter taste aversion in animals can be used as a tool in behavioural management of (wild) animals, for example to prevent herbivores from foraging on crops (Andelt, *et al* 1994; see for example Willoughby, *et al* 2011), and bitter taste additives have been used on poisoned slug pellets to prevent mammals from being poisoned (see for example Kleinkauf, *et al* 1999). In experimental research bitter taste additives are used as well, for example to elicit food avoidance behaviour in distinct behavioural tests (such as cognitive bias and decision making tests) (see for example Burman, *et al* 2009; Dwyer 2011; Koot, *et al* 2012) and to investigate mechanisms involved in bitter taste perception (see for example Geran and Travers 2011; Glendinning, *et al* 2008; Hallock, *et al* 2009). Quinine is a substance that has been used regularly in experimental contexts and has been shown to cause avoidance in several strains of rats and mice (Boughter Jr. and Whitney 1997; Boughter Jr., *et al* 2005; Boughter, *et al* 1992; Burman, *et al* 2009; Koot, *et al* 2012; Lush 1984). In previous experiments in our lab quinine was used as an aversive taste additive to almond pieces in an odour conditioning paradigm in mice. In this test set-up, where one odour was associated with a normal almond piece, which is highly palatable to mice, while a second odour was associated with a quinine soaked bitter tasting almond piece, mice rapidly learned to avoid almond pieces that tasted bitter (Boleij, *et al* 2012b). However, in follow-up experiments it appeared that several mice probably were able to differentiate between quinine soaked almond pieces and normal tasting almond pieces either by smell or some other non-taste factor, apart from the (to be) conditioned odour. Therefore, we decided to investigate if a different bitter substance might as well be suitable for the odour conditioning procedure. Denatonium benzoate (denatonium, brand name Bitrex®) is an extremely bitter-tasting substance with low toxicity that for example has been used to prevent ingestion of toxic chemicals in humans (Rowe, *et al* 2009). Denatonium has been shown to act via similar mechanisms as quinine (Dahl, *et al* 1997; Wilson, *et al* 2012) and to cause similar behavioural responses in rats (Brasser, *et al* 2005; Frank, *et al* 2004; Geran and Travers 2011; Spector and Kopka 2002). Also individuals of some strains of mice (C57BL6 and C57BL6 and 129 mixed background) have been found to avoid water treated with denatonium (Damak, *et al* 2006; Glendinning, *et al* 2008; Hallock, *et al* 2009). Still, different inbred strains may react differentially to a range of bitter substances (Boughter Jr., *et al* 2005; Boughter, *et al* 1992); for example, BALB/c mice have a stronger glossopharyngeal nerve (GL) response to a range of bitter substances other than quinine in comparison with C57BL6 mice (Tanimura, *et al* 1994).

In the present experiment an odour conditioning paradigm was used in order

to test differentiation between normal tasting and bitter tasting almond pieces in male BALB/cJ mice. In this procedure the animals were conditioned with either quinine or denatonium as a bitter taste additive. The goal was to compare the aversive effect of the two substances in order to evaluate denatonium as a potential alternative to quinine in this odour conditioning test procedure.

MATERIALS AND METHODS
ETHICAL NOTE
The experimental protocol (DEC number 2012.I.02.015) was approved by the Animal Experiments Committee of Utrecht University and University Medical Centre, The Netherlands. The Animal Experiments Committee based its decision on the EC Directive 86/609/EEC (Directive for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes). Furthermore, all animal experiments followed the 'Principles of Laboratory Animal Care' and refer to the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioural Research (National Research Council 2010).

ANIMALS AND HOUSING
All handling and experimental procedures with the mice were performed by well-trained members of the laboratory. 28 Male BALB/cJ (BALB/c) mice were obtained from Charles River laboratories (Margate, UK) and were 6-8 weeks of age at arrival. The experiment took place at the animal laboratory of the Netherlands Vaccine Institute (Bilthoven, The Netherlands) in a humidity (45%-50%) and temperature (22 ± 2°C) controlled room. The animals were housed individually in Eurostandard type 3 macrolon cages (40 cm x 26 cm x 20 cm) with standard cage bedding (Aspen chips) a plastic shelter (Mouse House Techniplast®) and facial tissue (Kleenex® Facial Tissue, Kimberly-Clark) as standard enrichment. Animals were housed under a reversed light/dark cycle (the dark period started at 6:00 h and lasted until 18:00 h, with red lights on) and radio music was turned on as background noise during the dark phase in the whole experimental period. During the first two weeks after arrival (habituation period) the animals were handled for ~3 minutes per mouse three times a week (between 9:00 h and 11:00 h) by the experimenter who also did behavioural testing and were weighed twice a week. All testing took place in the animals housing room and equipment was installed before the animals arrived.

ODOUR CONDITIONING PROCEDURE
ALMOND PIECES, BITTER STIMULI AND ODOUR STIMULI
Pieces of almond were used as rewards, mice eat these readily even if they are fed *ad libitum* (see for example Ohl, *et al* 2003). For the negative trials (NEG) almonds were soaked for 5 minutes in either 180 mM quinine hydrochloride (Sigma-Aldrich) or 10 mM denatonium benzoate (Sigma-Aldrich) both dissolved in demineralised water, and dried overnight. For the positive trials (POS) almond pieces were soaked for 5 minutes in demineralised water. Odour stimuli that were used were vanilla and apple odour (Micro-Plus, Stadtoldendorf, Germany), dissolved in distilled water (0.05%), since mice are attracted by those (e.g. Ohl, *et al* 2003). Both odours were dissolved in a low concentration because the stock solution is highly concentrated and similar concentrations were used previously (Boleij, *et al* 2012b).

TESTING PROCEDURE

Animals were tested during the dark (active) phase between 9:00 h and 16:00 h. Mice were habituated to eating the piece of almond (30 mg), by offering it with tweezers in the home cage on days 14, 15 and 16 after arrival. On day 19, 20 and 21 after arrival the testing procedure took place. The home cage test set-up was similar as described before (see Boleij *et al.*, 2012). During all trials the home-cage was placed on a table in front of a video camera (placed on the side of the odour cup) that was connected to a dvd-recorder (Panasonic). First of all, the enrichment and water bottle were removed from the home cage. Almond pieces were presented on a small petri dish (ø 5.5 cm). The odours were spread on a filter paper ø 5.5 cm) in an amount of 0.1 ml per odour that was positioned in the petri dish (Schellinck, *et al* 2001). The petri dish with the filter paper was covered by a lid with several holes to let the odours diffuse through the top; this dish is referred to as the odour cup (see also Boleij, *et al* 2012b). A trial started with placing this odour cup in the home cage. The training trials were terminated when the almond piece was eaten. Mice received six trials per day (with a one minute inter-trial interval), trials were ended when the whole almond piece was eaten and were ended with a cut-of time of two minutes when the almond piece was not eaten. During training in a positive (POS) trial the odour cup was presented with a normal tasting almond piece and in a negative (NEG) trial the odour cup was presented with a bitter tasting almond piece, and within groups half of the mice received apple as a POS conditioned stimulus and vanilla as a NEG conditioned stimulus (counterbalanced design). Mice in the denatonium group (n=14) received a denatonium covered almond piece in the NEG trials and mice in the quinine group received a quinine covered almond piece in the NEG trials. On the first day of testing the mice received four POS trials and two NEG trials (to get the animals used to running to the odour cup). On the second and third day mice received three POS and three NEG trials, we aimed to keep a balance between a higher amount of trials necessary to keep 50/50 chance of receiving either a positive or negative trial randomly and preventing that animals were less motivated in the end of the session (they were fed ad libitum). Trials were presented in a random order and mice were tested randomly each day.

BEHAVIOURS SCORED

Behaviour during testing was scored live using the computer program "The Observer" version 5.0 (Noldus b.v., Wageningen, the Netherlands). Behaviours were scored in a continuous way, i.e. all-occurrence recording of the behaviours of interest. The following behavioural parameters were measured: Latency until picking up and eating the almond piece, exploration (sniffing) of odour cup (latency and duration), locomotor activity: line crossings between front and back (latency and total number), general exploration: rearing (latency and total number), grooming (latency, total duration and total number).

STATISTICS

Statistics were performed with the computer programme SPSS 20.0 for Windows (SPSS Inc. IL, USA). Continuous data (latency and duration of behavioural parameters) are represented as mean ± standard error of the mean (SEM), and were first investigated for gaussianity using the Kolmogorov-Smirnov test. Homoscedasticity was tested by Levine's test. Discrete data on the ordinal scale (total number of behavioural parameters) are represented as median with interquartile range. Some of the parameters revealed

a non-parametric distribution and were either log-transformed (continuous data) or rank transformed (number data (Conover and Iman 1982)) to get a normal distribution. All data (except for the percentage of animals eating the whole almond piece) were subsequently analysed using linear mixed model analysis. If certain behaviours did not occur during the trial latencies were set at 120 s (maximum trial length). Before analyses, the most appropriate test for each parameter was defined by varying the linear mixed model test with or without random intercept and slope. Based on the value of the 2-log likelihood of the Chi-square distribution, the significantly best test on most parameters appeared to be a model with a random intercept and a fixed slope so this model was chosen to be used on all parameters. Furthermore, a backward strategy was adopted in which all non-significant interaction terms were removed. Positive and negative trials were analysed separately, because we were only interested in the effect of the bitter substance on positive and negative association learning. Fixed factors in the model were trial and bitter substance, and their interactions. The percentage of animals eating the whole almond piece in the negative trials was analysed using an Generalized Estimated Equations model using a loglink function (chi-square distribution taking repeated measurements into account). A probability value less than 0.05 (two-tailed) was considered as statistically significant.
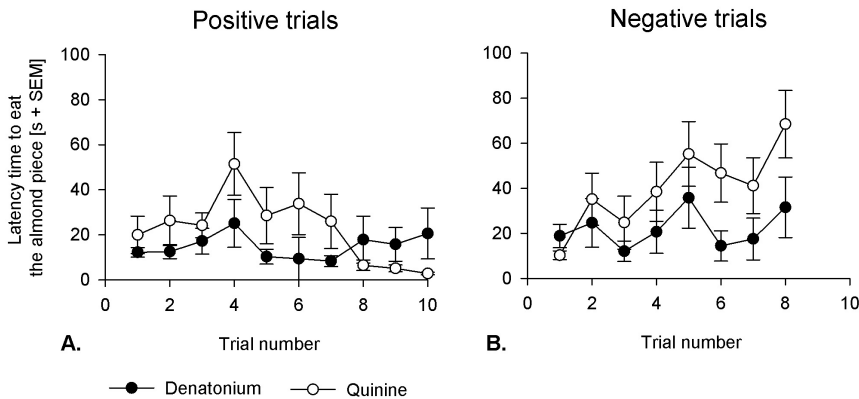
Fig. 1: Average latency (seconds ± SEM) from the start of the trial until mice started eating the presented almond piece in positive (A) and negative trails (B). A significant trial and trial x substance effect was found (p<0.01) for the positive trials and a general substance and trial effect in the negative trials (p=0.01).

RESULTS
EATING

In the positive trials no general effect of substance (used in the negative trials) on latency to start eating the almond piece was found ($F_{1,28}= 0.114$, p>0.05), however a significant trial ($F_{9,252}=2.537$, p= 0.008) and trial x substance ($F_{2,252}= 2.540$, p= 0.008) interaction effect was found. The significant interaction effect is probably due to different patterns of latencies to start eating the almond piece over time (see Fig. 1A): animals in the quinine group showed an increase in latency times towards the positive stimulus over the first four trials (first day) after having experienced bitter tasting almond pieces and after that showed a gradual

decrease in latency times to eat the almond piece over time when exposed to the positive stimulus, while animals in the denatonium group took on average 15-20 seconds to start eating the almond piece, and stayed at this level over time. In the negative trials a general effect of substance ($F_{1,28}$= 79.060, p= 0.010) and trial ($F_{7,196}$= 7.564, p= 0.010) was found, but no trial x stimulus interaction effect, so the interaction effect was removed from the model. Animals in the quinine group showed a clear increase in latency to eat the almond piece over time and had in general a higher latency to start eating the almond piece than animals in the denatonium group (see Fig. 1B).
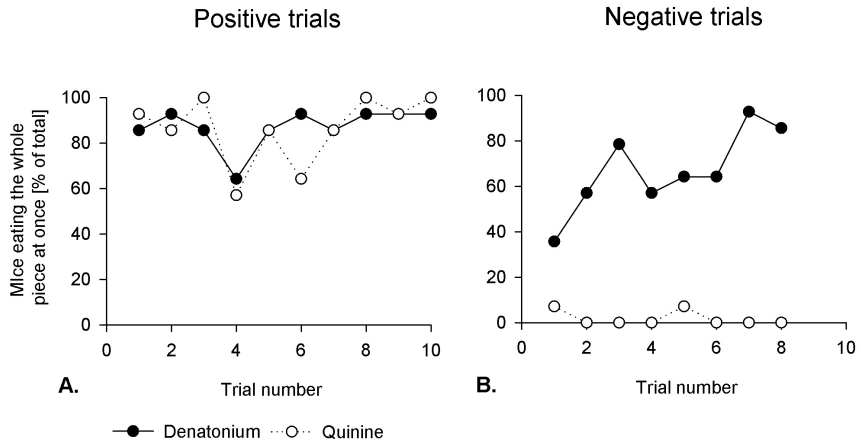
Fig. 2: Total percentage (%) of mice that ate the whole almond piece at once, without rejecting it, in the positive (A) and negative trials (B). In the negative trials general effects of trial, substance and substance x trial were found (p<0.001). Post hoc significant effects of substance were found in trials 2-9 (p<0.005)

To get an indication of the rejection of the bitter almond pieces also the percentages of animals eating the whole almond piece after picking it up for the first time were examined. In figure 2 clear differences between the denatonium and quinine groups in the negative trials appear; over time more mice in the denatonium group eat the whole almond piece immediately after picking it up for the first time, while almost all mice in the quinine group keep rejecting the almond piece after they have picked it up. Statistical analysis on the negative trials (general estimating equations) reveals a general effect of trial (Wald χ^2= 973.088, p<0.001), substance (Wald χ^2= 494.697, p<0.001) and a trial x substance interaction effect (Wald χ^2= 269.993, p<0.001). Post-hoc testing reveals that the percentage of mice eating the almond piece at once in the first trial is not predicted by substance (p>0.005, corrected α p<0.005 is significant), but that there is a significant effect of substance on the percentage of mice eating the whole almond piece in trial 2-9 (all p<0.001).

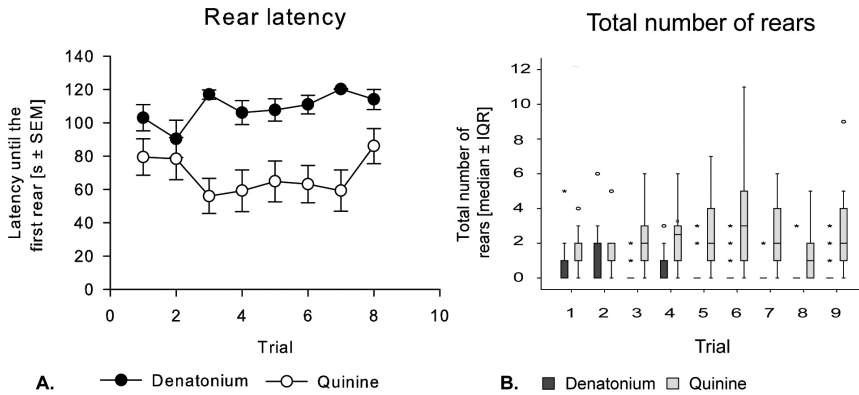Fig. 3: (A) Average latency from the start of the trial until the first rear was performed (seconds ± SEM) and (B) total number of rears [± SEM] in the negative trials. * and o indicate extreme cases within that time point. Rearing was performed sooner and more in the negative trials performed with quinine indicated by significant substance and substance x trail effects (p<0.05).

EXPLORATION

As an index of general exploration rearing behaviour of the mice was measured, see Fig. 3A and table S1. In both the positive and the negative trials rearing occurred sooner when quinine was used in the negative trials (positive: $F_{1,28}$= 9.217, p= 0.005; negative: $F_{1,28}$= 41.249, p= 0.000). No general trial effects (positive: $F_{9,252}$= 1.183, p> 0.05; negative $F_{7,196}$= 1.467, p> 0.05) were found but the bitter substance used had a different effect on latency to rear over time (increase in denatonium and decrease in quinine), since in both the positive and the negative trials a substance x trial interaction effect was found (positive:$F_{9,252}$= 2.182, p= 0.024; negative: $F_{7,196}$= 2.247, p= 0.032). Also the total number of rears was recorded, only the total number of rears in the negative trials is presented here (Fig. 3B) because rearing hardly occurred in the positive trials. For the total number of rears in the negative trials again a significant effect of substance ($F_{7,196}$=39.672, p= 0.000) and a significant substance x trial interaction effect ($F_{7,196}$= 2.357, p= 0.025) was found; i.e. mice exposed to quinine treated almond pieces reared more in the negative trials than mice exposed to denatonium treated almond pieces. The general trial effect was not significant ($F_{7,196}$= 1.467, p= 0.181).

OTHER BEHAVIOURS

For additional information on the other behavioural parameters scored during the trials (latency to cross the first line, latency and total time spent grooming and total time spent eating), see table S1.

DISCUSSION

This study was aimed at investigating if denatonium would be an alternative for quinine as a bitter taste additive to almond pieces in an odour conditioning test for mice. The results show that mice appear to respond less aversively towards denatonium than to quinine: the latency to eat the almond piece clearly increased less over time in response to denatonium when compared to quinine (Fig. 1B), with denatonium treated almond pieces being completely eaten by almost all the mice towards the end of the test period, while the quinine soaked almond pieces were either not completely eaten or entirely avoided (Fig. 2B).

This less aversive effect of denatonium was unexpected because previous publications report solutions of denatonium benzoate in a concentration similar to the one used here to be avoided by C57Bl/6J, DBA2 and C57Bl/6J;129Ola mice (Boughter Jr., *et al* 2005; Dotson, *et al* 2005; Glendinning, *et al* 2008; Hallock, *et al* 2009). However, those studies made use of different strains and different methods as have been used in the present study, i.e. two-bottle preference tests were applied in which denatonium was dissolved up to 30 mM in water or saccharin water. In contrast, the here used almond pieces are not only highly palatable for mice but have a high caloric value which, although the mice were fed ad libitum, may have had a motivating effect to ingest even the bitter almond pieces (Beeler, *et al* 2012). To our knowledge the use of denatonium as additive to food items such as almonds has not yet been investigated in a laboratory setting. However, some studies have been performed in wild rodent species, such as wood mice and grey squirrels, and these species have been reported to eat less of denatonium treated foods, but they seem to develop tolerance (i.e. habituate) to the substance over a two week period (Kleinkauf, *et al* 1999; Willoughby, *et al* 2011). A similar effect was found here, and suggests that BALB/cJ mice habituated to the bitter taste of denatonium but not that of quinine. In addition, the overall difference in avoidance between the substances suggests that the taste of denatonium was less aversive than quinine.

It might be suggested that BALB/c mice differ in sensitivity towards specific bitter substances from strains that have been investigated in 2-bottle preference tests (Boughter Jr., *et al* 2005; Dotson, *et al* 2005; Glendinning, *et al* 2008; Hallock, *et al* 2009). It is known that mice lacking the sucrose octa acetate (SOA) allele (Whitney and Harder 1986), have lower taste thresholds towards the bitter tasting SOA than mice that do possess this allele (tasters, e.g. SWR/J vs. non-tasters, e.g. C57Bl6). The SOA gene has also been implicated in the perception of quinine and denatonium (Boughter Jr. and Whitney 1997) and BALB/cByJ mice have been identified as demi-tasters, i.e. having a mediate sensitivity towards SOA (SOA(c) allele) (Capeless, *et al* 1994), and although it is known that BALB/c mice in general are sensitive to bitter tastants such as quinine and caffeine (Tanimura, *et al* 1994), and that they avoid licking ethanol (White, *et al* 2007), their sensitivity towards denatonium and their potential habituation to this taste has not been investigated before.

While it has been demonstrated that (Sprague-Dawley) rats are unable to discriminate between denatonium and quinine (Spector and Kopka 2002) and, moreover, that individual rats (Sprague-Dawley) showing high aversive reactions towards quinine reveal comparable reactions towards denatonium (Brasser, *et al* 2005), in mice no direct comparison between the substances has been made yet. An indirect indication that denatonium and quinine might not be perceived and processed similarly in mice may, however, be obtained from a study in which DBA/2 and C56/Bl6 animals were tested: C57Bl6 mice appeared to be

more sensitive towards quinine than DBA/2 mice, but both strains were equally sensitive towards denatonium (Boughter Jr., *et al* 2005). Further, another study showed that neurons in the brainstem respond differently towards a range of bitter substances (including a difference between quinine and denatonium) in Sprague-Dawley rats. On the other hand denatonium and quinine are suggested to work on similar receptors (Dahl, *et al* 1997) and have quite similar receptive fields in mice (Wilson, *et al* 2012). It thus seems unreasonable to assume a general insensitivity of BALB/cJ mice to denatonium. Instead, the habituation towards denatonium in the present study might for example be explained by the (too low) concentration of denationium used. The here chosen concentration was based on information from literature and, in addition, a pilot experiment in which this concentration was clearly avoided by BALB/cJ mice. Still, the differences in effects with quinine only developed more clearly over time and thus seems to be restricted to habituation related processes. It remains to be investigated if higher denatonium concentrations cause similar effects.

Other measures taken in the present study underline the conclusion that quinine treated almond pieces are experienced as being more aversive than denatonium treated almond pieces. For example the impact of having received a bitter tasting almond piece on the first day had an effect on the latency to eat the positively associated almond only in mice that experienced quinine soaked almonds (fig 1A), while that latency remained unchanged in the denatonium-treated group of mice. This suggests that the experience of quinine on the first day made the mice more hesitant to start eating the positive piece as well (generalization). After four trials the mice started to make an association between the odour (of either the almond or the conditioned odour) and the availability of a palatable almond piece, since latency times to start eating decreased again after that trial. This effect was not seen in the group that received denatonium treated almond pieces in the positive trials. The hypothesis that mice were using the smell of quinine (or the conditioned odour) to identify if the almond pieces were bitter seems to be further supported by the finding that mice in the quinine group performed explorative behaviour (i.e. rearing, Fig. 3) much earlier than animals that were tested on denatonium. Since rearing is performed to gain information on environmental cues (Archer and Birke 1983; Cowan 1983), the early performance of rearing in the quinine trials may be an indication that mice were able to identify if the almond pieces were bitter. BALB/c mice have excellent olfactory capacities (Restivo, *et al* 2006) and although quinine is reported to be odourless according the supplier, it cannot be excluded that these mice are able to detect some smell, or that the bitter taste was detected by bitter taste receptors that are expressed in the respiratory tract (Behrens and Meyerhof 2010).

CONCLUSIONS AND RECOMMENDATIONS

The main finding was that denatonium was not aversive enough for the mice to persistently induce avoidance of treated almond pieces. In contrast, quinine appears to be effective as an aversive taste with repeated exposure. It seems thus that BALB/cJ mice are more sensitive towards the taste of quinine than towards that of denatonium to which the animals habituate after repeated exposure, although initially responding with some aversion. Denatonium as used in the present concentration therefore seems to be of limited use as additive when aiming at persisting food aversion in mice. However, as quinine seems to be detectable by mice by means of other sensory systems than taste, this substance may be of limited use for distinct experimental approaches as well.

The underlying mechanisms involved in this difference in perception and habituation were not investigated and were beyond the scope of the present study, but we suggest to use quinine as a taste additive, at least in male BALB/c mice, if aiming at persistently making food items unpalatable. An alternative to quinine that may be more suitable when an odourless substance is required still needs to be identified.
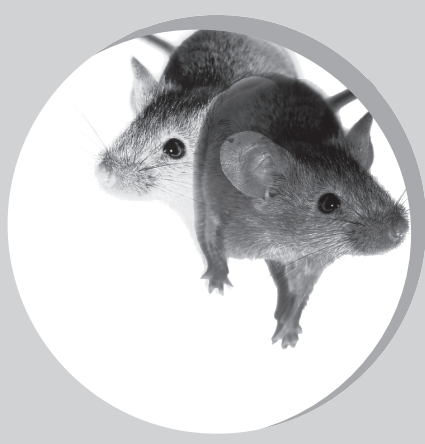
Table S1: Overview of the behavioural parameters scored. Continuous data are expressed as mean ± SEM and discrete data is expressed in median ± IQR. Significant effects (p<0.05) are shown in the column "statistics".

Behavioural parameter	Positive trials					Negative trials				
	Statistics	Quinine		Denatonium		Statistics	Quinine		Denatonium	
		trial 1	trial 11	trial 1	trial 11		trial 1	trial 9	trial 1	trial 9
Eating										
Latency [s]	T, SxT	20.00 ± 8.34	2.86 ± 0.52	12.23 ± 2.08	20.61 ± 11.29	S, T	10.28 ± 1.92	68.5 ± 14.97	18.84 ± 5.19	31.54 ± 13.46
Duration [s]	S, T	6.45 ± 0.88	8.88 ± 1.17	4.67 ± 0.42	8.57 ± 2.18	S*T	6.03 ± 0.93	15.25 ± 6.96	8.03 ± 2.07	5.62 ± 1.16
Animals eating the whole piece [%]	ns	92.9	100	85.7	92.8	S, SxT, T	7.1	0	35.7	85.7
Rearing										
Latency [s]	S, SxT	113.90 ± 6.10	120 ± 0	112.41 ± 7.59	120.0	S, S*T	79.39 ± 10.87	85.87 ± 10.57	102.89 ± 7.86	113.92 ± 7.07
Total number [nr]	ns	0 ± 0	0 ± 0	0 ± 0	0 ± 0.5		2 ± 1.5	1 ± 2.25	0 ± 1.25	0 ± 0
Line cross										
Latency [s]	S	2.58 ± 0.62	0.52 ± 0.23	3.36 ± 0.83	1.28 ± 0.40	ns	2.77 ± 1.09	0.78 ± 0.53	4.34 ± 1.51	1.07 ± 0.26
Grooming										
Latency [s]	S (trend)	113.65 ± 6.34	120 ± 0	114.54 ± 5.46	109.97 ± 8.21	S	87.83 ± 10.60	98.23 ± 8.82	95.55 ± 10.22	109.89 ± 8.19
Duration [s]	ns	0.17 ± 0.17	0 ± 0	0 ± 0	0.76 ± 0.63	S	2.72 ± 0.88	1.89 ± 0.76	1.53 ± 0.58	0.71 ± 0.51

S= substance effect, T=trial effect, SxT= substance*trial interaction effect

CHAPTER 7

EFFECTS OF ENVIRONMENTAL FACTORS ON JUDGEMENT BIAS IN MICE

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ABSTRACT

Judgement bias is a process by which the interpretation of ambiguous information is influenced by emotional states and traits. Elaborating on previous findings, in which we found that male BALB/c mice show a relatively negative judgement bias, here we investigated the effect of specific environmental conditions on responses in the judgement bias test in two separate experiments. In *experiment 1* the effect of the testing environment was investigated by comparing BALB/c mice that were either tested in the home cage or in a novel test cage, while in *experiment 2* the effect of extra enrichment in the home cage on performance in the judgement bias test was investigated. For both experiments mice were trained to associate one odour cue (either apple or vanilla) with a palatable almond piece and the other odour with an unpalatable (bitter) almond piece. In the test the reaction of the animals towards an ambiguous cue (a 50/50 mixture of both odour cues) was investigated. In *experiment 1* two groups of mice were tested, the "novel cage" group was trained and tested in an empty test cage and the "home cage" group in its' home cage. In *experiment 2* all mice were trained and tested on judgement bias in their home cage, and then assigned to either the "standard housed" (housing with tissue and shelter) or "enriched" group which received an enrichment item (toilet roll shaft with cotton wool and chocolate sprinkles), this item remained in the home cage and filled with new contents five times over ten days. After this period the animals were retrained for one day and then again tested on judgement bias. Results show that mice in *experiment 1* only revealed differentiation between the cues when tested in the home cage. In the novel cage a general behavioural inhibition was observed which can be related to increased state anxiety under these conditions. Thus, a home cage procedure might be more suitable to measure judgement bias. Due to methodological issues for *experiment 2* no conclusion on the effect of extra enrichment on the interpretation of the ambiguous cue could be drawn. Still, mice that had received enrichment decreased their responses towards the negative stimulus indicating that these mice were in a more positive emotional state than the standard housed mice. Further studies are necessary to solve methodological issues on the present judgement bias test.

INTRODUCTION

Emotional states and traits have influences on the interpretation of ambiguous information, an effect referred to as judgement bias. For example, people in a more positive emotional state interpret ambiguous information more optimistically, while people in a negative emotional state interpret ambiguous information more pessimistically (Eysenck, *et al* 1991; Mathews, *et al* 1989; Mathews, *et al* 1997). It appears that this effect is also measurable in non-human animals such as sheep (Destrez, *et al* 2012; Doyle, *et al* 2011; Sanger, *et al* 2011), pigs (Douglas, *et al* 2012), chickens (Salmeto, *et al* 2011), starlings (Bateson and Matheson 2007; Brilot, *et al* 2010; Brilot, *et al* 2009; Matheson, *et al* 2008), rats (Brydges, *et al* 2011; Burman, *et al* 2008; Burman, *et al* 2009; Enkel, *et al* 2010; Harding, *et al* 2004; Richter, *et al* 2012), dogs (Burman, *et al* 2011; Mendl, *et al* 2010a; Müller, *et al* 2012), and mice (Boleij, *et al* 2012b). In extrapolating of the findings in humans it has been proposed that the measurement of judgement bias in animals may offer some indication of their internal emotional state (Paul, *et al* 2005), and, importantly, of the animals' own emotional experience (Mendl, *et al* 2009).

In previous experiments we have found that an odour conditioning procedure can be used to investigate judgement biases in mice (Boleij, *et al* 2012b). In addition, it could be demonstrated that BALB/c mice show a negative judgement bias under aversive testing conditions (white light) as compared to less aversive testing conditions (red light), indicating that the affective state of the animals during testing affects judgement bias in mice. These results mirror findings in humans and in other non-human species, where anxiety has been shown to cause negative judgement biases (see for example Anderson, *et al* 2012a; Burman, *et al* 2009; Eysenck, *et al* 1991; Mathews, *et al* 1989; Mendl, *et al* 2010a). Considering the finding that aversive testing conditions might be of influence on the expressed judgement biases, it is likely that the exposure to a judgement bias test in a novel environment (which is a common procedure) may influence the expressed judgement bias in animals, as for example novel environment testing has been demonstrated to be aversive itself (Misslin, *et al* 1982; Misslin and Cigrang 1986; Pellow, *et al* 1985). BALB/c mice in particular show high initial anxiety in a novel environment (see for example: Brinks, *et al* 2007; Ducottet and Belzung 2004; Ennaceur 2011; Griebel, *et al* 2000; Ohl, *et al* 2001a; Salomons, *et al* 2010a), which is why the previous judgement bias test in this strain had been performed in the animals' home cage (Boleij, *et al* 2012b). It has, however, not yet been investigated what the actual effect of a novel test environment of judgement bias in BALB/c mice is.

Next to the testing environment, the home cage environment of animals is considered to influence their affective state, for example environmental enrichment has been shown to positively influence the overall well-being of laboratory animals (Smith and Corrow 2005). Moreover, environmental enrichment has been shown to influence emotional reactivity at different levels, including an increase in feeding motivation (Fernández-Teruel, *et al* 1997), a decrease in anxiety (Chapillon, *et al* 1999; Fernández-Teruel, *et al* 1997; but see van de Weerd, et al 1994) ,an increase in exploration of a novel environment (Fernández-Teruel, *et al* 1997), increased habituation (Amaral, *et al* 2008) and an improved ability to cope with aversively motivated tasks (Fernández-Teruel, *et al* 1997). Further, environmental enrichment has been shown to have positive effects on learning and neuronal growth (Van Praag, *et al* 2000) and mice that have been exposed to environmental enrichment at an early age have been

reported to reveal less stereotypic behaviours (Mason, *et al* 2007). Judgement bias studies seem to support the idea that environmental enrichment indeed positively influences emotional states since it has been found that environmental enrichment causes a positive judgement bias in rats (Brydges, *et al* 2011; Richter, *et al* 2012), starlings (Matheson, *et al* 2008) and pigs (Douglas, *et al* 2012), although no positive effect of enrichment on judgement bias was observed in dogs (Burman, *et al* 2011) and chickens (Wichman, *et al* 2012), which might be explained by differences in enrichment protocols between these studies. With the present experiments, we aimed at extending previous findings showing that light conditions during testing affected judgement bias in BALB/c mice (Boleij, *et al* 2012b) Here, we firstly explore whether judgment bias might be affected by testing in a novel environment when compared to home cage testing environment. The second part of the study is directed on the effects of environmental enrichment in the home cage on judgement bias performance in BALB/c mice.

MATERIALS AND METHODS
ETHICAL NOTE
The protocols of the experiments (DEC-DGK numbers 2011.I.08.080 and 2012.I.02.015) were peer reviewed by the scientific committee of our department and approved by the local Animal Experiments Committee. The Animal Experiments Committee based its decision on "De Wet op de Dierproeven" (The Dutch "Experiments on Animals Act" 1996) and on the "Dierproevenbesluit" (The Dutch "Experiments on Animals Decision", 1996). Both documents are available online at: htpp://wetten.overheid.nl. Further the animal experiments followed the "Principles of laboratory animal care" and refer to the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioural Research (National Research Council 2010).

ANIMALS AND GENERAL HOUSING CONDITIONS
Husbandry and experimental procedures were performed by well-trained members of the laboratory. 42 male BALB/cJ (BALB/c) mice were obtained from Charles River laboratories (Margate, UK) and were 6-8 weeks of age at arrival. Both experiments took place at the animal laboratory of the Netherlands Vaccine Institute (Bilthoven, The Netherlands) in a humidity (45%-50%) and temperature (22 ± 2°C) controlled room. The animals were housed individually in Eurostandard type 3 macrolon cages (40 cm x 26 cm x 20 cm) with standard cage bedding (Aspen chips) a plastic shelter (Mouse House Techniplast®) and facial tissue (Kleenex® Facial Tissue, Kimberly-Clark) as standard enrichment. Animals were housed under a reversed light/dark cycle (the dark period started at 6.00 and lasted until 18.00h, with red lights on) and radio music was turned on as background noise during the dark phase in the whole experimental period. During the first two weeks after arrival (habituation period) the animals were handled for ~3 minutes per mouse three times a week (between 9.00h and 11.00 h) by the experimenter who also did behavioural testing and were weighed twice a week. All testing took place in the animals housing room and equipment was installed before the animals arrived.

GENERAL EXPERIMENTAL PROCEDURES
ALMOND PIECES, BITTER STIMULI AND ODOUR STIMULI

Pieces of almond were used as rewards, mice eat these readily even if they are fed ad libitum (see for example (Ohl, *et al* 2003)). For the negative trials (NEG) almonds were soaked for 5 minutes in 180 mM quinine hydrochloride (Sigma-Aldrich) dissolved in demineralised water, and dried overnight. Odour stimuli that were used were vanilla and apple odour (Micro-Plus, Stadtoldendorf, Germany), dissolved in distilled water (0.05%), since mice are attracted by those (e.g. (Ohl, *et al* 2003)). Both odours were dissolved in a low concentration because the stock solution is highly concentrated and similar concentrations were used before (Boleij, *et al* 2012b).

APPARATUS

During training and test trials almond pieces were presented on a small petri dish (ø 5.5 cm). The odours were spread on a filter paper (ø 5.5 cm) in an amount of 0.1 ml per odour that was positioned in the petri dish (see (Boleij, *et al* 2012b)). The petri dish with the filter paper was covered by a lid with several holes to let the odours diffuse through the top. From now on this dish will be called the odour cup.

TRAINING AND TESTING

Animals were tested during the dark (active) phase between 9.00 h and 16.00 h. Mice were habituated to eating the piece of almond (30 mg), by offering it with tweezers in the home cage on days 14, 15 and 16 after arrival.

Briefly, a trial started with the placement of the home-cage on a table in front of a video camera (placed on the side of the odour cup) that was connected to a dvd-recorder (Panasonic). During preparation of the cage for the trials the mouse was temporarily placed in a small empty cage next to the home cage (or novel cage in experiment 1), for this purpose the mouse was gently approached and picked up by its tail base (the mice were already habituated to this procedure during the "habituation period", see above). Before each training or test session enrichment and the water bottle were removed from the home cage and the odour cup with the almond piece was placed one small side of the cage (see (Boleij, *et al* 2012b)), the trial was started with gently placing the mouse (picked up by the tail base) on the opposite end of the cage. Trials were ended when either the whole almond piece was eaten or when the cut-off time of two minutes was reached. The next trial started after an inter-trial interval of one minute. In between sessions of different mice the novel cage as well as the small cage used in the intertrial intervals were cleaned with a damp towel.

During training in a positive (POS) trial the odour cup or cylinder was presented with a normal tasting almond piece and in a negative (NEG) trial the odour cup or cylinder was presented with a bitter tasting almond piece, and within groups half of the mice received apple as a POS conditioned stimulus and vanilla as a NEG conditioned stimulus (counterbalanced design). All trials were presented in a random order and mice were tested randomly each day.

EXPERIMENT 1: COMPARISON HOME CAGE AND NOVEL CAGE TESTING

On day 19-23 after arrival the training and testing procedure took place, consisting of 6 trials per mouse per day (session). The animals were randomly assigned to the respective experimental group, that is either the home-cage tested group (home cage, n=14) or animals being tested in a novel environment

(novel cage, n=14). On the first day of training (day 19 after arrival) all mice received four POS trials and two NEG trials, on the second, third and fourth day mice received three POS and three NEG trials. In the test session (day 23 after arrival), in addition to two POS and two NEG trials, two ambiguous (MIX) trials were performed, in which a 50/50 mixture of both odours was presented with a palatable almond piece. The home cage group was trained and tested in the home cage, before each session enrichment and the water bottle were removed from the home cage and this cage was prepared for testing while the mouse was placed in a small empty cage next to the home cage. The novel cage group was trained and tested in an empty cage identical to the mouse's home cage. All other procedures were similar as that performed for the home cage group. Both groups were tested in the light (white light, provided by a desk lamp of approximately 120 lx, directed on the animal cage) since this test condition previously has been found to increase the differentiation between the POS and NEG cues in the test trial (Boleij, *et al* 2012b).

EXPERIMENT 2: COMPARISON STANDARD HOME CAGE AND HOME CAGE WITH EXTRA ENRICHMENT

In experiment 2, BALB/c mice (n=14) were standardly housed (with a plastic shelter and facial tissue as nesting material once a week) in the beginning of the experiment; on days 19-21 all mice were trained and on day 22 all animals were tested following similar procedures as described for experiment 1: on day one of training the mice received four POS trials and two NEG trials, on the second and third day mice received three POS and three NEG trials. In contrast to experiment 1, however, animals were now trained for three days only, since baseline performance appeared to be reached after three days of training in the first experiment. In the first test session (day 22 after arrival), in addition to two POS and two NEG trials and one ambiguous (MIX) trial was performed, in which a 50/50 mixture of both odours was presented with a palatable almond piece. In addition, in all trials three normal and three bitter almond pieces were put under the lid of the odour cup to create a stable background smell, because previous experiments revealed that mice might be able to smell the quinine on the almond pieces.

After the first test mice were divided into two groups, one group remained on standard housing conditions (control group, n=7) with facial tissue once a week and plastic shelter, while the other group (extra enriched, n=7) received an extra enrichment item every other day (a total of 5 times in ten days), a procedure that has been demonstrated positively to affect the emotional state in mice (Gross, *et al* 2011; La Mela, *et al* 2010; Van De Weerd, *et al* 1998). Here, we used a toilet roll shaft with cotton wool that contained several small pieces of milk chocolate (chocolate sprinkles, de Ruijter, the Netherlands), since mice are attracted by chocolate, and milk chocolate has been shown to be rewarding for mice in a place preference test (La Mela, *et al* 2010). The fact that the chocolate sprinkles are spread within the cotton wool gives the mice the opportunity to search for the food items, which could of additional enrichment value since searching for food is preferred above readily available food and is considered more rewarding in other species (see for example de Jonge, et a 2008; Inglis, *et al* 1997). In addition, the cotton wool was manipulated and used as additional nesting material. For mice, it has been shown that items that can be used as nesting material are preferred in order to build nests (Gross, *et al* 2011; Van De Weerd, *et al* 1998). Both groups received one retraining session with three POS and three NEG after

the 10 days of extra enrichment. One day later the mice were tested for the second time with again two POS, two NEG and one MIX trial.

BEHAVIOURS SCORED

Behaviour during testing was scored live using the computer program "The Observer" version 5.0 (Noldus b.v., Wageningen, the Netherlands). Behaviours were scored in a continuous way, i.e. all-occurrence recording of the behaviours of interest. The following behavioural parameters were measured: Latency until picking up and eating the almond piece, exploration (sniffing) of odour cup (latency and duration), locomotor activity: line crossings between front and back, i.e. the midline of the cage (latency and total number), general exploration: rearing (latency and total number), grooming (latency, total duration and total number), risk assessment: stretched attend postures (latency and total number).

CORTICOSTERONE

Blood was sampled to determine the influence of the procedures in experiment 1 and 2 on plasma corticosterone (pCORT) levels after testing. To this aim basal blood samples were taken 5 days before the training procedure started (BASAL) and another sample half an hour after the last test (POST-TEST). A third blood sample was obtained from the trunk blood (TRUNK) after decapitation, which was performed two hours after testing. All blood sampling took place in a separate room adjacent to the experimental room under red light conditions not to disturb the other animals. To prevent any influence of handling and blood sampling on pCORT, the procedures were done within a maximum of three minutes. Via tail vein incision (animals were placed on a cage lid on top of an empty cage, with this method it was not needed to physically restrain the animals, and the incision itself was made with a sharp razor blade close to the base of the tail) a small blood sample was collected (±50 µl) and stored in pre-chilled Microvette tubes (CB300, Sarstedt, Numbrecht, Germany) containing lithium heparin. Trunk blood after decapitation was collected in Minicollect tubes (1 ml Lithium Heparin, Greiner Bio-One GmbH, Kremsmünster, Austria). Blood samples were centrifuged (10 min at 20,000×g, 4°C) and stored at–20 °C until measurement. pCORT levels were measured by radioimmunoassay (RIA) according to the protocol of the supplier with an ImmuChemTM Double Antibody Corticosterone kit for rats and mice (MPI Biochemicals, Amsterdam, The Netherlands).

STATISTICS

Statistics were performed with the computer program SPSS 16.0.1 for Windows (SPSS Inc. IL, USA). Continuous data (plasma CORT, latency and duration of behavioural parameters) are represented as mean ± standard error of the mean (SEM), and were first investigated for gaussianity using the Kolmogorov-Smirnov test. Homoscedasticity was tested by Levine's test. Discrete data on the ordinal scale (total number of behavioural parameters) are represented as median with interquartile range. Some of the parameters revealed a non-parametric distribution and were either log-transformed (continuous data) or rank transformed (number data) to get a normal distribution (Conover and Iman 1982). The (transformed) data from experiment 1 was subsequently analysed with a repeated measurements (RM) ANOVA with group as between subject variable and stimulus as within subject variable, the test data from experiment 2 was analysed using a RM ANOVA with group as between subject variable and stimulus and test as within subject variable. (re-)Training data from both experiments was

analysed with separate RM ANOVA's for the positive and negative trials using trial number as within and group as between subject factor. Post-hoc testing was done using a Dunn-Ŝidák correction.

RESULTS
EXPERIMENT 1: HOME CAGE/NOVEL CAGE COMPARISON
TRAINING

In the positive trials mice tested in the home cage and in the novel cage showed a decrease in latency time to start eating the almond piece (trial effect: $F_{12,312}$= 4.453, p< 0.001) (Fig. 1A). Animals that were tested in the novel cage showed a different pattern of latency to start eating the almond piece over time; they had higher latency times in the beginning of the training period and reached latency times similar to those of the home cage group at the end the training period (trial x group effect: $F_{12,312}$= 2.075, p= 0.020 and group effect: $F_{1,26}$= 3.498, p= 0.073).
In the negative trials (Fig. 1B) the latency times to start eating the almond piece increased over time in both groups (trial effect: $F_{10,260}$= 16.978, p= 0.000), but no trial x group interaction effect was found ($F_{10,260}$= 0.916, p= 0.511). Home cage tested animals tended to be faster than novel cage tested animals (group effect: $F_{1,26}$= 3.189, p= 0.086), although the difference was relatively small and did not reach significance (Fig.1B).
Change of the other behavioural parameters over time in the training trials can be found in table S1. For the latency until the first odour cup exploration a significant trial effect (POS: $F_{12,31}$2= 4.930, p< 0.001; NEG: $F_{10,260}$= 2.565, p= 0.007), group x trial interaction (POS: $F_{12,312}$= 2.336, p= 0.011; NEG: $F_{10,260}$= 1.989, p= 0.038) and a group effects (POS: $F_{1,26}$= 10.032, p= 0.004; NEG: $F_{1,26}$= 14.096, p<0.001) were found, both in the positive and negative trials. Mice tested in a novel environment initially showed higher latency times until the first odour cup exploration, but decreased their latencies towards levels similar to those of the home cage group at the end of the training period. Similar effects were found for the latency until the first line cross (trial effect: POS: $F_{12,312}$= 7.499, p<0.001, NEG: $F_{10,260}$= 4.262, p< 0.001; trial x group interaction effect: POS: $F_{12,312}$= 2.054, p=0.026, NEG: $F_{10,260}$= 2.099, p= 0.026; group effect: POS: $F_{1,26}$= 30.720, p<0.001, NEG: $F_{1,26}$= 34,850, p<0.001), together indicating that the mice tested in a novel cage stayed longer on the side of the cage in which they were placed (table S1).
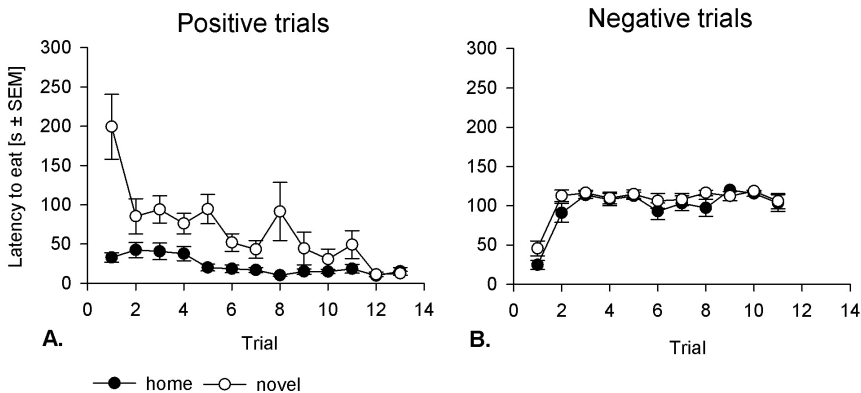

Fig. 1: Mean latency until eating the almond piece (seconds ± SEM) in the positive (A) and negative (B) trials during training of experiment 1.

Furthermore, significant differences between trials ($F_{12,312}$= 5.155, p<0.001) and groups ($F_{1,26}$= 11.305, p= 0.002) were found for the latency to rear in the positive trials (latency higher in the home cage group and increasing over time, see table S1), but not in the negative trials (trial effect: $F_{10,260}$= 1.761, p=0.078; group effect: $F_{1,26}$= 0.033, p> 0.05), for both positive and negative trials no significant interaction effects were found (p> 0.05). However, for the total number of rears in both the positive (trial effect: $F_{12,312}$= 13.990, p<0.001; trial x group effect: $F_{12,312}$= 5.650, p<0.001; group effect: $F_{1,26}$= 21,150, p<0.001) and negative trials (trial effect: $F_{10,260}$= 9.712, p<0.001; trial x group effect: $F_{10,260}$= 4.137, p<0.001; group effect: $F_{1,26}$= 23,427, p<0.001) significant factor and interaction effects were found; i.e. animals initially reared more in the novel cage, but decreased the number of rears towards the end of the training period.

As indication of arousal, grooming behaviour was measured (table S1). Animals decreased their latency to groom in both groups in the positive as well as the negative trials (trial effects: POS: $F_{12,312}$= 4.949, p< 0.001; NEG: $F_{10,260}$= 2.061, p<0.028). In the positive trials a significant trial x group interaction effect ($F_{12,312}$= 2.026, p= 0.030) and a trend for a group effect ($F_{1,26}$= 3.770, p= 0.063) for the latency until the first grooming event was found, while this was not the case in the negative trials (p> 0.05). In addition, the total duration of grooming was in general higher for mice tested in a novel cage only in the positive trials (group effect: $F_{1,26}$= 11.425, p= 0.002) but not in the negative trials (group effect: $F_{1,26}$= 0.379, p> 0.05), i.e. in the negative trials home cage tested animals groomed more than in the positive trials while there were no clear differences between positive and negative trials in the novel cage tested group. Also the duration of grooming changed over time in both positive and negative trials (trial effects: POS: $F_{12,312}$= 2.646, p= 0.003; NEG: $F_{10,260}$= 2.514, p= 0.007), however there is no clear increase or decrease seen over the whole training period. Trial x group interaction effects were not significant for the total duration of grooming (p> 0.05).

Risk assessment behaviour (stretched attend postures; table S1) only occurred in the beginning of training, so only the first trial was analysed on this behaviour. Since the first trial in the first session was always a positive trial, risk assessment had a higher occurrence in the first positive trial than in the first negative trial; animals tested in a novel cage showed more stretched attends than animals tested in the home cage only in the first positive trials (Mann-Whitney U= 15, p< 0.001) and showed a lower latency to perform the first stretched attend (Mann-Whitney U= 123, p<0.001).
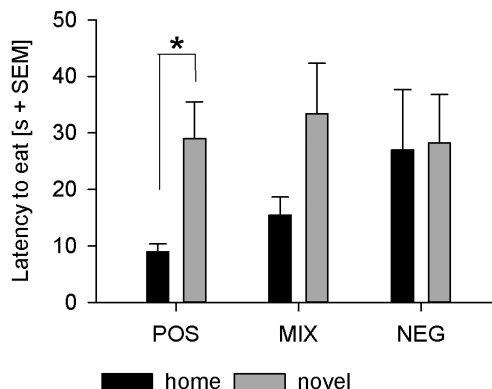

Fig. 2: Mean latency until eating the almond piece (seconds + SEM) in the test session of experiment 1. Values are averaged across the two trials, POS= positive, MIX= ambiguous and NEG= negative trial. * p<0.05

The mean latencies until the mice started eating the almond piece in the test session are presented in Fig. 2. There was no general difference between stimulus type and latency to start eating the almond piece in the test session ($F_{2,52}$= 1.526, p= 0.227), but a trend for a significant group x stimulus ($F_{2,52}$= 2.911, p= 0.063) and group effect ($F_{1,26}$= 3.062, p= 0.069) was found. Post-hoc testing revealed that the interaction effect can be explained by a significant difference between the home cage and novel cage tested group only in the POS trials (t= -3.090, p= 0.006, adjusted α, p<0.017 is significant).

There also appeared to be a general group difference in latency to cross the first line in the test session ($F_{1,26}$= 4.425, p= 0.045; Fig. 3). Mice tested in a novel cage showed a trend for a higher latency to cross the first line in the POS and NEG trial (POS: t= -2.513, p= 0.022; NEG: t= -2.332, p= 0.035, adjusted α, p<0.017 is significant), while this difference was not found by post-hoc testing towards the MIX stimulus (P>0.05).

The latency until the first rear in the test trials is presented in Fig. 4. Latency to rear differed between the stimulus type (stimulus effect: $F_{2,26}$= 19, 413, p<0.001) and there was a significant stimulus x group interaction effect found ($F_{2,26}$= 29.244, p<0.001), there was no general difference between groups in the latency until the first rear (group effect: $F_{1,26}$= 2.485). Post-hoc testing revealed that mice tested in a novel cage showed a lower latency to make the first rear only in the POS and MIX trials (POS: t= 3.018, p=0.006, MIX: t= -4.102, p= 0.001), but not in the negative trials (p>0.05). The total number of rears was only analysed in the first negative trial (presentation of bitter almond piece) since rearing hardly occurred in the other trials. There was a significant difference in total number of rears in this trial novel cage tested mice reared more (median ± IQR : 22 ± 13.25) than home cage tested mice (median ± IQR : 18 ± 9.75, t= -2.534, p= 0.018).

For total duration of grooming no significant effects were found (all p>0.05), see table S2.

Fig. 3: Mean latency until the first line was crossed (seconds + SEM) in the test session of experiment 1. Values are averaged across the two trials, POS= positive, MIX= ambiguous and NEG= negative trial. t1 p= 0.022, t2 p= 0.035 (p<0.017 is significant).
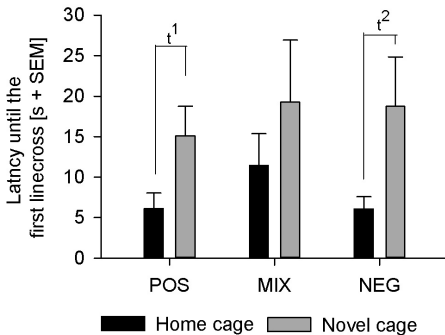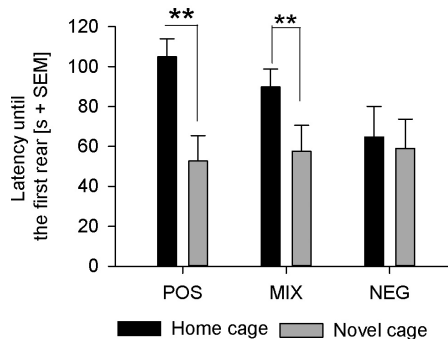
Fig. 4: Mean latency until the first rear (seconds + SEM) in the test session of experiment 1. Values are averaged across the two trials, POS= positive, MIX= ambiguous and NEG= negative trial. ** p<0.01
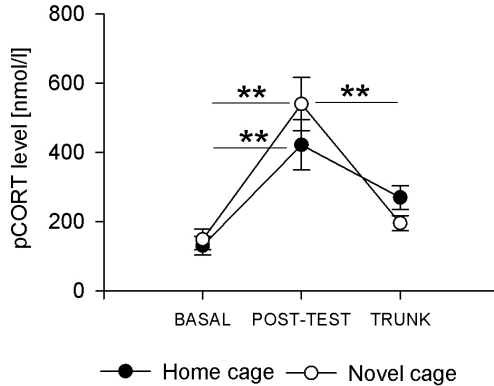
Fig. 5: Corticosterone levels in the blood plasma (pCORT, nmol/l ± SEM) 5 days before testing (BASAL), 30 minutes after testing (POST-TEST) and 2 hours after testing (TRUNK) in experiment 1. ** p<0.01

PCORT LEVELS

The measured pCORT levels in the blood are represented in Fig. 5. pCORT levels increased due testing in both groups ($F_{2,40}$= 17.931, p<0.001), however no significant sample x group interaction ($F_{2,40}$= 1.735, p= 0.189) or group effects were found ($F_{1,20}$= 0.012, p= 0.915). In the home cage test there was a significant increase in pCORT due to testing (BASAL-POSTTEST: t= -3.720, p= 0.005, adjusted α, p<0.017 is significant) and these values were still increased 2 hours after testing in comparison to baseline values (BASAL-TRUNK: t= -2.920, p= 0.014, adjusted α, p<0.017 is significant), while this was not the case for the novel cage group; i.e. pCORT values were significantly increased due to testing (BASAL-POSTTEST: t= -4.667, p= 0.001, adjusted α, p<0.017 is significant), however these values significantly decreased again 2 hours after testing (POSTTEST-TRUNK: t= 4.227, p= 0.001, adjusted α, p<0.017 is significant) and were not different between BASAL and TRUNK blood (BASAL-TRUNK: t= 1.357, p>0.05, adjusted α, p<0.017 is significant).

EXPERIMENT 2: EXTRA ENRICHMENT EFFECTS ON JUDGEMENT BIAS
TRAINING

The latency until eating the almond piece in the training of experiment 2 is presented in Fig. 6. The latency until eating the almond piece in the positive trials decreased over time, while the latency until eating the almond piece in the negative trials increased over time, reflected by a significant trial x stimulus interaction effect ($F_{1,91}$= 3.579, p<0.01) and a general difference between stimuli (stimulus effect: $F_{1,91}$= 4.930, p= 0.045) but not between trials ($F_{7,91}$= 3.579, p= 0.003). In the retraining session, which was performed after the extra enrichment treatment, the standardly housed group seemed to have a higher latency until eating the bitter tasting almond piece when compared to mice that received extra enrichment; however this effect was not significant (group effect: $F_{1,11}$= 1.397, p= 0.262). Finally, there was a significant stimulus effect ($F_{1,11}$= 37.851, p<0.001) but no significant interaction effects (all p>0.05).
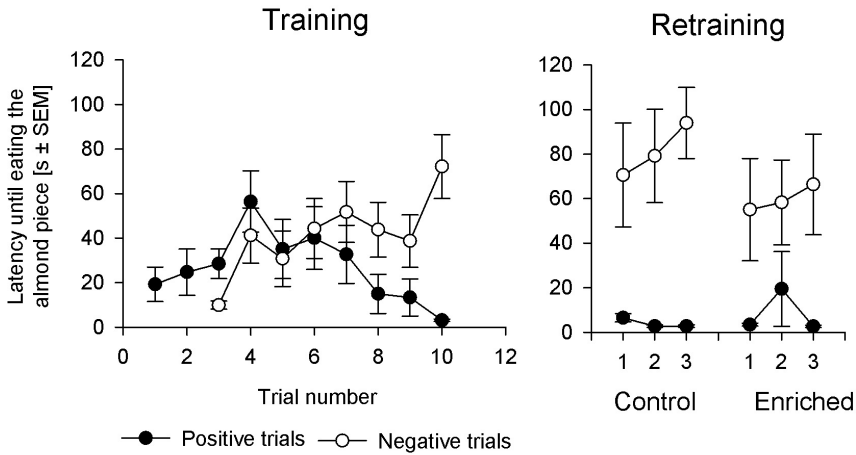
Fig. 6: Overview of the latency times to pick up and eat the almond piece in the positive and negative training trials of experiment 2. The left panel showes the first three days of training and the right panel the retraining session that was performed one day before the second test session.
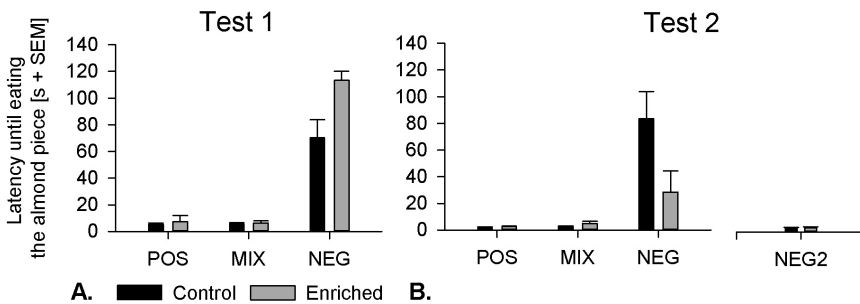
Fig. 7: Latency times to start eating the almond piece (seconds + SEM) in the test session of experiment 2 . Values are averaged across the two trials, POS= positive, MIX= ambiguous and NEG= negative trial. In the second test NEG was presented with a bitter almond piece and NEG2 with a palatable almond piece.

TEST

The mean latencies to start eating the almond piece in the first and the second test session are presented in Fig. 7, both test sessions were analysed together in a RM ANOVA. The results show a significant effect of test ($F_{1,12}$= 13.771, p=0.003), stimulus ($F_{2,24}$= 14.734, p=<0.001) and a significant test x stimulus x group interaction effect ($F_{2,24}$=4.826, p= 0.021) all other separate and interaction effects were not significant (all p>0.05). Post-hoc analysis revealed that the significant interaction effect can be explained by a decrease in latency to eat the almond piece in the enriched group when the first test was compared with the second one (t=3.362, p= 0.015, corrected α: p<0.017 is significant).

The latency until the first line cross (see table S3) showed a trend for a decrease in the second test session in comparison with the first test session ($F_{1,12}$= 3.572,

p= 0.083). In addition, there was a trend for a difference between stimuli in the latency to start exploring the odour cup (see table 3, $F_{2,24}$= 3.369, p= 0.075) and a significant test x stimulus interaction effect ($F_{2,24}$= 4.755, p= 0.018) indicating that this difference followed a different pattern between both test sessions.

The total duration of odour cup exploration was higher in the negative trial ($F_{2,24}$= 25.432, p<0.001) and was almost different between groups ($F_{1,12}$= 4.430, p= 0.057); enriched mice show a higher total duration of odour cup exploration than standardly housed mice (see table S3).

On the parameters grooming and rearing only analyses were performed on the first negative session in both tests, since the positive trials were ended when the almond piece was eaten. The latency until the first rear and the total number of rears in the first negative trial (table S3) were significantly different between the first and the second test (test effect: $F_{1,12}$= 7.487, p= 0.018), mice showed a higher latency to rear and a lower total number of rears in the second test. There were no significant differences on rearing behaviour between groups and there were no group x test interaction effects (all p>0.05). There was a significant decrease in the total time spent grooming as well as a significant increase in latency until the first grooming bout in the first negative trial in mice that were tested for the second time (total duration: $F_{1,12}$= 8.182, p= 0.014; latency: $F_{1,12}$= 4.754, p= 0.05), all group and group x test interaction effects were not significant (p>0.05).
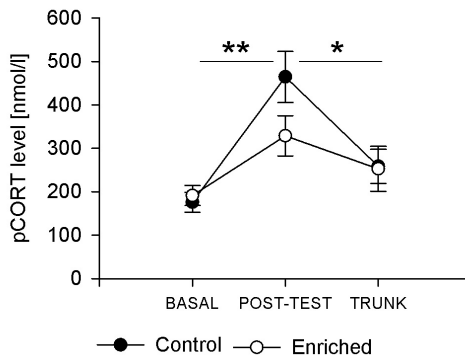
Fig. 8: Corticosterone levels in the blood plasma (pCORT, nmol/l ± SEM) 5 days before testing (BASAL), 30 minutes after testing (POST-TEST) and 2 hours after testing (TRUNK) in experiment 2. ** p<0.01, *p<0.05

CORTICOSTERONE

The pCORT levels are represented in Fig. 8. There was a significant difference in pCORT levels between samples ($F_{2,44}$= 13.090, p<0.001). The second sample which was taken 30 minutes after testing contained higher pCORT than either the basal sample and the sample taken 2 hours after the test (BASAL-POST-TEST: t= 5.216, p<0.001; POST-TEST-TRUNK: t= 2.778, p=0.010, corrected p<0.017 is significant). Although mice that had received the extra enrichment seem to have higher pCORT this effect was not significantly different and there were no significant sample x group interaction effects found (all p>0.05).

DISCUSSION
EXPERIMENT 1

Overall, the results reveal significant differences between BALB/c mice tested on judgement bias either in their home cage or in a novel cage. During training, mice tested in a novel cage showed considerably higher latencies to start eating the almond piece in the positive trials and reached latency times similar to those

mice tested in the home cage only towards the end of the training period (Fig. 1A). Further, mice from the novel-cage group showed a high latency to perform the first line crossing, a higher latency to explore the odour cup, and more stretched attends than the home cage group during the training period (table S1). Together these behaviours indicate that the mice tested in a novel cage were behaviourally inhibited in response to the unfamiliar (potentially dangerous) environment, which is in line with extensive findings on neophobia in mice (Belzung and Griebel 2001; see Lister 1990; Ohl; Treit 1985). More specifically, endocrine as well as behavioural responses indicate that forced placement into a novel environment is stressful for rats and mice and induces anxiety (Chapillon, *et al* 1999; Misslin, *et al* 1982; Misslin and Cigrang 1986; Pellow, *et al* 1985; Tang, *et al* 2012). Risk assessment behaviour in particular is known to be performed by rodents in response to a novel (potentially) threatening situation and is thought to be performed to gain more information on the potential threat (Blanchard, *et al* 2003; Cruz, *et al* 1994; Rodgers, *et al* 1997). Once an environment is assessed to be safe, risk assessment decreases, which is in accordance with the finding in the present study that risk assessment was only performed in the first trial in a novel unfamiliar environment. Further, the suppression of both food exploration and intake seen in the animals tested in the novel cage represents another indication of increased anxiety (Merali, *et al* 2003; Rodgers, *et al* 2002), and we show here that once the animals become more familiar with the environment food intake suppression decreases, indicating that the mice habituated to the testing situation. Both experimental groups of mice, however, showed a rapid increase in latency to start eating the bitter almond piece in the negative trials, which indicates that all animals learned to avoid the almond pieces very quickly.

During the test session, mice tested in a novel cage do not appear to differ in reaction towards the three odour-stimuli (Fig. 2). In contrast, BALB/c mice tested in their home cage seem to be able to differentiate between all three odours, as found before (Boleij, *et al* 2012b). The lack of differentiation in the group tested in the novel cage can possibly be explained by the fact that mice in the novel cage not only revealed an increased behavioural inhibition, but as well a more clear increase of pCORT levels due to testing (Fig. 5 and table S2) clearly indicating that the novel-cage constitutes a more aversive test condition than the home cage. Thus, although we found a clear differentiation between the cues during the training phase in both groups (which was performed in the dark) the additional white light in the test session inhibits the mice tested in a novel cage more persistently than mice tested in the home cage. In effect, it appears that the testing condition 'novelty' entirely masks the behavioural expression of a cognitive bias as it occurred in the home-cage tested animals.

While mice tested in the home cage clearly differentiated between the odour stimuli (Fig. 1), their interpretation of the ambiguous stimulus appeared to be neither positive nor negative. In an earlier experiment, BALB/c mice tested under white light conditions showed a negative judgement bias, that is, their response towards the ambiguous stimulus was comparable to that towards the negative stimulus. These findings were in accordance with reports on negative judgement bias in rats that were tested under bright light conditions while being trained in the dark (Burman, *et al* 2009). The lack of a clear negative bias found in the present experiment might be explained by the prolonged training procedure of six trials per day that was chosen to equalize the length of training and test sessions, respectively, while previously a between animal comparison was performed using

only two trials per day (Boleij, *et al* 2012b). The present procedure is likely to have resulted in a better habituation of the animals towards both the test situation and the negatively associated odours (Gray and McNaughton 1983; O'Keefe 1999) resulting in a decreased anxiety (see for example Salomons, *et al* 2010afor BALB) and, therefore, a lower level of state anxiety may have shifted their judgement bias towards a more positive interpretation of the ambiguous stimulus (as is known in humans, see for example Eysenck, *et al* 1991). In conclusion, home cage testing seems more suitable for testing judgement bias in mice when compared to novel-cage testing. Further refinements however are necessary in order to induce a more negative judgement bias in the test session.

EXPERIMENT 2

In the second experiment the effects of enriched housing conditions on judgement bias tested in the home cage were investigated. Surprisingly, both experimental conditions resulted in a positive judgement bias, i.e. mice responded with an equal short latency to both the positive and the ambiguous odour, while response latencies towards the negative cue were significantly higher (Fig. 7) . However, the enriched group revealed shorter latency times to start eating the negatively cued almond piece, which implies that these mice seemed to assess a negative stimulus to be less aversive.

Similar to experiment 1, all animals learned to avoid the bitter almond pieces over time (Fig. 6A) and clearly differentiated between positive and negative cues (Fig. 6B). Nevertheless, mice showed a similar reaction towards positive and ambiguous cue in the test-session (Fig. 7A), which suggests that the animals might have differentiated between the presented almond pieces by other cues than the odour-cues. It remains unclear though what that cue might be since we attempted to mask any unintended cue that might be associated with the taste-additive by positioning both a bitter and a normal almond under the lid of each odour cup next to the either normal (POS and MIX) or bitter (NEG1) almond pieces on top of the lid. In addition, to confirm that mice used other cues to identify if the almond piece was bitter, we decided to include an additional trial by presenting a second negative cue (NEG2 in Fig. 7B) in the second test presenting a normal almond piece (instead of a bitter almond) on top of the lid and a bitter and normal almond beneath the lid of the food cup. The results show that indeed reactions towards this second negative stimulus were not different from the positive and ambiguous stimulus. This finding remains puzzling since the manufacturer reports quinine to be odourless and the taste additive has been used in a variety of studies in rodents before (e.g. Burman, *et al* 2009; Dwyer 2011; Koot, *et al* 2012). Still, to our knowledge no direct investigation on differentiation based on odour of quinine has been done before in laboratory animals. Though we cannot exclude that the mice are able to identify the almond pieces based on other cues than odour (e.g. colour, structure), or that that the bitter taste can be sensed via bitter taste receptors that are expressed in the airway tract (Behrens and Meyerhof 2010), it remains unclear why a more differentiated response had been found in our other experiments (see for example Burman, *et al* 2009). However, it seems unavoidable to conclude that for the present set-up only preliminary conclusions can be drawn based on the relative reactions of the mice towards the presentation of a bitter almond piece in the two test sessions (NEG trials).

Here, mice in the enriched group showed lower latency times to start eating the almond piece in the negative trial of the second test when compared to the first

test, which may be interpreted as a more "positive" assessment of the negative cue. Such an effect may be explained by a decrease in anxiety as induced by the testing procedure, given that anxiolytic effects of environmental enrichment in BALB mice have been reported before (Chapillon, *et al* 1999; Roy, *et al* 2001; but see van de Weerd, *et al* 1994). The occurrence of such an anxiolytic effect by environmental enrichment is further supported by findings on HPA-axis responsiveness, as for example rats and mice exposed to a mildly stressful testing situation have been reported to show lower ACTH and pCORT responses than control animals (Roy, *et al* 2001; Simpson and Kelly 2011) indicating that environmental enrichment increases the ability of animals to cope with a stressful situation. pCORT responses of mice provided with extra enrichment in the present experiment also seemed lower than those of standardly housed animals (Fig. 9) though this effect was not significant, which may be due to the high individual variation in pCORT levels. Another explanation for the more positive bias is that environmental enrichment may affect feeding motivation (Fernández-Teruel, *et al* 1997; Monosevitz 1970) or reward sensitivity (Richter, *et al* 2012). Which seems a likely explanation since in another judgement bias experiment in rats it has for example been found that environmental enrichment increased milk intake and reduced the latency to choose for a food reward (Richter, *et al* 2012). Irrespective of the described methodological concerns, our results suggest that mice from the enriched group were either more positive or less anxious than the mice in the standardly housed group. Several other judgement bias studies have found that environmental enrichment positively biases the interpretation of ambiguous cues in rats (Brydges, *et al* 2011; Richter, *et al* 2012), pigs (Douglas, *et al* 2012) and starlings (Matheson, *et al* 2008). However, studies on dogs and laying hens (Burman, *et al* 2011; Wichman, *et al* 2012) have failed to find effects. It is difficult to explain such differences or to draw conclusions from them, since most studies did not include appropriate control measures emotional or motivational factors that might confound judgement bias performance in animals.

We conclude that further studies are necessary to develop and validate tests for judgement bias in animal species. For the judgement bias test used here it might be proposed to adapt the procedure such that the mice may not be able to smell the taste additive. This could for example be achieved by implementing a method using active responses or separating the presentation of the conditioned stimulus with the unconditioned stimulus.

Table S1: Overview of behaviour performed in the first and last trial by mice tested in the home or novel cage of experiment 1. Continuous data is represented as mean ± SEM and numerical data as median ± IQR. Statistical significant effects: P= in positive trials, N= in negative trials, T= trial effect, TxG= trial*group effect and G= group effect. **p<0.01, *p<0.05

SUPPLEMENTARY INFORMATION

Behavioural category and parameters	Statistics	Home cage				Novel cage			
		Positive trials		Negative trials		Positive trials		Negative trials	
		trial 1	trial 13	trial 1	trial 11	trial 1	trial 13	trial 1	trial 11
Eating									
Latency until picking up the almond piece [sec]	P: T**, TxG**, G** / N: T**, G*	32.2 ± 6.0	13.3 ± 4.6	24.0 ± 5.8	101.1 ± 10.7	192.7 ± 42.5	12.3 ± 3.3	45.1 ± 9.5	105.8 ± 9.7
Latency until eating the almond piece [sec]	P: T**, TxG*, G (p=0.073) / N: T**, G (p=0.086)	32.8 ± 6.0	15.1 ± 4.7	24.5 ± 5.8	103.4 ± 10.7	199.4 ± 41.2	12.7 ± 2.5	45.6 ± 9.5	105.3 ± 9.7
Exploration									
Latency until the first rear [sec]	P: T*, G* / N: T (p=0.078)	93.6 ± 12.0	107.3 ± 9.1	13.8 ± 3.7	22.9 ± 3.1	26.2 ± 4.4	97.1 ± 12.3	29.2 ± 10.8	15.5 ± 3.7
Total number of rears [nr.]	P: T**, TxG**, G** / N: T**, TxG**, G**	0.5 ± 3.5	0 ± 5.5	5 ± 4.5	8 ± 16.3	2 ± 6.3	0 ± 12.3	5 ± 8.25	14 ± 8.5
Latency until odour cup exploration [sec]	P: T**, TxG*, G** / N: T**, TxG*, G**	12.5 ± 2.5	12.1 ± 4.7	12.8 ± 3	9.7 ± 2.8	102.1 ± 35.5	11.1 ± 2.5	35.4 ± 10.6	26.6 ± 9.2
Total duration of odour cup exploration [sec]	P: T** / N: T**, TxG*, G**	7.1 ± 0.9	1.8 ± 0.4	6.2 ± 1.5	20.8 ± 4.9	30.6 ± 11.5	1.7 ± 0.3	4.8 ± 1.0	5.7 ± 1.5
Risk assessment									
Latency until the first streched attend (only trial 1) [sec]	P: G	112.2 ± 7.8	120 ± 0	120 ± 0	120 ± 0	23.9 ± 11.0	120 ± 0	110.7 ± 7.2	120 ± 0
Total number of streched attends (only trial 1) [nr.]	P: G	0 ± 0	0 ± 0	0 ± 0	0 ± 0	12.5 ± 18.8	0 ± 0	0 ± 0	0 ± 0
Locomotion									
Total number of line crosses [nr.]	P: T**, G (p=0.053) / N: T**, TxG**, G**	0.5 ± 3.5	0 ± 5.5	5 ± 4.5	8 ± 6.3	3 ± 8.3	1 ± 0.25	3 ± 2.25	7 ± 4.75
Latency until first line cross [sec]	P: T**, TxG*, G** / N: T**, TxG*, G**	19.6 ± 8.26	11.1 ± 4.7	9.1 ± 2.7	7.16 ± 2.34	78.8 ± 16.0	10.3 ± 2.6	26.6 ± 7.0	14.6 ± 3.5
Arousal									
Total time spent grooming [sec]	P: T**, G** / N: T**	0 ± 0	1.2 ± 0.9	1.3 ± 0.4	2.3 ± 0.7	3.0 ± 1.1	1.5 ± 0.7	2.7 ± 0.7	4.2 ± 0.9
Latency until first self groom [sec]	P: T**, TxG*, G (p=0.063) / N: T	120 ± 0	112.1 ± 7.9	84.1 ± 11.8	76.1 ± 11.0	120 ± 0	88.4 ± 13.9	77.2 ± 10.6	43.6 ± 12.1

Table S2: Overview of the behavioural data scored in the test session of experiment 1. Continuous data is represented as mean ± SEM and numerical data as median ± IQR. Values are averaged across the two trials, POS= positive, MIX=ambiguous and NEG= negative trials. Statistical significant effects: S= stimulus effect, G=group effect, S*G is Stimulus*group interaction effect. *p<0.05, **p<0.01

Behavioural category and parameters	Statistics	Home cage			Novel cage		
		POS	MIX	NEG	POS	MIX	NEG
Eating							
Latency until picking up the almond piece [sec]	trend: S*G (p=0.057) & G (p=0.071)	8.54 ± 1.45	15.01 ± 3.19	26.66 ± 10.71	28.40 ± 6.56	32.83 ± 8.87	27.84 ± 8.51
Latency until eating the almond piece [sec]	trend: S*G (p=0.063) & G (p=0.069)	8.96 ± 1.43	15.45 ± 3.18	26.98 ± 10.68	29.02 ± 6.53	33.45 ± 8.87	28.27 ± 8.51
Exploration							
Latency until the first rear [sec]	S** & SxG**	104.93 ± 9.01	89.77 ± 9.03	39.27 ± 7.49	52.73 ± 12.73	57.66 ± 12.98	33.52 ± 7.16
Total number of rears [nr.]	S**, G**	0 ± 0	0 ± 0	18 ± 9.75	1 ± 3	1 ± 3.5	22 ± 13.25
Latency until odour cup exploration [sec]	S*	23.67 ± 7.01	17.57 ± 5.17	7.53 ± 1.36	29.24 ± 6.22	23.10 ± 8.10	17.39 ± 3.98
Total duration of odour cup exploration [sec]	ns	1.33 ± 0.19	1.75 ± 0.19	19.00 ± 11.58	1.70 ± 0.27	2.83 ± 0.63	3.73 ± 0.49
Locomotion							
Total number of line crosses [nr.]	ns	0 ± 0	1 ± 0	1 ± 1	1 ± 1	1 ± 1	1 ± 0.25
Latency until first line cross [sec]	G*	6.13 ± 1.36	11.46 ± 3.04	5.62 ± 1.26	15.11 ± 3.30	19.26 ± 6.99	18.74 ± 5.28
Arousal							
Total time spent grooming [sec]	ns	2.47 ± 0.53	2.92 ± 0.68	4.57 ± 1.75	6.04 ± 1.48	5.74 ± 2.08	5.18 ± 0.91
Latency until first self groom [sec]	G*	116.18 ± 3.83	108.95 ± 7.82	90.06 ± 6.01	97.35 ± 9.13	103.61 ± 9.26	79.17 ± 8.21

Table S3: Overview of the behavioural data scored in the first and second test session of experiment 2. Continuous data is represented as mean ± SEM and numerical data as median ± IQR. Values are averaged across the two trials, POS= positive, MIX= ambiguous and NEG= negative trials. Statistical significant effects: S= stimulus effect, G= group effect, S*G is Stimulus*group interaction effect. *p<0.05, **p<0.01

Behavioural category and parameters	Statistics	Control group						Enriched group					
		Test 1			Test 2			Test 1			Test 2		
		POS	MIX	NEG	POS	MIX	NEG	POS	MIX	NEG	POS	MIX	NEG
Eating													
Latency until picking up the almond piece [sec]	T**, S**, TxSxG*	13.0±9.4	5.0±1.2	73.8±22.4	2.0±0.2	2.4±0.4	83.2±20.5	6.7±4.7	6.0±1.3	69.8±23.7	1.9±0.3	4.1±1.5	28.3±15.8
Latency until eating the almond piece [sec]	T**, S**, TxSxG*	5.2±1.1	5.3±1.1	73.4±22.1	2.3±0.1	2.7±0.4	83.4±20.4	15.5±8.9	6.4±1.4	103.2±16.8	2.5±0.4	5.0±1.5	28.5±15.8
Exploration													
Latency until the first rear [sec]	T*	111.5±8.5	106.1±13.9	35.0±5.8	120±0	120±0	68.0±17.4	103.5±9.1	97.1±15.0	32.8±8.6	120±0	120±0	80.9±13.7
Total number of rears [nr.]	T**	0±0	0±0	6±5	0±0	0±0	2±3	0±0	0±2	2±3	0±0	0±0	2±2
Latency until odour cup exploration [sec]	TxS*, S (trend)1	1.9±0.6	1.7±0.7	1.8±0.5	0.6±0.2	0.3±0.3	1.5±0.4	0.7±0.4	2.9±0.9	1.4±0.2	0.5±0.3	0.8±0.5	3.9±2.0
Total duration of odour cup exploration [sec]	S**, G (trend)2	0.8±0.2	2.4±1.0	13.1±1.1	0.2±0.1	0.1±0.1	11.6±3.9	5.0±3.1	5.5±4.0	15.1±4.2	0.2±0.1	0.9±0.7	15.3±3.8
Locomotion													
Latency until first line cross [sec]	T (trend)3	1.3±0.7	0.2±0.1	0.1±0.0	0.2±0.1	0.4±0.2	0.2±0.1	8.7±8.6	0.8±0.3	0.1±0.1	0.1±0.0	0.2±0.1	0.4±0.3
Arousal													
Total time spent grooming [sec]	T*	0±0	0±0	4.3±0.9	0±0	0±0	0.7±0.5	1.3±0.7	0.4±0.4	3.2±1.3	0±0	0±0	2.0±0.0
Latency until first self groom [sec]	T*	120±0	120±0	46.7±15.0	120±0	120±0	11.3±12.9	102.9±8.1	109.2±10.8	87.4±13.5	120±0	120±0	79.3±16.7
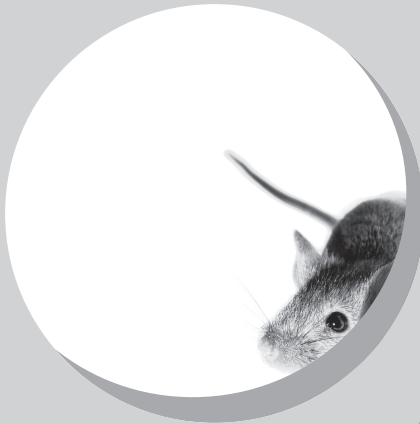
CHAPTER 8

GENERAL DISCUSSION

This thesis aims at developing a better understanding on how mice perceive their own emotional state. Next to extending on previous research on the adaptive capacities in laboratory mice, we aimed at approaching the emotional perceptions of mice by establishing a behavioural test for the assessment of judgement bias. In addition to behavioural observations, different physiological and central nervous parameters were investigated in order to get a first indication of the underlying mechanisms that may regulate emotional perception in mice.

HABITUATION AS INDICATOR OF ADAPTIVE CAPACITIES

The ability of an animal to adapt to a certain environmental challenge is reflected by its behavioural response, which is guided by emotional and cognitive processes. For example a decrease of an initial anxiety responses over time is suggested to indicate an adaptive response while a lack of decrease or increase of anxiety may indicate a non-adaptive response (Ohl, *et al* 2008; Salomons, *et al* 2009).

In extending previous findings in 129P3 mice, (Salomons, *et al* 2010; Salomons, *et al* 2010a) the results presented in chapter 2 (Boleij, *et al* 2012a) indicate that mice of different 129 substrains (129P2/OlaHsd, 129X1/J, 129S2/SvPas and 129S2/SvOlaHsd) are susceptible for the development of non-adaptive anxiety, although the different substrains have their own unique behavioural profiles. In addition to these findings on habituation, others have found that some 129 substrains, including the 129X1 and 129P3 substrains (Camp, *et al* 2009; Hefner, *et al* 2008), are also impaired in fear extinction. Therefore, 129 substrains might be proposed as a model for non-adaptive (pathological) anxiety, since both impairments of habituation and fear extinction are characteristic of some human anxiety disorders (Beck, *et al 2005*). The brain areas that seem to be involved in these non-adaptive responses point towards circuits that are crucial for the integration of emotional and cognitive information, and it has been hypothesised that the behavioural profile in 129 substrains might be the result of an impaired cognitive control of emotional processes (Salomons, *et al* 2010a).

The experiments on 129 substrains suggest a genetic susceptibility of this strain to development non-adaptive anxiety. However, it is also known that in addition to genetic factors environmental influences (gene x environment interactions) are of relevance for the development and onset of psychopathologies (Arborelius, *et al* 1999; Leonardo and Hen 2008). Nevertheless, the results of chapter 3 show that at least in mice from the CD1 outbred strain environmental stress alone does not result in the development of persistent non-adaptive anxiety behaviour: although the animals had experienced prolonged social instability stress during adolescence and early adulthood, they did not show an impairment of habituation towards a novel environment one week after termination of the stress protocol (Figs. 5, 6 & 7 page 46), irrespective of the fact that measures taken during the stress period indicated that the mice did experience chronic stress (Fig. 2 page 43 Fig. 3 page 44, Fig. 9 page 49 and table 1 page 45).

Social instability during the adolescence and young adulthood of mice and rats has been shown to cause increases in HPA-axis activity, resulting in an increase in plasma corticosteroid levels (Baranyi, *et al* 2005; Haller, *et al* 1999; Maslova, *et al* 2010; Schmidt, *et al* 2007; Schmidt, *et al* 2010; Sterlemann, *et al* 2008). The release of corticosteroids into the bloodstream as a result of stress is adaptive since it prepares the body to react appropriately towards the source of stress (De Kloet, *et al* 2005). Even prolonged stress does not necessarily disrupt this adaptive response in that the dynamic nature of internal systems enable an

organism adequately to respond to environmental challenges and to shift internal set-points within a considerable range, a process called allostasis (Korte 2001; Korte, *et al* 2005; McEwen 2007). Decreased levels of GR mRNA expression in the hippocampus of previously stressed CD1-mice (Fig. 9 page 49) indicate that indeed a minor shift of internal set-points may have taken place but is likely to have remained within an adaptive range (Korte, *et al* 2005) since no significant increases in pCORT and no differences in MR expression were found, i.e. it is thought that actually an imbalance of MR and GR results in an increased susceptibility to disease (De Kloet, *et al* 1998; Oitzl, *et al* 2010). In addition, the fact that CD1-mice did show appropriate behavioural habituation in a mildly stressful environment one week after termination of the social stress procedure shows that any internal changes remained within the adaptive range of the animals. In contrast, 129P3 animals are more susceptible towards stress exposure, considering that exposure to a chronic mild stress procedure intensified the lack of habituation in these mice (Salomons, *et al* 2010b) indicating that adaptive capacities were exceeded. Interestingly, even in less susceptible CD1 mice there seemed to be a sub-population of mice showing different behavioural responses (reflected by an increased variation) indicating that chronic stress might have affected these individuals more persistently ("responders"). Thus, in both stress vulnerable strains and more vulnerable individuals similar environmental challenges might be expected to exceed adaptive capacities and to result in the development of non-adaptive behaviour.

Overall, it can be concluded that impaired habituation might be indicative of exceeded adaptive capacities and might be a valuable "behavioural tool" to investigate emotional dysfunctions in animals.

ANIMAL WELFARE CONSIDERATIONS

Animal welfare can be considered guaranteed when an animal is able to adapt behaviourally and physiologically towards environmental challenges (Korte, *et al* 2007; Ohl and van der Staay 2012; Salomons, *et al* 2009). We show here that anxiety and stress responses, often taken as indicative of "bad" welfare (e.g. the five freedoms, see Brambell 1965), are not necessarily detrimental to animal welfare after stress exposure has been terminated (chapter 3), since animals still may be able to adequately respond to environmental challenges when subsequently tested. Thus it is not negative experiences per se that are detrimental to an animals' welfare state, but instead, welfare states should be considered along the line of an animals' adaptive capacities. Based on this description some concern might be raised for the housing and treatment of mice with a 129 background, since these mice are characterized by a lack of ability to adapt (Boleij, *et al* 2012a; Salomons, *et al* 2010; chapter 2 and Salomons, *et al* 2010a). 129 mice thus might be especially sensitive towards environmental challenges they encounter under standard laboratory housing conditions. Further research is necessary to evaluate if common laboratory procedures fall within the range of adaptive capacities of commonly used mouse strains, such as the 129 substrains.

JUDGEMENT BIAS AS INDICATOR FOR EMOTIONAL PERCEPTIONS

Though our elaborations so far have resolved in a better understanding of how we might assess whether environmental challenges exceed the adaptive capacities in mice, the examination of non-adaptive responses still does not allow us to

draw any conclusion on how the animal itself might *perceive* its emotional state. Recently, the assessment of cognitive biases, such as judgement bias, in animals has become of developing interest, since such biases are cognitive expressions of internal emotional states (i.e. "emotional perceptions") (Eysenck, *et al* 1991; Mathews, *et al* 1989; Paul, *et al* 2005). Internal emotional perceptions are considered to be closely related to how animals experience their living conditions (Duncan 1993; Fraser and Duncan 1998), hence a reliable reflection of these perceptions might provide more information about an animal's state of welfare (Mendl, *et al* 2009).Thus in addition to gaining more information on emotional processes in itself, by demonstrating that judgement bias tests are reliably reflecting the animals' internal emotional states, effects of laboratory and husbandry practices on animal welfare might be investigated.

Following the studies on habituation profiles outlined in the section above, the investigation of judgement bias in mouse strains showing adaptive and non-adaptive emotional responses, respectively, might provide more information on how this relates to the perception of their emotional state. In this line of reasoning behavioural responses in 129P3 (non-adaptive habituation profile) and BALB/c mice (adaptive habituation profile) towards ambiguous odour cues were investigated (chapter 4). The results show that BALB/c mice readily learn to differentiate between different odours (Fig. 2; page 69), in that they approach an odour associated with the presentation of a palatable almond and are slower to respond towards an odour associated with an unpalatable almond piece (Boleij, *et al* 2012b)). Further, BALB/c mice show intermediate reactions towards a mixture of both odours (Fig. 2; page 69) (Boleij, *et al* 2012b)). In contrast, 129P3 mice did not reveal a similar discrimination in the test (Fig. 2; page 69), which may be related to the short training procedure (i.e. a consequence of their impaired ability to habituate), or it might as well be a reflection of an improper ability to integrate emotional and cognitive information, as has been suggested before for this strain . However, while further validations of the testing procedure in animals of the 129-strain did not seem useful at this stage, promising first results were found in BALB/c mice and further judgement bias experiments were performed in this strain.

Irrespective of some methodological problems (see section "methodological considerations on judgement bias testing" below), the subsequent experiments in BALB/c mice (chapter 4 and 5) provided some evidence of a causal relation between the animal's emotional state and the expressed judgement bias: Firstly, the animals' emotional state was experimentally manipulated by using more (white light) or less (red light) aversive test conditions. More aversive white-light conditions did not only result in higher pCORT levels (Fig. 6; page 73), and a previously demonstrated increase in anxiety related behaviour in BALB/c mice (Salomons, *et al* 2010a), but induced a negative judgement bias as well (Fig. 3; page 70). Secondly, the effects of treatment with the anxiolyticum diazepam in the following experiment supported this first indication of a causal relation between internal emotional state and cognitive bias in mice. This pharmacological manipulation of the animals' internal state resulted in some indication of a less negative judgement bias in BALB/c mice (Fig. 2; page 95). Thirdly, in chapter 7 it was shown that a novel testing environment obscured possible judgement bias effects in that it resulted in a generalized behavioural inhibition (Misslin, *et al* 1982; Misslin and Cigrang 1986). Overall, these findings strengthen suggestions in the scarce literature on an actual causal relationship between emotional state and judgement bias in animals (in rats see Anderson,

et al 2012b; Burman, *et al* 2009).

Internal emotional states underlying the expression of judgement biases are regulated in the brain and behavioural findings on changes in judgement bias in mice should translate back into differences at the central nervous level as well. Therefore, a first inventory on the activation of potentially involved brain areas (see chapter 4 and box 1) was done using immunohistochemical analysis of the immediate early gene c-Fos. c-Fos is expressed at low levels already under basal conditions but is upregulated by neuronal activation induced by changes in afferent input and/or changes in external stimulation (Kovács 2008; Luckman, *et al* 1994). Thus, c-Fos expression can be used as a marker for (unspecific) neuronal activity following experimental challenges such as exposure to conditioned stimuli (Hess, *et al* 1997; e.g. Holahan and White 2004; Nordquist, *et al* 2003). The analysis of BALB/c brains revealed that especially the amygdala and the (dorso) lateral septum (LS), brain areas known to be involved in the regulation of anxiety (Davis and Whalen 2001; Kirk 1998; LeDoux 2003; McNaughton and Gray 2000; Millan 2003; Pratt 1992), might be involved in the processing of ambiguous information by these animals, in that negative judgement bias was paralleled by a decrease in c-Fos in BALB/c mice that were exposed to an ambiguous cue in comparison with mice exposed to a positive or negative cue (Fig. 5; page 72 and Fig. 1 box 1; page 85).

From studies in humans it can be concluded that the amygdala is likely to be involved in the estimation of the (emotional) value of a presented cue (Blasi, *et al* 2009; Herry, *et al* 2007; Hess, *et al* 1997; Hsu, *et al* 2005; Whalen 1998), see also fig 1 in the introduction, while the (dorso) lateral septum is probably more involved in linking this emotional value to contextual information (see Sheehan, *et al* 2004 for a review) and is thought to be part of the Behavioural Inhibition System, acting as a "comparator system" (Gray 1982; McNaughton and Gray 2000). The LS and the amygdala are closely connected, and the LS is hypothesized to play a regulatory role on amygdala output of emotional information by inhibiting anxiety and fear related responses (Sheehan, *et al* 2004): For example, in a conditioning procedure rats exposed to a conditioned stimulus associated with shock show a decrease in firing rate of the LS while exposure to a safety stimulus increases firing in this area (Thomas, *et al* 1991). Since we found a decrease in c-Fos expression in response to an ambiguous stimulus in BALB/c mice, the increase in behavioural inhibition indeed seemed to be mirrored by decreased neuronal activation in the corresponding brain area (Fig. 5; page 72 and Fig. 1; box 1; page 85). The mechanism of action behind the ability of diazepam to decrease negative judgement bias can be hypothesized to be mediated via the LS, since it has been found that diazepam infusions into the LS inhibit the amygdala and decrease anxiety (Stevens, *et al* 1987). In addition, BALB/c mice do show a decrease in c-Fos expression in the LS while 129P3 mice reveal no differences between the cues (Fig. 5; page 72), stressing that the inability of 129P3 mice to show appropriate responses in the judgement bias test might be related to the cognitive regulation of emotional responses as hypothesized before to be involved in their inability to habituate in the mHB (Salomons, *et al* 2010a).

Finally, the fact that at the level of the brain BALB/c mice appear to react differently towards the ambiguous cue when compared to either the positive or negative cue in terms of neuronal activation in these specific areas supports the idea that the behavioural response measured in the judgment bias test indeed might reflect the integration of emotional-cognitive processes and, thus, might give us some indication of the emotional perceptions of a mouse. Future studies

on changes in such emotional perception and on differences in emotional perception between individuals and/or groups of animals might thus profit from a more detailed evaluation of the LS as a core area in the integration of emotional and cognitive information and inhibitory mechanisms on the expression of emotions (Drugan, *et al* 1986; Pesold and Treit 1996).

INDIVIDUAL DIFFERENCES

A general theme coming back in all chapters of this thesis is the inter-individual variance in behavioural and physiological response under standardized testing conditions, even within genetically homogeneous populations of mice.
In judgment bias testing it was found that mice react differently and might use different strategies to identify if almond pieces are bitter reflected by a high variation within the groups (chapter 4-7) and, in addition, treatment with diazepam resulted in responder and non-responder effects (chapter 5), while also in the CD1 outbred strain differences responder/non-responder effects towards chronic social stress were found. Moreover, a high variability in pCORT responses occurred in all experiments (chapter 2-7), indicating differences in HPA-axis responses between individual mice.
It is possible that the observed individual differences can be related to the existence of differences in coping strategies within the tested populations of mice. Koolhaas *et al.* (Koolhaas, *et al* 1999) describe that at least two different coping strategies exist within a large range of species. An active coping style is characterized by the fight/flight response and is aimed at removing themselves from the source of threat or dealing with the stressor by attack (Benus, *et al* 1991), animals using this strategy are driven by territorial control and aggression.
In contrast, reactive coping style is characterized by low levels of aggression and immobility in response to a stressor in this way reducing the emotional impact of the stressor (Benus, *et al* 1991). In addition, animals with a reactive coping style have a higher HPA axis reactivity (see Koolhaas, *et al* 1999 for a review). Both strategies can be adaptive depending on the specific environmental situation, however the reactive coping strategy is considered more flexible than the proactive strategy (Koolhaas, *et al* 1999; Korte, *et al* 2005). In wild housemice both strategies have been demonstrated (Benus, *et al* 1991) and are suggested to exist in CD1 mice as well (Yen, *et al* 2012). Furthermore, both phenotypes appear to be influenced by genetic as well as environmental influences and their interactions (see for example in rats: Brunelli and Hofer 2007; De Boer, *et al* 2003; in birds: Schoech, *et al* 2011; in mice: Van oortmerssen, *et al* 1984; in salmon: Vaz-Serrano, *et al* 2011 and Korte, *et al* 2005 for a review). In the judgement bias test differences in coping style within experimental groups could possibly translate into high inter-individual variance in approach latencies and how animals cope with negative consequences. The latter for example might result in different responses towards pharmacological treatments, while in exploring a novel environment reactive coping animals might show higher avoidance behaviour and, in contrast, actively coping animals might perform more escape behaviour. In essence, thus, different coping strategies within experimental groups of mice would be likely to result in opposing responses towards environmental challenges and, therefore, would offer a possible explanation for high inter-individual variation in distinct behavioural and physiological data.
Often genetically homogeneous inbred strains are being used for experiments as they are supposed to produce a lower inter-individual variation than outbred strains. Still, a variety of publication counteract this presupposition (Caldji, *et*

al 2004; e.g. Crabbe, *et al* 1999; Erhardt, *et al* 2009; Veĭko, *et al* 2007), arguing that such variation rather is the result of gene x environment interactions on the expression of the behaviours than of genes alone (Kafkafi, *et al* 2005; van der Staay, *et al* 2010; Öbrink, *et al* 2000). Thus, variance in experimental results to some degree can be reduced by standardization of procedures as for example standardization of housing and feeding conditions, controlling ambient room conditions (Baker 2011). However, what the experimenter cannot standardize are the conditions and management procedures at the breeder, and it is maternal factors and early rearing conditions that play an important role in the development of emotional reactivity of animals (Holmes, *et al* 2005).

METHODOLOGICAL CONSIDERATIONS ON JUDGEMENT BIAS TESTING

While our initial odour conditioning studies were promising for the development of a test for judgement bias in mice, some problems regarding the test set-up occurred along the way that might have obscured the possible effects of some manipulations on actual judgement biases.

Firstly, it appeared that the mice seemed to be able to identify a bitter almond piece irrespective of the odour cues used. Although the taste additives used have been reported to be odour-free as reported by the supplier, it appeared that the mice were able to 'smell' quinine itself on the almond pieces in the negative trials (the "unconditioned" stimulus), instead of using the (to be) conditioned odour as a cue. However, in our first experiments (chapter 4) this problem did not occur, in contrast, BALB/c mice correctly differentiated between the different odour mixtures (see Fig. 2 and 3; page 69-70). In this experiment, however, short training procedures were used and different groups of mice were exposed to the positive, ambiguous and negative odours, respectively, during training, while, in the test session all the odour cues (including the negative odour) were combined with a non-bitter almond piece. In contrast, for the purpose of investigating the effect of diazepam on negative judgement biases (chapter 5), a within animal comparison was used, that is, in the test session animals were exposed to all stimuli two times and the negative trials were performed with bitter pieces to prevent within session learning. Via this design only one group per dose of diazepam was necessary instead of three subgroups for each dose, reducing the number of animals by threefold. However, when compared to the first experiment, in the test session much higher avoidance in the negative trials was found than in the experiments described in chapter 4, suggesting that it was the quinine on the almond pieces that caused this difference. Interestingly, others used similar tests set-ups for judgement bias testing before, but did not report such intended effects (Burman, *et al* 2011). Still, the following experiments (described in chapter 7) again confirmed our notion, since reactions towards the odour conditioned with the bitter piece were much lower when presented with a normal almond piece (fig 7 ; page 128).

We also tried to improve the test by using a different bitter taste additive, denatonium benzoate (chapter 6). However, this substance appeared not to be suitable at all for the judgement bias test since mice habituated to its taste. The further use of quinine for judgement bias tests in mice thus demands some more methodological studies, since no literature is available on the ability of mice to detect quinine based on other sensory cues than taste. While it is generally assumed that tastants used in experiments are identified and perceived by taste only, a human study has shown that odours of these compounds are used by

some individuals to identify the quality of a taste solution, though this effect for quinine is not very strong in humans (Mojet, *et al* 2005). Thus it may well be possible that the mice were able to smell the quinine, given the very good olfactory capabilities of BALB/c mice (Restivo, *et al* 2006). Another possibility is that the bitter taste was detected by bitter taste receptors that are expressed in the respiratory tract (Behrens and Meyerhof 2010). However, both explanations are contradicted by the fact that we placed bitter pieces as well as normal tasting almond pieces under the lid of the odour cup in the experiments described in chapter 7, thus creating a basal background odour containing quinine. It remains unclear what kind of sense may then be used because it is also unlikely that the mice were able to identify the quality by their visual appearance, i.e. albino BALB/c mice do not have a good visual ability (Brown and Wong 2007; Roullet and Lassalle 1995). This aspect therefore remains subject for further research. Finally, it is important to note that there are some general issues involved in animal judgement bias testing that remain to be solved in further research. Firstly, most experiments described until now assume the involvement of distinct emotional states as subject of investigation. For example, chronic stress and removal of enrichment are assumed to cause a negative affective state (see for example: Bateson and Matheson 2007; Harding, *et al* 2004), while cage enrichment is hypothesized to induce a positive affective state (see for example: Brydges, *et al* 2011; Douglas, et al 2012; Matheson, *et al* 2008; Richter, *et al* 2012). However, to whether indeed the effects of distinct emotional states are being modelled in a judgement bias test and, further, whether results may be of translational value, some validity criteria should to be considered (McKinney Jr. and Bunney Jr. 1969):

1) Face validity refers to the behavioural and physiological responses that are observed in the goal species should be the same in the animal model. Behavioural responses seen in judgement bias tests indeed seem to be similar to behaviour in humans (Anderson, *et al* 2012a) and across species (Mendl, *et al* 2009), but physiological responses have not yet been correlated with the expressed judgement bias behaviour.

2) Predictive validity refers to the sensitivity of the model to pharmacological agents that are also effective in the goal species. Literature shows that this aspect is of increasing interest, for example the effect of anxiolytics and antidepressants are being investigated, such as diazepam in mice (used here in chapter 5), lambs (Destrez, *et al* 2012) and rats (Anderson, *et al* 2012b) and reboxetrine and fluoxetine in rats (Anderson, *et al* 2012b; Enkel, *et al* 2010). Further studies can also be suggested to investigate the effect of anxiogenic drugs and drugs that induce a positive mood.

3) Construct validity includes the underlying mechanisms that cause the observed effects, these should be similar between animal judgement bias tests. The construct validity of an animal model however is difficult to prove, especially when being related to human (psychopathological) behaviour, since the constructs underlying such phenomena are usually the subject of the research. Thus, in fact it is the construct validity itself that constitutes the research question. Still, for example c-Fos data as described in chapter 2 and box 1 suggests that similar brain areas might be involved in mice as in humans. Further research could unravel more of these mechanisms.

Next to the questions of the validity of the judgement bias test it is important to consider that the behavioural outcome that is measured in this test might be influenced by other internal drivers than emotions alone. The drivers aspects of

motivation may play a role for the execution of the behaviour of mice during the test; for example an animal that is satiated might not be motivated to approach an potential food reward at all (Mendl, *et al* 2009), and certain drug treatments can affect motivation as well (Anderson, *et al* 2012b). In addition, often animals are exposed to multiple ambiguous trials in a test session and this procedure might lead to within session learning effects, i.e. the animals may learn that this stimulus is resulting in a certain outcome and as a consequence the stimulus is becoming less ambiguous over time. Finally, several manipulations that are used to induce a positive or negative emotional state are also influencing cognition and may influence the measured responses: for example housing condition before and the state of arousal during the test can affect cognition (Bradley, *et al* 1992; Van Praag, *et al* 2000), and certain pharmacological treatments might affect cognition as well (Beuzen and Belzung 1995).

## TEST IMPROVEMENTS AND SUGGESTIONS FOR FURTHER JUDGEMENT BIAS RESEARCH

Despite the complexity of the tested processes and the methodological problems in testing judgement bias in mice, the experiments so far are providing us with some insight in the emotional perception of animals. Such knowledge is of relevance for both translational purposes and, especially, for considerations on animal welfare. For future studies on judgement bias in mice we would, however, suggest to try and improve the test set-up, for example by removing the potential influence of quinine on the measured responses, perhaps by separating the conditioned odours from the almond pieces (i.e. first presenting the odour cue, for example in a start box, and presenting the almond piece in another part of the test so the mouse can decide to approach or avoid the food item). Another option may be to implement an operant response in the test procedure in order to let the mice actively choose the expected outcome. An advantage of the latter is possibly that a more directly linked cognitive-emotional effect can be observed. However, operant procedures take a longer time for the animals to be trained.

Further, to reduce potentially confounding motivational influences on the behavioural responses assessed in the test a different reward item might be proposed. Almond pieces are not only highly palatable but also have a high caloric value and, thus, mice are highly motivated to obtain the nutrients, which possibly positively biases responses measured in the judgement bias test. Palatable food items with a lower caloric value, such as sugar pellets, might be suitable as well, though it is of relevance that the mice are still willing to work for the reward without being food-restricted since food restriction constitutes a stressor (see (Nakamura, *et al* 1990)).

CONCLUDING REMARKS

In conclusion, our results show that behavioural habituation is a useful measurement in order to differentiate between adaptive behavioural responses and non-adaptive responses. This again may help to assess whether an animal is able to cope with environmental challenge or whether its adaptive capacities are being exceeded by such challenges. We in addition show that the measurement of judgement biases in mice might provide more information on the animals' perception of its own emotional state, although further research needs to be done to support this notion and to refine the testing procedure. Still, first steps have been taken in establishing a method for the assessment of adaptive capacities in mice as well as their perception of emotional states, which may help in gaining a better understanding of the (dys)regulation of emotions in these animals and, thus, in the management of their welfare.
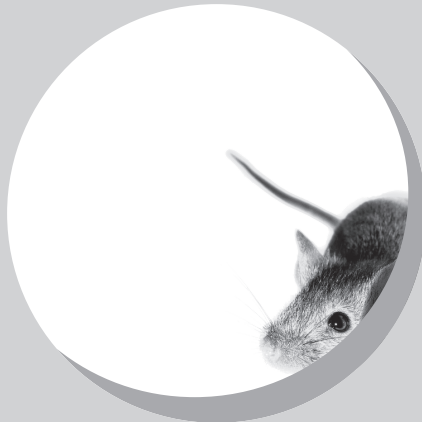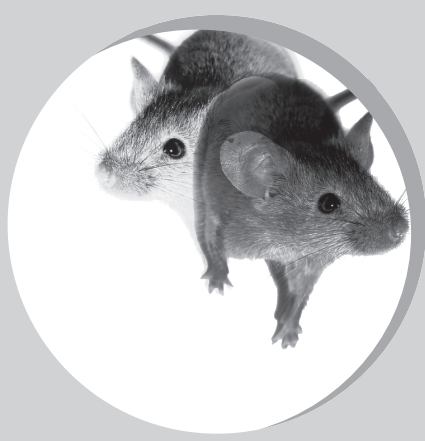
REFERENCES

A

Abramov U, Puussaar T, Raud S, Kurrikoff K, Vasar E (2008). Behavioural differences between C57BL/6 and 129S6/SvEv strains are reinforced by environmental enrichment. Neuroscience Letters 443: 223-227.

Akana SF, Strack AM, Hanson ES, Dallman MF (1994). Regulation of activity in the hypothalamo-pituitary-adrenal axis is integral to a larger hypothalamic system that determines caloric flow. Endocrinology 135: 1125-1134.

Amaral OB, Vargas RS, Hansel G, Izquierdo I, Souza DO (2008). Duration of environmental enrichment influences the magnitude and persistence of iths behavioral effects in mice. Physiology & Behavior 93: 388-394.

Andelt WF, Burnham KP, Baker DL (1994). Effectiveness of capsaicin and bitrex repellents for deterring browsing by captive mule deer. Journal of Wildlife Management 58: 330-334.

Anderson MH, Hardcastle C, Munafò MR, Robinson ESJ (2012a). Evaluation of a novel translational task for assessing emotional biases in different species. Cognitive, Affective and Behavioral Neuroscience 12: 373-381.

Anderson MH, Munafò MR, Robinson ESJ (2012b). Investigating the psychopharmacology of cognitive affective bias in rats using an affective tone discrimination task. Psychopharmacology (Berl) 1-13.

Arborelius L, Owens MJ, Plotsky PM, Nemeroff CB (1999). The role of corticotropin-releasing factor in depression and anxiety disorders. Journal of Endocrinology 160: 1-12.

Archer J and Birke LIA (1983): Some issues and problems in the study of animal exploration. In: J. Archer and L. I. A. Birke (eds). Exploration in animals and humans. Van Nostrand Reinhold: Berkshire, England. pp 1.

Arzt E and Holsboer F (2006). CRF signaling: molecular specificity for drug targeting in the CNS. Trends in Pharmacological Sciences 27: 531-538.

B

Baker M (2011). Animal models: Inside the minds of mice and men. Nature 475: 123-128.

Baranyi J, Bakos N, Haller J (2005). Social instability in female rats: The relationship between stress-related and anxiety-like consequences. Physiology and Behavior 84: 511-518.

Barnard CJ and Hurst JL (1996). Welfare by design: The natural selection of welfare criteria. Animal Welfare 5: 405-433.

Barnett JL and Hemsworth PH (1990). The validity of physiological and behavioural measures of animal welfare. Applied Animal Behaviour Science 25: 177-187.

Bartlang MS, Neumann ID, Slattery DA, Uschold-Schmidt N, Kraus D, Helfrich-Förster C, Reber SO (2012). Time matters: Pathological effects of repeated psychosocial stress during the active, but not inactive, phase of male mice. The Journal of Endocrinology 215: 425-437.

Bartolomucci A, Palanza P, Parmigiani S, Pederzani T, Merlot E, Neveu PJ, Dantzer R (2003). Chronic psychosocial stress down-regulates central cytokines mRNA. Brain Research Bulletin 62: 173-178.

Bartolomucci A, Pederzani T, Sacerdote P, Panerai AE, Parmigiani S, Palanza P (2004). Behavioral and physiological characterization of male mice under chronic psychosocial stress. Psychoneuroendocrinology 29: 899-910.

Bateson M, Brilot B, Nettle D (2011). Anxiety: An evolutionary approach. Canadian Journal of Psychiatry 56: 707-715.

Bateson M and Matheson SM (2007). Performance on a categorisation task suggests that removal of environmental enrichment induces 'pessimism' in captive European starlings (Sturnus vulgaris). Animal Welfare 16: 33-36.

Bateson M, Desire S, Gartside SE, Wright GA (2011). Agitated Honeybees Exhibit Pessimistic Cognitive Biases. Current Biology 21: 1070-1073.

Beck AT, Emery G, Greenberg RL (2005). Anxiety disorders and phobias: A cognitive perspective. Basic Books: New York.

Beeler JA, Mccutcheon JE, Cao ZFH, Murakami M, Alexander E, Roitman MF, Zhuang X (2012). Taste uncoupled from nutrition fails to sustain the reinforcing properties of food. European Journal of Neuroscience 36: 2533-2546.

Behrens M and Meyerhof W (2010). Oral and extraoral bitter taste receptors. Results and Problems in Cell Differentiation 52: 87-99.

Belzung C and Berton F (1997). Further pharmacological validation of the BALB/c neophobia in the free exploratory paradigm as an animal model of trait anxiety. Behavioural Pharmacology 8: 541-548.

Belzung C and Griebel G (2001). Measuring normal and pathological anxiety-like behaviour in mice: a review. Behavioural Brain Research 125: 141-149.

Benus RF, Bohus B, Koolhaas JM, Van Oortmerssen GA (1991). Heritable variation for aggression as a reflection of individual coping strategies. Experientia 47: 1008-1019.

Berger-Tal O and Avgar T (2012). The glass is half-full: Overestimating the quality of a novel environment is advantageous. PLoS ONE 7: e34578. .

Bernard C (1865). Introduction a l'Etude de la Medecine Experimentale (Introduction to the study of experimental medicine).

Berridge KC and Peciña S (1995). Benzodiazepines, appetite, and taste palatability. Neuroscience and Biobehavioral Reviews 19: 121-131.

Bethell EJ, Holmes A, MacLarnon A, Semple S (2012). Cognitive bias in a non-human primate: Husbandry procedures influence cognitive indicators of psychological well-being in captive rhesus macaques. Animal Welfare 21: 185-195.

Beuzen A and Belzung C (1995). Link between emotional memory and anxiety states: A study by principal component analysis. Physiology and Behavior 58: 111-118.

Bishop SJ (2007). Neurocognitive mechanisms of anxiety: an integrative account. Trends in Cognitive Sciences 11: 307-316.

Blanchard CD, Griebel G, Blanchard RJ (2003). Conditioning and residual emotionality effects of predator stimuli: some reflections on stress and emotion. Progress in Neuro-Psychopharmacology & Biological Psychiatry 27: 1177-1185.

Blanchard CD, Hynd AL, Minke KA, Minemoto T, Blanchard RJ (2001). Human defensive behaviors to threat scenarios show parallels to fear- and anxiety-related defense patterns of non-human mammals. Neuroscience and Biobehavioral Reviews 25: 761-770.

Blanchard DC, Griebel G, Blanchard RJ (2003). The Mouse Defense Test Battery: Pharmacological and behavioral assays for anxiety and panic. European Journal of Pharmacology 463: 97-116.

Blanchard DC, Spencer RL, Weiss SM, Blanchard RJ, McEwen B, Sakai RR (1995). Visible burrow system as a model of chronic social stress: Behavioral and neuroendocrine correlates. Psychoneuroendocrinology 20: 117-134.

Blanchard RJ, Yudko EB, John Rodgers R, Caroline Blanchard D (1993). Defense system psychopharmacology: An ethological approach to the pharmacology of fear and anxiety. Behavioural Brain Research 58: 155-165.

Blasi G, Hariri AR, Alce G, Taurisano P, Sambataro F, Das S, Bertolino A, Weinberger DR, Mattay VS (2009). Preferential Amygdala Reactivity to the Negative Assessment of Neutral Faces. Biological Psychiatry 66: 847-853.

Bodyak N and Slotnick B (1999). Performance of mice in an automated olfactometer: Odor detection, discrimination and odor memory. Chemical Senses 24: 637-645.

Bogacz R (2007). Optimal decision-making theories: linking neurobiology with behaviour. Trends in Cognitive Sciences (Regul Ed) 11: 118-125.

Boissy A (1995). Fear and fearfulness in animals. Quarterly Review of Biology 70: 165-191.

Boissy A, Arnould C, Chaillou E, Désiré L, Duvaux-Ponter C, Greiveldinger L, Leterrier C, Richard S, Roussel S, Saint-Dizier H, Meunier-Salaün MC, Valance D, Veissier I (2007a). Emotions and cognition: A new approach to animal welfare. Animal Welfare 16: 37-43.

Boissy A, Manteuffel G, Jensen MB, Moe RO, Spruijt B, Keeling LJ, Winckler C, Forkman B, Dimitrov I, Langbein J, Bakken M, Veissier I, Aubert A (2007b). Assessment of positive emotions in animals to improve their welfare. Physiology and Behavior 92: 375-397.

Boleij H, Salomons AR, van Sprundel M, Arndt SS, Ohl F (2012a). Not all mice are equal: welfare implications of behavioural habituation profiles in four 129 mouse substrains. PLoS One 7: e42544.

Boleij H, van't Klooster J, Lavrijsen M, Kirchhoff S, Arndt SS, Ohl F (2012b). A test to identify judgement bias in mice. Behavioural Brain Research 233: 45-54.

Bolivar VJ (2009). Intrasession and intersession habituation in mice: From inbred strain variability to linkage analysis. Neurobiology of Learning and Memory 92: 206-214.

Bothe GWM, Bolivar VJ, Vedder MJ, Geistfeld JG (2005). Behavioral differences among fourteen inbred mouse strains commonly used as disease models. Comparative Medicine 55: 326-334.

Bothe GWM, Bolivar VJ, Vedder MJ, Geistfeld JG (2004). Genetic and behavioral differences among five inbred mouse strains commonly used in the production of transgenic and knockout mice. Genes, Brain and Behavior 3: 149-157.

Boughter Jr. JD, Raghow S, Nelson TM, Munger SD (2005). Inbred mouse strains C57BL/6J and DBA/2J vary in sensitivity to a subset of bitter stimuli. BMC Genetics 6: .

Boughter Jr. JD and Whitney G (1997). Behavioral specificity of the bitter taste gene soa. Physiology and Behavior 63: 101-108.

Boughter JD, Harder DB, Capeless CG, G.Whitney (1992). Polygenic determination of quinine aversion among mice. Chemical Senses 17: 427-434.

Bracke MBM and Hopster H (2006). Assessing the importance of natural behavior for animal welfare. Journal of Agricultural and Environmental Ethics 19: 77-89.

Bradley MM, Greenwald MK, Petry MC, Lang PJ (1992). Remembering Pictures: Pleasure and Arousal in Memory. Journal of Experimental Psychology: Learning, Memory, and Cognition 18: 379-390.

Brambell FWR (1965). Report of the Technical Committee to Enquire into the Welfare of Animals kept under Intensive Livestock Husbandry Systems.

Brasser SM, Mozhui K, Smith DV (2005). Differential covariation in taste responsiveness to bitter stimuli in rats. Chemical Senses 30: 793-799.

Brilot BO, Asher L, Bateson M (2010). Stereotyping starlings are more 'pessimistic'. Animal Cognition 13: 721-731.

Brilot BO, Normandale CL, Parkin A, Bateson M (2009). Can we use starlings' aversion to eyespots as the basis for a novel`cognitive bias' task? Applied Animal Behaviour Science 118: 182-190.

Brinks V, van der Mark M, de Kloet R, Oitzl M (2007). Emotion and cognition in high and low stress sensitive mouse strains: a combined neuroendocrine and behavioral study in BALB/c and C57BL/6J mice. Frontiers in Behavioral Neuroscience 1: 8.

Britton DR and Britton KT (1981). A sensitive open field measure of anxiolytic drug activity. Pharmacology Biochemistry and Behavior 15: 577-582.

Brown RE and Wong AA (2007). The influence of visual ability on learning and memory performance in 13 strains of mice. Learning and Memory 14: 134-144.

Brunelli SA and Hofer MA (2007). Selective breeding for infant rat separation-induced ultrasonic vocalizations: Developmental precursors of passive and active coping styles. Behav Brain Res 182: 193-207.

Brydges NM, Leach M, Nicol K, Wright R, Bateson M (2011). Environmental enrichment induces optimistic cognitive bias in rats. Animal Behaviour 81: 169-175.

Burman OHP, Parker R, Paul ES, Mendl M (2008). A spatial judgement task to determine background emotional state in laboratory rats, Rattus norvegicus. Animal Behaviour 76: 801-809.

Burman OHP, Parker RMA, Paul ES, Mendl MT (2009). Anxiety-induced cognitive bias in non-human animals. Physiology and Behavior 98: 345-350.

Burman O, McGowan R, Mendl M, Norling Y, Paul E, Rehn T, Keeling L (2011). Using judgement bias to measure positive affective state in dogs. Appl Anim Behav Sci 132: 160-168.

C

Cahill L and McGaugh JL (1996). Modulation of memory storage. Current Opinion in Neurobiology 6: 237-242.

Caldji C, Diorio J, Anismam H, Meaney MJ (2004). Maternal behavior regulates benzodiazepine/GABAA receptor subunit expression in brain regions associated with fear in BALBC/c and C57BL/6 mice. Neuropsychopharmacology 29: 1344-1352.

Camp M, Norcross M, Whittle N, Feyder M, D'Hanis W, Yilmazer-Hanke D, Singewald N, Holmes A (2009). Impaired Pavlovian fear extinction is a common phenotype across genetic lineages of the 129 inbred mouse strain. Genes, Brain and Behavior 8: 744-752.

Capeless CG, Boughter JD, Whitney G (1994). Hydrolysis of sucrose octa-acetate: Qualitative differences in taster and demistaster avoidance phenotypes. Chemical Senses 19: 595-607.

Casellas J (2011). Inbred mouse strains and genetic stability: A review. Animal 5: 1-7.

Chan CKY and Lovibond PF (1996). Expectancy Bias in Trait Anxiety, Journal of Abnormal Psychology 105: 637-647.

Chapillon P, Manneché C, Belzung C, Caston J (1999). Rearing environmental enrichment in two inbred strains of mice: 1. effects on emotional reactivity. Behavior Genetics 29: .

Chesler EJ, Wilson SG, Lariviere WR, Rodriguez-Zas SL, Mogil JS (2002). Identification and ranking of genetic and laboratory environment factors influencing a behavioral trait, thermal nociception, via computational analysis of a large data archive. Neuroscience and Biobehavioral Reviews 26: 907-923.

Clément Y, Calatayud F, Belzung C (2002). Genetic basis of anxiety-like behaviour: A critical review. Brain Research Bulletin 57: 57-71.

Clément Y, Guisquet AML, Venault P, Chapouthier G, Belzung C (2009). Pharmacological alterations of anxious behaviour in mice depending on both strain and the behavioural situation. PLoS ONE 4: e7745.

Conover WJ and Iman RL (1982). Analysis of covariance using the rank transformation. Biometrics 38: 715-724.

Cook MN, Bolivar VJ, McFadyen MP, Flaherty L (2002). Behavioral differences among 129 substrains: Implications for knockout and transgenic mice. Behavioral Neuroscience 116: 600-611.

Cosquer B, Kuster N, Cassel J- (2005). Whole-body exposure to 2.45 GHz electromagnetic fields does not alter 12-arm radial-maze with reduced access to spatial cues in rats. Behavioural Brain Research 161: 331-334.

Cowan PE (1983): Exploration in small mammals: ethology and ecology. In: J. Archer and L. I. A. Birke (eds). Exploration in animals and humans. Van Nostrand Reinhold: Berkshire, England. pp 147.

Crabbe JC, Wahlsten D, Dudek BC (1999). Genetics of mouse behavior: Interactions with laboratory environment. Science 284: 1670-1672.

Crusio WE (2001). Genetic dissection of mouse exploratory behaviour. Behavioural Brain Research 125: 127-132.

Cruz APM, Frei F, Graeff FG (1994). Ethopharmacological analysis of rat behavior on the elevated plus-maze. Pharmacology Biochemistry and Behavior 49: 171-176.

D

Dahl M, Erickson RP, Simon SA (1997). Neural responses to bitter compounds in rats. Brain Research 756: 22-34.

Damak S, Rong M, Yasumatsu K, Kokrashvili Z, Pérez CA, Shigemura N, Yoshida R, Mosinger Jr. B, Glendinning JI, Ninomiya Y, Margolskee RF (2006). Trpm5 null mice respond to bitter, sweet, and umami compounds. Chemical Senses 31: 253-264.

Davis M and Whalen PJ (2001). The amygdala: Vigilance and emotion. Molecular Psychiatry 6: 13-34.

Dawkins MS (2008). The science of animal suffering. Ethology 114: 937-945.

Dawkins MS (2006). A user's guide to animal welfare science. Trends in Ecology and Evolution 21: 77-82.

De Boer SF, Van der Vegt BJ, Koolhaas JM (2003). Individual variation in aggression of feral rodent strains: A standard for the genetics of aggression and violence? Behavior Genetics 33: 485-501.

De Kloet ER, Joëls M, Holsboer F (2005). Stress and the brain: From adaptation to disease. Nature Reviews Neuroscience 6: 463-475.

De Kloet ER, Vreugdenhil E, Oitzl MS, Joëls M (1998). Brain corticosteroid receptor balance in health and disease. Endocrine Reviews 19: 269-301.

De Souza EB (1990). Neuroendocrine effects of benzodiazepines. Journal of Psychiatric Reseach 24: 111-119.

De Visser L, Van Den Bos R, Kuurman WW, Kas MJH, Spruijt BM (2006). Novel approach to the behavioural characterization of inbred mice: Automated home cage observations. Genes, Brain and Behavior 5: 458-466.

Debus G and Janke W (1980). Methods and methodological considerations in measuring anti-anxiety effects of tranquilizing drugs. Progress in Neuro-psychopharmacology 4: 391-404.

Destrez A, Deiss V, Belzung C, Lee C, Boissy A (2012). Does reduction of fearfulness tend to reduce pessimistic-like judgment in lambs? Applied Animal Behaviour Science 139: 233-241.

Dirks A, Fish EW, Kikusui T, Gugten Jvd, Groenink L, Olivier B, Miczek KA (2002). Effects of corticotropin-releasing hormone on distress vocalizations and locomotion in maternally separated mouse pups. Pharmacology, Biochemistry and Behavior 72: 993-999.

Dotson CD, Roper SD, Spector AC (2005). PLCβ2-independent behavioral avoidance of prototypical bitter-tasting ligands. Chemical Senses 30: 593-600.

Douglas C, Bateson M, Walsh C, Bédué A, Edwards SA (2012). Environmental enrichment induces optimistic cognitive biases in pigs. Applied Animal Behaviour Science 139: 65-73.

Doyle RE, Hinch GN, Fisher AD, Boissy A, Henshall JM, Lee C (2011). Administration of serotonin inhibitor p-Chlorophenylalanine induces pessimistic-like judgement bias in sheep. Psychoneuroendocrinology 36: 279-288.

Drugan RC, Skolnick P, Paul SM, Crawley JN (1986). Low doses of muscimol produce anticonflict actions in the lateral septum of the rat. Neuropharmacology 25: 203-205.

Ducottet C, Aubert A, Belzung C (2004). Susceptibility to subchronic unpredictable stress is related to individual reactivity to threat stimuli in mice. Behavioural Brain Research 115: 291-299.

Ducottet C and Belzung C (2004). Behaviour in the elevated plus-maze predicts coping after subchronic mild stress in mice. Physiology and Behaviour 81: 417-426.

Ducottet C, Griebel G, Belzung C (2003). Effects of the selective nonpeptide corticotropin-releasing factor receptor 1 antagonist antalarmin in the chronic mild stress model of depression in mice. Prog Neuropsychopharmacol Biol Psychiatry 27: 625-631.

Duncan IJH (1993). Welfare is to do with what animals feel. Journal of Agricultural and Environmental Ethics 6: 8-14.

Dwyer DM (2011). Lesions of the basolateral, but not central, amygdala impair flavour-taste learning based on fructose or quinine reinforcers. Behavioural Brain Research 220: 349-353.

E

Eisenstein EM and Eisenstein D (2006). A behavioral homeostasis theory of habituation and sensitization: II. Further developments and predictions. Reviews in the Neurosciences 17: 533-557.

Enkel T, Gholizadeh D, Von Bohlen Und Halbach O, Sanchis-Segura C, Hurlemann R, Spanagel R, Gass P, Vollmayr B (2010). Ambiguous-cue interpretation is biased under stress-and depression-like states in rats. Neuropsychopharmacology 35: 1008-1015.

Ennaceur A (2011). Omission of the habituation procedure in the acquisition of a working memory task - evidence from Balb/c, C57/BL6J, and CD-1 mice. Behavioural Brain Research 223: 203-210.

Erhardt A, Müller MB, Rödel A, Welt T, Ohl F, Holsboer F, Keck ME (2009). Consequences of chronic social stress on behaviour and vasopressin gene expression in the PVN of DBA/2OlaHsd mice-influence of treatment with the CRHR1-antagonist R121919/NBI 30775. Journal of Psychopharmacology 23: 31-39.

Erickson K and Schulkin J (2003). Facial expressions of emotion: A cognitive neuroscience perspective. Brain and Cognition 52: 52-60.

Etkin A (2010). Functional neuroanatomy of anxiety: A neural circuit perspective. Current Topics in Behavioral Neurosciences 2010: 251-277.

Everitt BJ, Cardinal RN, Parkinson JA, Robbins TW (2003). Appetitive behavior: Impact of amygdala-dependent mechanisms of emotional learning. Annals of the New York Academy of Sciences 985: 233-250.

Eysenck MW, Mogg K, May J, Richards A, Mathews A (1991). Bias in Interpretation of Ambiguous Sentences Related to Threat in Anxiety. Journal of Abnormal Psychology 100: 144-150.

F

Fernández-Teruel A, Escorihuela RM, Castellano B, González B, Tobeña A (1997). Neonatal handling and environmental enrichment effects on emotionality, novelty/reward seeking, and age-related cognitive and hippocampal impairments: Focus on the roman rat lines. Behavior Genetics 27: 513-526.

File SE and Wardill AG (1975). Validity of head dipping as a measure of exploration in a modified hole board. Psychopharmacologia 44: 53-59.

Frank ME, Bouverat BP, MacKinnon BI, Hettinger TP (2004). The distinctiveness of ionic and nonionic bitter stimuli. Physiology and Behavior 80: 421-431.

Franklin KGB and Paxinos G (1997): The mouse brain in stereotactic coordinates. Academic press.

Fraser D and Duncan IJH (1998). 'Pleasures', 'pains' and animal welfare: Toward a natural history of affect. Animal Welfare 7: 383-396.

G

Gamallo A, Villanua A, Trancho G, Fraile A (1986). Stress adaptation and adrenal activity in isolated and crowded rats. Physiology and Behavior 36: 217-221.

Garcia AMB, Cardenas FP, Morato S (2005). Effect of different illumination levels on rat behavior in the elevated plus-maze. Physiology and Behavior 85: 265-270.

Geran LC and Travers SP (2011). Glossopharyngeal nerve transection impairs unconditioned avoidance of diverse bitter stimuli in rats. Behavioral Neuroscience 125: 519-528.

Glendinning JI (1994). Is the bitter rejection response always adaptive? Physiology and Behavior 56: 1217-1227.

Glendinning JI, Yiin Y, Ackroff K, Sclafani A (2008). Intragastric infusion of denatonium conditions flavor aversions and delays gastric emptying in rodents. Physiology and Behavior 93: 757-765.

Goldman-Rakic PS (1995). Architecture of the prefrontal cortex and the central executive. Annals of the New York Academy of Sciences 769: 71-83.

Gorka Z, Moryl E, Papp M (1996). Effect of chronic mild stress on circadian rhythms in the locomotor activity in rats. Pharmacology Biochemistry and Behavior 54: 229-234.

Goto SH, Conceição IM, Ribeiro RA, Frussa-Filho R (1993). Comparison of anxiety measured in the elevated plus-maze, open-field and social interaction tests between spontaneously hypertensive rats and Wistar EPM-1 rats. Brazilian Journal of Medical and Biological Research 26: 965-969.

Gray JA (1982): The neuropsychology of anxiety. Oxford University Press New York Clarendon Press Oxford.

Gray JA and McNaughton N (1983). Comparison between the behavioural effects of septal and hippocampal lesions: A review. Neuroscience and Biobehavioral Reviews 7: 119-188.

Griebel G, Belzung C, Misslin R, Vogel E (1993). The free-exploratory paradigm: An effective method for measuring neophobic behaviour in mice and testing potential neophobia-reducing drugs. Behavioural Pharmacology 4: 637-644.

Griebel G, Belzung C, Perrault G, Sanger DJ (2000). Differences in anxiety-related behaviours and in sensitivity to diazepam in inbred and outbred strains of mice. Psychopharmacology (Berl) 148: 164-170.

Griebel G, Simiand J, Steinberg R, Jung M, Gully D, Roger P, Geslin M, Scatton B, Maffrand J-, Soubrié P (2002). 4-(2-chloro-4-methoxy-5-methylphenyl)-N-[(1S)-2-cyclopropyl-1- (3-fluoro-4-methylphenyl) ethyl]5-methyl-N-(2-propynyl)-1, 3-thiazol-2-amine hydrochloride (SSR125543A), a potent and selective corticotrophin-releasing factor 1 receptor antagonist. II. Characterization in rodent models of stress-related disorders. The Journal of pharmacology and experimental therapeutics 301: 333-345.

Grill HJ and Norgren R (1978). The taste reactivity test. I. Mimetic responses to gustatory stimuli in neurologically normal rats. Brain Research 143: 263-279.

Groenewegen HJ, Wright CI, Uylings HB (1997). The anatomical relationships of the prefrontal cortex with limbic structures and the basal ganglia. Journal of Psychopharmacology 11: 99-106.

Gross AN, Engel AKJ, Würbel H (2011). Simply a nest? Effects of different enrichments on stereotypic and anxiety-related behaviour in mice. Applied Animal Behaviour Science 134: 239-245.

Gross JJ (1999). Emotion regulation: Past, present, future. Cognition and Emotion 13: 551-573.

H

Hall CS (1934). Emotional behavior in the rat. I. Defecation and urination as measures of individual differences in emotionality. Journal of Comparative Psychology 18: 385-403.

Haller J, Fuchs E, Halász J, Makara GB (1999). Defeat is a major stressor in males while social instability is stressful mainly in females: towards the development of a social stress model in female rats. Brain Research Bulletin 50: 33-39.

HHallock RM, Tatangelo M, Barrows J, Finger TE (2009). Residual chemosensory capabilities in double P2X2/P2X3 purinergic receptor null mice: intraoral or postingestive detection? Chemical Senses 34: 799-808.

Hamann SB, Ely TD, Grafton ST, Kilts CD (1999). Amygdala activity related to enhanced memory for pleasant and aversive stimuli. Nature Neuroscience 2: 289-293.

Harding EJ, Paul ES, Mendl M (2004). Animal behaviour: cognitive bias and affective state. Nature 427: 312.

Hefner K, Whittle N, Juhasz J, Norcross M, Karlsson R-, Saksida LM, Bussey TJ, Singewald N, Holmes A (2008). Impaired fear extinction learning and cortico-amygdala circuit abnormalities in a common genetic mouse strain. Journal of Neuroscience 28: 8074-8085.

Herry C, Bach DR, Esposito F, Di Salle F, Perrig WJ, Scheffler K, Lüthi A, Seifritz E (2007). Processing of temporal unpredictability in human and animal amygdala. Journal of Neuroscience 27: 5958-5966.

Hesen W, Karst H, Meijer O, Cole TJ, Schmid W, De Kloet ER, Schütz G, Joëls M (1996). Hippocampal cell responses in mice with a targeted glucocorticoid receptor gene disruption. Journal of Neuroscience 16: 6766-6774.

Hess US, Gall CM, Granger R, Lynch G (1997). Differential patterns of c-fos mRNA expression in amygdala during successive stages of odor discrimination learning. Learning and Memory 4: 262-283.

Hettema JM, Neale MC, Kendler KS (2001). A review and meta-analysis of the genetic epidemiology of anxiety disorders. American Journal of Psychiatry 158: 1568-1578.

Hogg S (1996). A review of the validity and variability of the elevated plus-maze as an animal model of anxiety. Pharmacology Biochemistry and Behavior 54: 21-30.

Holahan MR and White NM (2004). Amygdala c-Fos induction corresponds to unconditioned and conditioned aversive stimuli but not to freezing. Behavioural Brain Research 152: 109-120.

Holmes A, le Guisquet AM, Vogel E, Millstein RA, Leman S, Belzung C (2005). Early life genetic, epigenetic and environmental factors shaping emotionality in rodents. Neuroscience and Biobehavioral Reviews 29: 1335-1346.

Hsu M, Bhatt M, Adolphs R, Tranel D, Camerer CF (2005). Neuroscience: Neural systems responding to degrees of uncertainty in human decision-making. Science 310: 1680-1683.

Hymel KA and Sufka KJ (2012). Pharmacological reversal of cognitive bias in the chick anxiety-depression model. Neuropharmacology 62: 161-166.

J

Jensen P (2010). Domestication, selection, behaviour and welfare of animals - Genetic mechanisms for rapid responses. Animal Welfare 19: 7-9.

K

Kafkafi N, Benjamini Y, Sakov A, Elmer GI, Golani I (2005). Genotype-environment interactions in mouse behavior: A way out of the problem. Proceedings of the National Academy of Sciences U S A 102: 4619-4624.

Kalueff AV and Tuohimaa P (2004). Grooming analysis algorithm for neurobehavioural stress research. Brain Research Protocols 13: 151-158.

Kas MJH and Van Ree JM (2004). Dissecting complex behaviours in the post-genomic era. Trends in Neurosciences 27: 366-369.

Kendler KS, Prescott CA, Myers J, Neale MC (2003). The structure of genetic and environmental risk factors for common psychiatric and substance use disorders in men and women. Archives General Psychiatry 60: 929-937.

Kiefer SW, Hill KG, Kaczmarek HJ (1998). Taste reactivity to alcohol and basic tastes in outbred mice. Alcoholism: Clinical and Experimental Research 22: 1146-1151.

Kim D, Chae S, Lee J, Yang H, Shin HS (2005). Variations in the behaviors to novel objects among five inbred strains of mice. Genes Brain and Behavior 4: 302-306.

Kirk IJ (1998). Frequency modulation of hippocampal theta by the supramammillary nucleus, and other hypothalamo-hippocampal interactions: Mechanisms and functional implications. Neuroscience and Biobehavioral Reviews 22: 291-302.

Kleinkauf A, Macdonald DW, Tattersall FH (1999). A bitter attempt to prevent non-target poisoning of small mammals. Mammal Review 29: 201-204.

Knierim U and Winckler C (2009). On-farm welfare assessment in cattle: Validity, reliability and feasibility issues and future perspectives with special regard to the Welfare Quality® approach. Animal Welfare 18: 451-458.

Koob GF and Le Moal M (2001). Drug addiction, dysregulation of reward, and allostasis. Neuropsychopharmacology 24: 97-129.

Koolhaas JM, Bartolomucci A, Buwalda B, de Boer SF, Flügge G, Korte SM, Meerlo P, Murison R, Olivier B, Palanza P, Richter-Levin G, Sgoifo A, Steimer T, Stiedl O, van Dijk G, Wöhr M, Fuchs E (2011). Stress revisited: A critical evaluation of the stress concept. Neuroscience and Biobehavioral Reviews 35: 1291-1301.

Koolhaas JM, Korte SM, De Boer SF, Van Der Vegt BJ, Van Reenen CG, Hopster H, De Jong IC, Ruis MA, Blokhuis HJ (1999). Coping styles in animals: current status in behavior and stress-physiology. Neuroscience and Biobehavioral Reviews 23: 925-935.

Koolhaas JM, Meerlo P, De Boer SF, Strubbe JH, Bohus B (1997). The temporal dynamics of the stress response. Neuroscience and Biobehavioral Reviews 21: 775-782.

Koot S, Zoratto F, Cassano T, Colangeli R, Laviola G, Van Den Bos R, Adriani W (2012). Compromised decision-making and increased gambling proneness following dietary serotonin depletion in rats. Neuropharmacology 62: 1640-1650.

Kopp C, Vogel E, Misslin R (1999). Comparative study of emotional behaviour in three inbred strains of mice. Behavioural Processes 47: 161-174.

Korte SM (2001). Corticosteroids in relation to fear, anxiety and psychopathology. Neuroscience and Biobehavioral Reviews 25: 117-142.

Korte SM, Koolhaas JM, Wingfield JC, McEwen BS (2005). The Darwinian concept of stress: Benefits of allostasis and costs of allostatic load and the trade-offs in health and disease. Neurosci Biobehav Rev 29: 3-38.

Korte SM, Olivier B, Koolhaas JM (2007). A new animal welfare concept based on allostasis. Physiology and Behavior 92: 422-428.

Kovács KJ (2008). Measurement of immediate-early gene activation- c-fos and beyond. Journal of Neuroendocrinology 20: 665-672.

Kuipers SD, Trentani A, Den Boer JA, Ter Horst GJ (2003). Molecular correlates of impaired prefrontal plasticity in response to chronic stress. Journal of Neurochemistry 85: 1312-1323.

L

La Mela I, Latagliata EC, Patrono E, Puglisi-Allegra S, Ventura R (2010). Olfactory priming reinstates extinguished chocolate-induced conditioned place preference. Appetite 54: 237-240.

Laarakker MC, Ohl F, Van Lith HA (2008). Chromosomal assignment of quantitative trait loci influencing modified hole board behavior in laboratory mice using consomic strains, with special reference to anxiety-related behavior and mouse chromosome 19. Behavior Genetics 38: 159-184.

Landgraf R and Wigger A (2002). High vs low anxiety-related behavior rats: An animal model of extremes in trait anxiety. Behavior Genetics 32: 301-314.

Lazarus RS (1982). Thoughts on the relations between emotion and cognition. The American Psychologist 37: 1019-1024.

LeDoux J (2003). The emotional brain, fear, and the amygdala. Cellular and Molecular Neurobiology 23: 727-738.

Lee AW, Emsley JG, Brown RE, Hagg T (2003). Marked differences in olfactory sensitivity and apparent speed of forebrain neuroblast migration in three inbred strains of mice. Neuroscience 118: 263-270.

Lee C and Rodgers RJ (1990). Antinociceptive effects of elevated plus-maze exposure: influence of opiate receptor manipulations. Psychopharmacology (Berl) 102: 507-513.

Leonard BE (2007). HPA and immune axes in stress: Involvement of the serotonergic system. NeuroImmunoModulation 13: 268-276.

Leonardo ED and Hen R (2008). Anxiety as a developmental disorder. Neuropsychopharmacology 33: 134-140.

Lister RG (1985). The amnesic actions of benzodiazepines in man. Neuroscience and Biobehavioral Reviews 9: 87-94.

Lister RG (1990). Ethologically-based animal models of anxiety disorders. Pharmacology and Therapeutics 46: 321-340.

Livesey PJ (1986): Learning and Emotion: a biological synthesis. Lawrence Erlbaum Associates, publishers: Hillsdale, New Jersey.

Luckman SM, Dyball REJ, Leng G (1994). Induction of c-fos expression in hypothalamic magnocellular neurons requires synaptic activation and not simply increased spike activity. Journal of Neuroscience 14: 4825-4830.

Ludbrook J (1991). On making multiple comparisons in clinical and experimental pharmacology and physiology. Clinical and Experimental Pharmacology and Physiology 18: 379-392.

Lush IE (1984). The genetics of tasting in mice III. Quinine. Genetical Research, Cambridge 44: 151-160.

MacLeod AK, Tata P, Kentish J, Jacobsen H (1997). Retrospective and Prospective Cognitions in Anxiety and Depression. Cognition and Emotion 11: 467-479.

M

MacLeod AK and Byrne A (1996). Anxiety, Depression, and the Anticipation of Future Positive and Negative Experiences. Journal of Abnormal Psychology 105: 286-289.

Makino J, Kato K, Maes FW (1991). Temporal structure of open-field behavior in inbred strains of mice. The Japanese Psychological Reserch 33: 145-152.

Marquis J, Killcross S, Haddon JE (2007). Inactivation of the prelimbic, but not infralimbic, prefrontal cortex impairs the contextual control of response conflict in rats. European Journal of Neuroscience 25: 559-566.

Marti O, Gavalda A, Gomez F, Armario A (1994). Direct evidence for chronic stress-induced facilitation of the adrenocorticotropin response to a novel acute stressor. Neuroendocrinology 60: 1-7.

Maslova LN, Bulygina VV, Amstislavskaya TG (2010). Prolonged social isolation and social instability in adolescence in rats: Immediate and long-term physiological and behavioral effects. Neuroscience and Behavioral Physiology 40: 955-963.

Mason G, Clubb R, Latham N, Vickery S (2007). Why and how should we use environmental enrichment to tackle stereotypic behaviour? Applied Animal Behaviour Science 102: 163-188.

Matheson SM, Asher L, Bateson M (2008). Larger, enriched cages are associated with `optimistic' response biases in captive European starlings (Sturnus vulgaris). Applied Animal Behaviour Science 109: 374-383.

Mathews A and Mackintosh B (1998). A cognitive model of selective processing in anxiety. Cognitive Therapy and Research 22: 539-560.

Mathews A, Mackintosh B, Fulcher EP (1997). Cognitive biases in anxiety and attention to threat. Trends in Cognitive Sciences (Regul Ed) 1: 340-345.

Mathews A and MacLeod C (1994). Cognitive approaches to emotion and emotional disorders. Annual Review of Psychology. 45: 25-50.

Mathews A, Richards A, Eysenck M (1989). Interpretation of Homophones Related to Threat in Anxiety States. Journal of Abnormal Psychology 98: 31-34.

McDonald AJ, Mascagni F, Guo L (1996). Projections of the medial and lateral prefrontal cortices to the amygdala: a Phaseolus vulgaris leucoagglutinin study in the rat. Neuroscience 71: 55-75.

McEwen (2008). Understanding the potency of stressful early life experiences on brain and body function. Metabolism Clinical and Experimental 57: S11-S15.

McEwen BS (2007). Physiology and neurobiology of stress and adaptation: Central role of the brain. Physiological Reviews 87: 873-904.

McEwen BS and Wingfield JC (2003). The concept of allostasis in biology and biomedicine. Hormones and Behavior 43: 2-15.

McKinney Jr. WT and Bunney Jr. WE (1969). Animal model of depression. I. Review of evidence: implications for research. Archives of General Psychiatry 21: 240-248.

McNaughton N (1997). Cognitive dysfunction resulting from hippocampal hyperactivity - A possible cause of anxiety disorder? Pharmacology Biochemistry and Behavior 56: 603-611.

McNaughton N and Gray JA (2000). Anxiolytic action on the behavioural inhibition system implies multiple types of arousal contribute to anxiety. Journal of Affective Disorders 61: 161-176.

Mendl M, Brooks J, Basse C, Burman O, Paul E, Blackwell E, Casey R (2010a). Dogs showing separation-related behaviour exhibit a 'pessimistic' cognitive bias. Current Biology 20: R839-R840.

Mendl M, Burman OHP, Paul ES (2010b). An integrative and functional framework for the study of animal emotion and mood. Proceedings of the Royal Society B: Biological Sciences 277: 2895-2904.

Mendl M, Burman OHP, Parker RMA, Paul ES (2009). Cognitive bias as an indicator of animal emotion and welfare: Emerging evidence and underlying mechanisms. Applied Animal Behaviour Science Special Issue: Animal Suffering and Welfare 118: 161-181.

Merali Z, Levac C, Anisman H (2003). Validation of a simple, ethologically relevant paradigm for assessing anxiety in mice. Biological Psychiatry 54: 552-565.

Meyer U, Van Kampen M, Isovich E, Flügge G, Fuchs E (2001). Chronic psychological stress regulates the expression of both GR and MR mRNA in the hippocampal formation of tree shrews. Hippocampus 11: 329-336.

Millan MJ (2003). The neurobiology and control of anxious states. Progress in Neurobiology 70: 83-244.

Mills DS (2008). Recognising the nature of problem behaviour. Veterinary Journal 176: 127-128.

Mineur YS, Prasol DJ, Belzung C, Crusio WE (2003). Agonistic behavior and unpredictable chronic mild stress in mice. Behavior Genetics 33: 513-519.

Mineur YS, Belzung C, Crusio WE (2006). Effects of unpredictable chronic mild stress on anxiety and depression-like behavior in mice. Behavioural Brain Research 175: 43-50.

Misslin R and Cigrang M (1986). Does neophobia necessarily imply fear or anxiety? Behavioural Processes 12: 45-50.

Misslin R, Herzog F, Koch B, Ropartz P (1982). Effects of isolation, handling and novelty on the pituitary-adrenal response in the mouse. Psychoneuroendocrinology 7: 217-221.

Mogg K and Bradley BP (1998). A cognitive-motivational analysis of anxiety. Behaviour Research and Therapy 36: 809-848.

Mojet J, Köster EP, Prinz JF (2005). Do tastants have a smell? Chemical Senses 30: 9-21.

Monosevitz M (1970). Early environmental enrichment and mouse behavior. Journal of comparative and physiological psychology 71: 459-466.

Montkowski A, Poettig M, Mederer A, Holsboer F (1997). Behavioural performance in three substrains of mouse strain 129. Brain Research 762: 12-18.

Muigg P, Hoelzl U, Palfrader K, Neumann I, Wigger A, Landgraf R, Singewald N (2007). Altered Brain Activation Pattern Associated With Drug-Induced Attenuation of Enhanced Depression-Like Behavior in Rats Bred for High Anxiety. Biological Psychiatry 61: 782-796.

Muigg P, Scheiber S, Salchner P, Bunck M, Landgraf R, Singewald N (2009). Differential Stress-Induced Neuronal Activation Patterns in Mouse Lines Selectively Bred for High, Normal or Low Anxiety. PLoS ONE 4: e5346.

Müller CA, Riemer S, Rosam CM, Schößwender J, Range F, Huber L (2012a). Brief owner absence does not induce negative judgement bias in pet dogs. Animal Cognition 1-5.

Müller CA, Riemer S, Rosam CM, Schößwender J, Range F, Huber L (2012b). Brief owner absence does not induce negative judgement bias in pet dogs. Animal Cognition 1-5.

N

Nakamura K, Aoike A, Hosokawa T, Rokutan K, Koyama K, Nishi Yoshida YA, Kawai K (1990). Effect of food-restriction stress on immune response in mice. Journal of Neuroimmunology 30: 23-29.

Nash JR and Nutt DJ (2005): Pharmacotherapy of anxiety. In: F. Holsboer and A. Ströhle (eds). Anxiety and Anxiolytic Drugs. Springer-Verlag: Berlin Heidelberg, Germany. pp 469.

National Research Council (2010): Guide for the care and use of laboratory animals. National Academies Press: Washington, DC.

Nguyen NK, Keck ME, Hetzenauer A, Thoeringer CK, Wurst W, Deussing JM, Holsboer F, Müller MB, Singewald N (2006). Conditional CRF receptor 1 knockout mice show altered neuronal activation pattern to mild anxiogenic challenge. Psychopharmacology (Berl) 188: 374-385.

Nordquist RE, Pennartz CMA, Uylings HBM, Joosten RNJMA, Jonker AJ, Groenewegen HJ, Voorn P (2003). C-fos activation patterns in rat prefrontal cortex during acquisition of a cued classical conditioning task. Behavioural Brain Research 146: 65-75.

Nygren TE, Isen AM, Taylor PJ, Dulin J (1996). The influence of positive affect on the decision rule in risk situations: Focus on outcome (and especially avoidance of loss) rather than probability. Organizational behavior and human decision processes 66: 59-72.

O

Öbrink KJ, Rehbinder C, Waller M (2000). Animal definition: A necessity for the validity of animal experiments? Lab Animal 34: 121-130.

Ohl F Animal models of anxiety. Handbook of experimental pharmacology: 35-69

Ohl F, Arndt SS, van der Staay FJ (2008). Pathological anxiety in animals. Veterinary Journal 175: 18-26.

Ohl F, Holsboer F, Landgraf R (2001). The modified hole board as a differential screen for behavior in rodents. Behavior Research Methods 33: 392-397.

Ohl F, Roedel A, Binder E, Holsboer F (2003). Impact of high and low anxiety on cognitive performance in a modified hole board test in C57BL/6 and DBA/2 mice. European Journal of Neuroscience 17: 128-136.

Ohl F, Roedel A, Storch C, Holsboer F, Landgraf R (2002). Cognitive performance in rats differing in their inborn anxiety. Behavioral Neuroscience 116: 464-471.

Ohl F, Sillaber I, Binder E, Keck ME, Holsboer F (2001a). Differential analysis of behavior and diazepam-induced alterations in C57BL/6N and BALB/c mice using the modified hole board test. Journal of Psychiatric Research 35: 147-154.

Ohl F, Toshi N, Wigger A, Henniger MSH, Landgraf R (2001b). Dimensions of emotionality in a rat model of innate anxiety. Behavioral Neuroscience 115: 429-436.

Ohl F and van der Staay FJ (2012). Animal welfare: At the interface between science and society. Veterinary Journal 192: 13-19.

Oitzl MS, Champagne DL, van der Veen R, de Kloet ER (2010). Brain development under stress: Hypotheses of glucocorticoid actions revisited. Neuroscience & Biobehavioral Reviews 34: 853-866.

O'Keefe J (1999). Do hippocampal pyramidal cells signal non-spatial as well as spatial information? Hippocampus 9: 352-364.

P

Padoa-Schioppa C and Assad JA (2006). Neurons in the orbitofrontal cortex encode economic value. Nature 441: 223-226.

Panksepp J (1998). Affective neuroscience: The foundations of human and animal emotions.

Parrott AC and Kentridge R (1982). Personal constructs of anxiety under the 1,5-benzodiazepine derivative clobazam related to trait-anxiety levels of the personality. Psychopharmacology (Berl) 78: 353-357.

Patel JB, Stengel J, Malick JB, Enna SJ (1984). Neurochemical characterstics of rats distinguished as benzodiazepine responders and non-responders in a new conflict test. Life Sciences 34: 2647-2653.

Paul ES, Harding EJ, Mendl M (2005). Measuring emotional processes in animals: the utility of a cognitive approach. Neuroscience & Biobehavioral Reviews 29: 469-491.

Pellow S, Chopin P, File SE, Briley M (1985). Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. Journal of Neuroscience Methods 14: 149-167.

Pesold C and Treit D (1996). The neuroanatomical specificity of the anxiolytic effects ofintra-septal infusions of midazolam. Brain Research 710: 161-168.

Pomerantz O, Terkel J, Suomi SJ, Paukner A (2012). Stereotypic head twirls, but not pacing, are related to a 'pessimistic'-like judgment bias among captive tufted capuchins (Cebus apella). Animal Cognition 15: 689-698.

Pothion S, Bizot J, Trovero F, Belzung C (2004). Strain differences in sucrose preference and in the consequences of unpredictable chronic mild stress. Behavioural Brain Research 155: 135-146.

Pratt JA (1992). The neuroanatomical basis of anxiety. Pharmacology and Therapeutics 55: 149-181.

Pratte M and Jamon M (2009). Detection of social approach in inbred mice. Behavioural Brain Research 203: 54-64.

R

Rago L, Kiivet R, Haro J (1986). Variation in Behavioral-Response to Baclofen - Correlation with Benzodiazepine Binding-Sites in Mouse Forebrain. Naunyn-Schmiedeberg's Archives of Pharmacology 333: 303-306.

Ramboz S, Oosting R, Amara DA, Kung HF, Blier P, Mendelsohn M, Mann JJ, Brunner D, Hen R (1998). Serotonin receptor 1A knockout: An animal model of anxiety-related disorder. Proceedings of the National Academy of Sciences of the United States of America 95: 14476-14481.

Razzoli M, Carboni L, Andreoli M, Michielin F, Ballottari A, Arban R (2011). Strain-specific outcomes of repeated social defeat and chronic fluoxetine treatment in the mouse. Pharmacology Biochemistry and Behavior 97: 566-576.

Restivo L, Chaillan FA, Ammassari-Teule M, Roman FS, Marchetti E (2006). Strain differences in rewarded discrimination learning using the olfactory tubing maze. Behavior Genetics 36: 923-934.

Retana-Márquez S, Bonilla-Jaime H, Vázquez-Palacios G, Domínguez-Salazar E, Martínez-García R, Velázquez-Moctezuma J (2003). Body weight gain and diurnal differences of corticosterone changes in response to acute and chronic stress in rats. Psychoneuroendocrinology 28: 207-227.

Richards A and French CC (1992).
An anxiety-related bias in semantic activation when processing threat/neutral homographs. Quarterly Journal of Experimental Psychology Section A: Human Experimental Psychology 45: 503-525.

Richardson DK, Reynolds SM, Cooper SJ, Berridge KC (2005). Endogenous opioids are necessary for benzodiazepine palatability enhancement: Naltrexone blocks diazepam-induced increase of sucrose-'liking'. Pharmacology Biochemistry and Behavior 81: 657-663.

Richter SH, Schick A, Hoyer C, Lankisch K, Gass P, Vollmayr B (2012). A glass full of optimism: Enrichment effects on cognitive bias in a rat model of depression. Cognitive, Affective and Behavioral Neuroscience 12: 527-542.

Rodgers RJ, Boullier E, Chatzimichalaki P, Cooper GD, Shorten A (2002). Contrasting phenotypes of C57BL/6JOlaHsd, 129S2/SvHsd and 129/SvEv mice in two exploration-based tests of anxiety-related behaviour. Physiology and Behavior 77: 301-310.

Rodgers RJ, Cao B-, Dalvi A, Holmes A (1997). Animal models of anxiety: An ethological perspective. Brazilian Journal of Medical and Biological Research 30: 289-304.

Rodgers RJ, Cole JC, Cobain MR, Daly P, Doran PJ, Eells JR, Wallis P (1992). Anxiogenic-like effects of fluprazine and eltoprazine in the mouse elevated plus-maze: profile comparisons with 8-OH-DPAT, CGS 12066B, TFMPP and mCPP. Behavioral Pharmacology 3: 621-634.

Rodgers RJ and Johnson NJT (1995). Factor analysis of spatiotemporal and ethological measures in the murine elevated plus-maze test of anxiety. Pharmacology Biochemistry and Behavior 52: 297-303.

Rolls ET (1992): Neurophysiology and functions of the primate amygdala. In J.P. Aggleton (Ed.) The amygdala: Neurobiological aspects of emotion, memory and mental dysfunction. In: Anonymous Wiley-Liss: New-York. pp 43-165.

Roman FS, Marchetti E, Bouquerel A, Soumireu-Mourat B (2002). The olfactory tubing maze: a new apparatus for studying learning and memory processes in mice. Journal of Neuroscience Methods 117: 173-181.

Roullet P and Lassalle JM (1995). Radial maze learning using exclusively distant visual cues reveals learners and nonlearners among inbred mouse strains. Physiology and Behavior 58: 1189-1195.

Rowe RE, Sheskey PJ, Quinn ME (2009): Handbook of pharmaceutical excipients. In: Anonymous 6th ed. Pharmaceutical Press: London.

Roy M-, Neale MC, Pedersen NL, Mathe AA, Kendler KS (1995). A twin study of generalized anxiety disorder and major depression. Psychological Medicine 25: 1037-1049.

Roy V, Belzung C, Delarue C, Chapillon P (2001). Environmental enrichment in BALB/c mice: Effects in classical tests of anxiety and exposure to a predatory odor. Physiology and Behavior 74: 313-320.

Rushen J (1991). Problems associated with the interpretation of physiological data in the assessment of animal welfare. Applied Animal Behaviour Science 28: 381-386.

S

Sachser N, Dürschlag M, Hirzel D (1998). Social relationships and the management of stress. Psychoneuroendocrinology 23: 891-904.

Salmeto AL, Hymel KA, Carpenter EC, Brilot BO, Bateson M, Sufka KJ (2011a). Cognitive bias in the chick anxiety-depression model. Brain Research 1373: 124-130.

Salmeto AL, Hymel KA, Carpenter EC, Brilot BO, Bateson M, Sufka KJ (2011b). Cognitive bias in the chick anxiety-depression model. Brain Res 1373: 124-130.

Salomons AR, Arndt SS, Ohl F (2009). Anxiety in relation to animal environment and welfare. Scandinavian Journal of Laboratory Animal Science 36: 37-45.

Salomons AR, Arndt SS, Ohl F (2012). Impact of anxiety-profiles on cognitive performance in BALB/c and 129P2 mice. Cognitive, Affective and Behavioral Neuroscience 12:794-803.

Salomons AR, Bronkers G, Kirchhoff S, Arndt SS, Ohl F (2010). Behavioural habituation to novelty and brain area specific immediate early gene expression in female mice of two inbred strains. Behavioural Brain Research 215: 95-101.

Salomons AR, Pinzon NE, Boleij H, Kirchhoff S, Arndt SS, Nordquist RE, Lindemann L, Jaeschke G, Spooren W, Ohl F (2012). Differential effects of diazepam and MPEP on habituation and neuro-behavioural processes in inbred mice. Behavioral and Brain Functions 8: 30.

Salomons AR, van Luijk JAKR, Reinders NR, Kirchhoff S, Arndt SS, Ohl F (2010a). Identifying emotional adaptation: behavioural habituation to novelty and immediate early gene expression in two inbred mouse strains. Genes, Brain and Behavior 9: 1-10.

SSalomons AR, Kortleve T, Reinders NR, Kirchhoff S, Arndt SS, Ohl F (2010b). Susceptibility of a potential animal model for pathological anxiety to chronic mild stress. Behavioral Brain Research 209: 241-248.

Sanger ME, Doyle RE, Hinch GN, Lee C (2011). Sheep exhibit a positive judgement bias and stress-induced hyperthermia following shearing. Applied Animal Behaviour Science 131: 94-103.

Schellinck HM, Forestell CA, LoLordo VM (2001). A simple and reliable test of olfactory learning and memory in mice. Chemical Senses 26: 663-672.

Schmidt M, Oitzl MS, Levine S, de Kloet ER (2002). The HPA system during the postnatal development of CD1 mice and the effects of maternal deprivation. Developmental Brain Research 139: 39-49.

Schmidt MV, Scharf SH, Liebl C, Harbich D, Mayer B, Holsboer F, Müller MB (2010a). A novel chronic social stress paradigm in female mice. Hormones and Behavior 57: 415-420.

Schmidt MV, Scharf SH, Sterlemann V, Ganea K, Liebl C, Holsboer F, Müller MB (2010b). High susceptibility to chronic social stress is associated with a depression-like phenotype. Psychoneuroendocrinology 5: 635- 643.

Schmidt MV, Sterlemann V, Ganea K, Liebl C, Alam S, Harbich D, Greetfeld M, Uhr M, Holsboer F, Müller MB (2007). Persistent neuroendocrine and behavioral effects of a novel, etiologically relevant mouse paradigm for chronic social stress during adolesence. Psychoneuroendocrinology 32: 417-429.

Schmidt MV, Sterlemann V, Müller MB (2008). Chronic stress and individual vulnerability. Annals of the New York Academy of Sciences 1148: 174-183.

Schmidt MV, Trümbach D, Weber P, Wagner K, Scharf SH, Liebl C, Datson N, Namendorf C, Gerlach T, Kühne C, Uhr M, Deussing JM, Wurst W, Binder EB, Holsboer F, Müller MB (2010). Individual stress vulnerability is predicted by short-term memory and AMPA receptor subunit ratio in the hippocampus. Journal of Neuroscience 30: 16949-16958.

Schoech SJ, Rensel MA, Heiss RS (2011). Short- and long-term effects of developmental corticosterone exposure on avian physiology, behavioral phenotype, cognition, and fitness: A review. Current Zoology 57: 514-530.

Schwabe L, Dalm S, Schächinger H, Oitzl MS (2008). Chronic stress modulates the use of spatial and stimulus-response learning strategies in mice and man. Neurobiology of Learning Memory 90: 495-503.

Schweizer MC, Henniger MSH, Sillaber I (2009). Chronic Mild Stress (CMS) in mice: Of anhedonia, "anomalous anxiolysis", and activity. PLoS ONE 4: 1-11.

Scott TR and Mark GP (1987). The taste system encodes stimulus toxicity. Brain Research 414: 197-203.

Shader RI and Greenblatt DJ (1993). Use of benzodiazepines in anxiety disorders. New England Journal of Medicine 328: 1398-1405.

Sheehan TP, Chambers RA, Russell DS (2004). Regulation of affect by the lateral septum: implications for neuropsychiatry. Brain Research Reviews 46: 71-117.

Sik A, van Nieuwehuyzen P, Prickaerts J, Blokland A (2003). Performance of different mouse strains in an object recognition task. Behavioural Brain Research 147: 49-54.

Silva MTA and Calil HM (1975). Screening hallucinogenic drugs: systematic study of three behavioral tests. Psychopharmacologia 42: 163-171.

Simpson EM, Linder CC, Sargent EE, Davidsson MT, Mobraaten LE, Sharp JJ (1997). Genetic variation among 129 substrains and its importance for targeted mutagenesis in mice. Nature genetics 16: .

Simpson J and Kelly JP (2011). The impact of environmental enrichment in laboratory rats-Behavioural and neurochemical aspects. Behavioural Brain Research 222: 246-264.

Singewald GM, Nguyen NK, Neumann ID, Singewald N, Reber SO (2009). Effect of chronic psychosocial stress-induced by subordinate colony (CSC) housing on brain neuronal activity patterns in mice. Stress 12: 58-69.

Slattery DA, Uschold N, Magoni M, Bar J, Popoli M, Neumann ID, Reber SO (2012). Behavioural consequences of two chronic psychosocial stress paradigms: anxiety without depression. Psychoneuroendocrinology 37: 702-714.

Smith AL and Corrow DJ (2005). Modifications to husbandry and housing conditions of laboratory rodents for improved well-being. ILAR Journal 46: 140-147.

Spector AC and Kopka SL (2002). Rats fail to discriminate quinine from denatonium: Implications for the neural coding of bitter-tasting compounds. Journal of Neuroscience 22: 1937-1941.

Steiner JE (1979). Human Facial Expressions in Response to Taste and Smell Stimulation. Advances in Child Development and Behavior 13: 257-295.

Sterlemann V, Ganea K, Liebl C, Harbich D, Alam S, Holsboer F, Müller MB, Schmidt MV (2008). Long-term behavioral and neuroendocrine alterations following chronic social stress in mice: Implications for stress-related disorders. Hormones and Behavior 53: 386-394.

Sterling P and Eyer J (1988). Allostasis: A new paradigm to explain arousal pathology. Handbook of Life Stress, Cognition and Health 629-649.

Stevens DR, Gallagher JP, Shinnick-Gallagher P (1987). In vitro studies of the role of γ-aminobutyric acid in inhibition in the lateral septum of the rat. Synapse 1: 184-190.

Strekalova T, Spanagel R, Bartsch D, Henn FA, Gass P (2004). Stress-induced anhedonia in mice is associated with deficits in forced swimming and exploration. Neuropsychopharmacology 29: 2007-2017.

Sullivan PF, Neale MC, Kendler KS (2000). Genetic epidemiology of major depression: Review and meta-analysis. American Journal of Psychiatry 157: 1552-1562.

T

Tang AC, Reeb-Sutherland BC, Romeo RD, McEwen BS (2012). Reducing behavioral inhibition to novelty via systematic neonatal novelty exposure: The influence of maternal hypothalamic-pituitary-adrenal regulation. Biological Psychiatry 72: 150-156.

Tang X and Sanford LD (2005). Home cage activity and activity-based measures of anxiety in 129P3/J, 129X1/SvJ and C57BL/6J mice. Physiology and Behavior 84: 105-115.

Tanimura S, Shibuya T, Ishibashi T (1994). Neural responses of the glossopharyngeal nerve to several bitter stimuli in mice. Comparative Biochemistry and Physiology - A Physiology 108: 189-194.

Telzer EH, Mogg K, Bradley BP, Mai X, Ernst M, Pine DS, Monk CS (2008). Relationship between trait anxiety, prefrontal cortex, and attention bias to angry faces in children and adolescents. Biological Psychology 79: 216-222.

Thiebot M- (1985). Some evidence for amnesic-like effects of benzodiazepines in animals. Neuroscience and Biobehavioral Reviews 9: 95-100.

Thomas E, Yadin E, Strickland CE (1991). Septal unit activity during classical conditioning: A regional comparison. Brain Research 547: 303-308.

Thompson RF and Spencer WA (1966). Habituation: A model phenomenon for the study of neuronal substrates of behavior. Psychology Reviews 73: 16-43.

Tordoff MG (2007). Taste solution preferences of C57BL/6J and 129X1/SvJ mice: Influence of age, sex, and diet. Chemical Senses 32: 655-671.

Tordoff MG, Alarcon LK, Lawler MP (2008). Preferences of 14 rat strains for 17 taste compounds. Physiology and Behavior 95: 308-332.

Treit D (1985). Animal models for the study of anti-anxiety agents: A review. Neuroscience and Biobehavioral Reviews 9: 203-222.

Tsetsenis T, Ma X-, Lo Iacono L, Beck SG, Gross C (2007). Suppression of conditioning to ambiguous cues by pharmacogenetic inhibition of the dentate gyrus. Nature Neuroscience 10: 896-902.

U

Ulrich-Lai YM, Figueiredo HF, Ostrander MM, Choi DC, Engeland WC, Herman JP (2006). Chronic stress induces adrenal hyperplasia and hypertrophy in a subregion-specific manner. American Journal of Physiology - Endocrinology and Metabolism 291: E965-E973.

V

Valle FP (1970). Effects of strain, sex, and illumination on open-field behavior of rats. American Journal of Psychology 83: 103-111.

van de Weerd HA, Baumans V, Koolhaas JM, van Zutphen LF (1994). Strain specific behavioural response to environmental enrichment in the mouse. Journal of Experimental Animal Science 36: 117-127.

Van De Weerd HA, Van Loo PLP, Van Zutphen LFM, Koolhaas JM, Baumans V (1998). Strength of preference for nesting material as environmental enrichment for laboratory mice. Applied Animal Behaviour Science 55: 369-382.

van der Staay FJ, Arndt SS, Nordquist RE (2010). The standardization-generalization dilemma: a way out. Genes, Brain and Behavior 9: 849-855.

Van Oortmerssen GA, Benus I, Dijk DJ (1984). Studies in wild house mice: Genotype-environment interactions for attack latency. Netherlands Journal of Zoology 35: 155-169.

Van Praag H, Kempermann G, Gage FH (2000). Neural Consequences of environmental enrichment. Nature Reviews Neuroscience 1: 191-198.

Vaz-Serrano J, Ruiz-Gomez ML, Gjøen HM, Skov PV, Huntingford FA, Øverli Ø, Höglund E (2011). Consistent boldness behaviour in early emerging fry of domesticated Atlantic salmon (Salmo salar): Decoupling of behavioural and physiological traits of the proactive stress coping style. Physiology and Behavior 103: 359-364.

Veenema AH, Meijer OC, de Kloet ER, Koolhaas JM (2003). Genetic selection for coping style predicts stressor susceptibility. Journal of Neuroendocrinology 15: 256-267.

Veĭko NN, Shubaeva NO, Malashenko AM, Beskova TB, Agapova RK, Liapunova NA (2007). Ribosomal genes in inbred mouse strains: interstrain and intrastrain variations of copy number and extent of methylation. Genetika 43: 1226-1238.

von Cramon DY, Markowitsch HJ, Schuri U (1993). The possible contribution of the septal region to memory. Neuropsychologia 31: 1159-1180.

W

Webster AJF (1994): Animal welfare: A cool eye towards Eden. Blackwell Science ltd.: Londen, UK.

Whalen PJ (1998). Fear, vigilance, and ambiguity: Initial neuroimaging studies of the human amygdala. Current Directions in Psychological Science 7: 177-188.

White TL, Dishaw LV, Sheehe PR, Youngentob SL (2007). The relationship between PROP and ethanol preferences: An evaluation of 4 inbred mouse strains. Chemical Senses 32: 847-853.

Whitney G and Harder DB (1986). Single-locus control of sucrose octaacetate tasting among mice. Behavior Genetics 16: 559-574.

Wichman A, Keeling LJ, Forkman B (2012). Cognitive bias and anticipatory behaviour of laying hens housed in basic and enriched pens. Applied Animal Behaviour Science 140: 62-69.

Wilkinson CJ (1985). Effects of diazepam (valium) and trait anxiety on human physical aggression and emotional state. Journal of Behavioral Medicine 8: 101-114.

Willner P, Towell A, Sampson D, Sophokleous S, Muscat R (1987). Reduction of sucrose preference by chronic unpredictable mild stress, and its restoration by a tricyclic antidepressant. Psychopharmacology 93: 358-364.

Willoughby IH, Jinks RL, Morgan GW, Pepper H, Budd J, Mayle B (2011). The use of repellents to reduce predation of tree seed by wood mice (Apodemus sylvaticus L.) and grey squirrels (Sciurus carolinensis Gmelin). European Journal of Forest Research 130: 601-611.

Wilson DM, Boughter JD, Lemon CH (2012). Bitter taste stimuli induce differential neural codes in mouse brain. PLoS ONE 7: e41597.

Wong AA and Brown RE (2007). Age-related changes in visual acuity, learning and memory in C57BL/6J and DBA/2J mice. Neurobiology of Aging 28: 1577-1593.

Wong R (1994). Response latency of gerbils and hamsters to nuts flavoured with bitter-tating substances. The quarterly journal of experimental psychology 47B: 173-186.
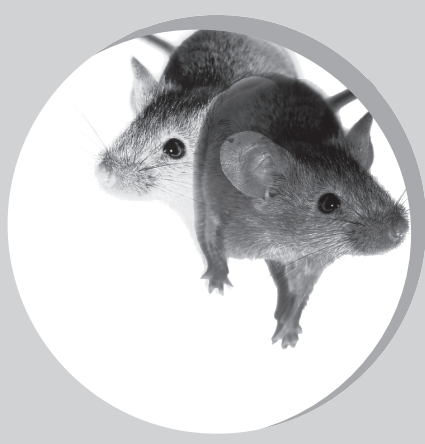
Y

Yeates JW and Main DCJ (2008). Assessment of positive welfare: A review. Veterinary Journal 175: 293-300.

Yen Y-, Mauch CP, Dahlhoff M, Micale V, Bunck M, Sartori SB, Singewald N, Landgraf R, Wotjak CT (2012). Increased levels of conditioned fear and avoidance behavior coincide with changes in phosphorylation of the protein kinase B (AKT) within the amygdala in a mouse model of extremes in trait anxiety. Neurobiology of Learning and Memory 98: 56-65.

Z

Zheng QY, Johnson KR, Erway LC (1999). Assessment of hearing in 80 inbred strains of mice by ABR threshold analyses. Hearing Research 130: 94-107.

NEDERLANDSE SAMENVATTING

Het doel van het onderzoek dat is beschreven in dit proefschrift was om emotionele percepties in muizen te onderzoeken. Allereerst is er op basis van voorgaand onderzoek gekeken naar de mate waarin bepaalde muizenstammen in staat zijn om zich aan te passen aan hun omgeving en wat de invloed van negatieve ervaringen daarop is. Ten tweede, is er een aanzet gemaakt om een test voor "judgement bias" te ontwikkelen voor muizen.

HABITUATIE ALS INDICATOR VOOR ADAPTATIE EN WELZIJN

Het vermogen van een dier om zich aan te passen aan zijn omgeving wordt gereflecteerd door de vertoonde gedragsrespons. Het is bijvoorbeeld adaptief voor een dier om zich angstig te gedragen in een onbekende, potentieel gevaarlijke omgeving. Echter, als blijkt dat de omgeving veilig is zou het angstgedrag af moeten nemen, een effect dat habituatie wordt genoemd. Als een dier niet in staat is om te habitueren aan veranderde omgevingsomstandigheden kan zijn gedrag omschreven worden als niet adaptief of pathologisch. Verscheidene welzijnsconcepten beschrijven onder andere het aanpassingsvermogen van een individu als belangrijk aspect: zolang een dier in staat is zich aan te passen aan zijn omgeving en op die manier in staat is een positieve emotionele staat te bereiken is het algehele welzijn niet aangetast. Door habituatie van gedrag te onderzoeken in muizen zou men dus een indruk kunnen krijgen van het aanpassingsvermogen, waarbij een gebrek aan habituatie gezien kan worden als een eigenschap van het dier welke op de lange termijn tot een welzijnsaantasting zou kunnen leiden.

In studies voorafgaand aan het huidige onderzoek is gevonden dat 129P3/J muizen niet in staat zijn om te habitueren aan een testomgeving (de modified hole board test, mHB) als ze er herhaaldelijk aan bloot worden gesteld, in tegenstelling tot BALB/cJ muizen welke initieel een verhoging van angstgedrag laten zien maar wel snel habitueren. Naast de 129P3/J substam blijkt uit de experimenten beschreven in hoofdstuk 2 dat ook muizen van andere 129 substammen (de 129X2/J, 129P2/OlaHsd, 129S2/SvPasCrl en 129S2/SvHsd stam) een soortgelijke respons vertonen in dezelfde testomgeving. Dit suggereert dat de genetische achtergrond van deze dieren een rol speelt bij de ontwikkeling van een niet-adaptieve angstrespons, al zijn er wel substam specifieke verschillen gevonden op andere gedragsparameters. Op twee manieren is dit resultaat belangrijk: ten eerste zouden specifieke 129 stammen (129P3 en 129P2) een potentieel diermodel kunnen zijn voor niet-adaptieve (pathologische) angst bij andere dieren of mensen; het is namelijk belangrijk dat het diermodel dezelfde symptomen vertoont als de doeldiersoort en dit is een karakteristiek dat ook bij mensen met pathologische angst voorkomt. Daarnaast impliceert een gebrek aan aanpassingsvermogen dat het welzijn van 129 muizenstammen misschien in het geding kan komen onder gestandaardiseerde laboratorium huisvesting, omdat zij meer moeite kunnen hebben (dan bijvoorbeeld BALB/c muizen) zich aan te passen aan onbekende omgevingen en stimuli, bijvoorbeeld na transport of bij het verschonen van de kooien. Dit praktische aspect dient nog onderzocht te worden, maar het geeft wel aan dat de ene muis niet hetzelfde is als de andere muis en dat richtlijnen omtrent huisvesting wellicht meer specifiek beschreven dienen te worden voor muizenstammen welke een aangetast aanpassingsvermogen hebben om hun welzijn te verbeteren.

Naast genetische aanleg is het bekend dat negatieve omgevingsfactoren, zoals situaties welke chronische stress veroorzaken, een belangrijke rol kunnen spelen bij het ontwikkelen van psychische aandoeningen zoals angststoornissen. Om te onderzoeken of het non-adaptieve angstprofiel dat was gevonden in de 129 muizenstammen ook geïnduceerd kan worden door chronische stress, is er in hoofdstuk 3 een andere veelgebruikte muizenstam (CD1) onderzocht. De groepssamenstellingen van experimentele groepen werd gedurende 7 weken gewisseld in de adolescente periode, hierdoor waren de muizen niet in staat een stabiele rangorde te vormen wat chronische sociale stress (CSS) veroorzaakt. De controle groep bleef gedurende deze periode gehuisvest in stabiele sociale groepen. Vervolgens (na beëindiging van CSS) werden de dieren herhaaldelijk getest in de mHB om een indruk te krijgen van hun adaptieve capaciteiten. Naast gedragsobservaties in de mHB zijn fysiologische en externe indicatoren van stress gemeten in het experiment, dit omvatte het lichaamsgewicht, vachtconditie, het bepalen van stresshormoon corticosteron in bloedplasma en het bepalen van glucocorticoïd (GR) en mineralocorticoïd (MR) receptor mRNA in hersengebieden betrokken bij stress. Via deze fysiologische parameters kon worden bevestigd dat de dieren inderdaad het protocol als stressvol ervoeren, echter na beëindiging van het protocol lieten de dieren geen aantasting van hun adaptieve capaciteiten zien in vergelijking met de controle groep. Deze bevinding geeft aan dat negatieve ervaringen niet per se een aantasting van het welzijn geven op de lange termijn (als de stressvolle periode is beëindigd), echter de hogere variatie in de gestreste groep geeft wel aan dat sommige dieren (responders) meer gevoelig zijn voor de effecten van stress op gedrag, dit effect zal verder onderzocht moeten worden voor bevestiging.

"JUDGEMENT BIAS" ALS INDICATOR VOOR EMOTIONELE PERCEPTIES

Het aanpassingsvermogen geeft echter nog geen uitsluitsel erover hoe dieren hun eigen emotionele toestand ervaren (emotionele percepties). Judgement bias is een proces waarbij de interpretatie van ambigue informatie (informatie waarvan de betekenis onduidelijk is) wordt beïnvloed door de emotionele staat, dit cognitieve proces is dus een indirecte afgeleide van de interne toestand van dieren. Recente studies geven aan dat judgement bias een proces is dat in meerdere diersoorten gevonden kan worden en indirect informatie kan verstrekken over de eigen perceptie van hun interne emotionele toestanden. Manieren om judgement bias in dieren te kunnen meten kunnen dus aanvulling geven op de kennis over emotionele toestanden in dieren en daarmee ook bijdragen aan het verbeteren van pre-klinisch onderzoek naar psychische stoornissen en ons begrip over dierenwelzijn. Ondanks dat er in verscheidene diersoorten al manieren zijn ontwikkeld om judgement bias te onderzoeken is dit tot nu toe nog niet gedaan in muizen, maar wel relevant voor deze diersoort omdat ze veel gebruikt wordt in laboratorium onderzoek. Het tweede onderdeel van dit proefschrift was daarom om judgement bias in muizen te onderzoeken. Gebaseerd op de studies naar habituatie is er in hoofdstuk 4 onderzoek gedaan naar judgement bias van 129P3 en BALB/c muizen, welke respectievelijk non-adaptief en adaptief angst gerelateerd gedrag vertonen in de mHB. De test om judgement bias te kunnen meten bestond uit een geurconditionering waarbij tijdens de trainingsperiode in de positieve trials een geurbakje (met appel of vanille geur) werd aangeboden samen met een lekkere amandel en in de negatieve trials een geurbakje met de andere geur samen met een

vieze (met kinine behandelde) amandel in de thuiskooi. In de test werd, naast de reacties van de dieren op de positieve en negatieve stimulus, het gedrag van de dieren als reactie op het aanbod van een mix van de twee geconditioneerde geuren onderzocht (ambigue stimuli) en daarbij werd de latentietijd tot het oppakken en eten van de amandel gebruikt als indicatie van interpretatie van de aangeboden stimulus. Deze eerste test toonde aan dat BALB/c muizen de conditionering goed leerden en intermediaire reacties vertoonden als zij werden blootgesteld aan ambigue stimuli. Dit impliceerde dat de gebruikte test geschikt kon zijn als test voor judgement bias in deze stam. 129P3 muizen vertoonden echter geen indicatie dat ze de geurassociaties met de positive en negative stimulus hadden geleerd wat te maken zou kunnen hebben met het beperkte aanpassingsvermogen van deze stam, waardoor judgement bias niet op deze manier gemeten kan worden. Verdere experimenten in BALB/c muizen, beschreven in hoofdstuk 4, geven aan dat een verhoogd angstniveau, welke geïnduceerd werd door de muizen onder wit licht te testen, een negatieve judgement bias tot gevolg heeft. Tevens is er in hoofdstuk 4 en 5 gevonden dat dit een effect is dat waarschijnlijk veroorzaakt wordt door deze negatieve emotionele toestand omdat het stresshormoon corticosteron verhoogd was en we aanwijzingen hebben gevonden dat het toedienen van een angst reducerende drug (diazepam) de bias minder negatief maakt.

Echter zijn er ook methodologische problemen gevonden, met name dat de bittere amandel (met kinine behandeld) mogelijk detecteerbaar is voor de muizen. In hoofdstuk 7 hebben wij inderdaad aanwijzingen gevonden dat dit het geval is, er is echter niet bekend welk zintuig de muizen gebruiken. In hoofdstuk 6 hebben we geprobeerd of dit probleem was op te lossen door een andere bittere smaakstof (denatonium benzoaat) te gebruiken, echter is er gevonden dat dit geen goed alternatief is voor kinine omdat de muizen aan de smaak habitueren.
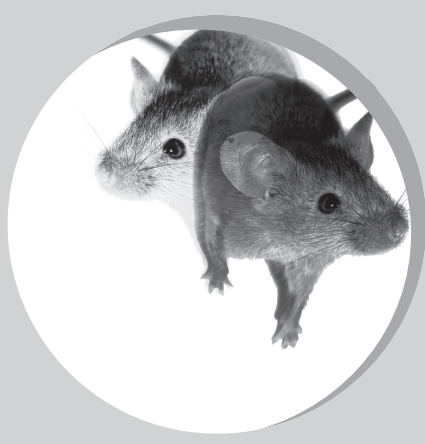
Los van dit methodologisch probleem, duiden de data op het niveau van het centraal zenuwstelsel (beschreven in hoofdstuk 4) erop dat het gemeten fenomeen een afspiegeling kan zijn van emotionele percepties, omdat de neurale marker c-Fos (indicatief voor neuron activiteit) verschilt tussen ambigue en positieve en negatieve stimuli juist in hersengebieden die relevant zijn voor het verwerken van emotionele informatie en gebieden welke een link vormen tussen emotionele en cognitieve processen. Dit ondersteunt het idee dat de gedragsresponsen die gemeten zijn in de judgement bias test een afspiegeling zijn van emotionele en cognitieve processen en een indruk zouden kunnen geven van emotionele percepties in muizen.

In hoofdstuk 8 worden de bevindingen met elkaar bediscussieerd en verbanden gelegd tussen de verschillende studies. Naast de conclusies en discussie met betrekking tot adaptieve capaciteiten en judgement bias beschrijven we dat er in bijna alle studies individuele verschillen gevonden zijn die mogelijk verklaard kunnen worden door verschillende strategieën (passief of actief coping) die individuele dieren hebben. Verder bediscussiëren we de beperkingen die deze en andere judgement bias testen nog hebben en wat mogelijk volgende stappen zijn om dit te verbeteren.

CONCLUSIES

Samenvattend kunnen we concluderen dat het meten van habituatie waardevol kan zijn om adaptieve gedragsresponsen te kunnen onderscheiden van niet-adaptieve gedragsresponsen. Dit zou kunnen helpen bij het vaststellen of een dier kan omgaan met zijn omgeving of dat het aanpassingsvermogen van een dier is overschreden. Bovendien hebben we gevonden dat het meten van "judgement bias" in muizen meer kennis zou kunnen verschaffen over hoe een dier zijn emotionele toestand ervaart, alhoewel er meer onderzoek nodig is om dit verder te onderbouwen en verdere verfijning van de testprocedure nodig is. Het beschreven onderzoek kan gezien worden als basis voor het verder ontwikkelen van methoden om adaptieve capaciteiten van muizen en hun emotionele percepties te kunnen duiden, met als uiteindelijke doel meer inzicht te krijgen in de (dys)regulatie van emoties in deze dieren en het bewaken van hun welzijn.

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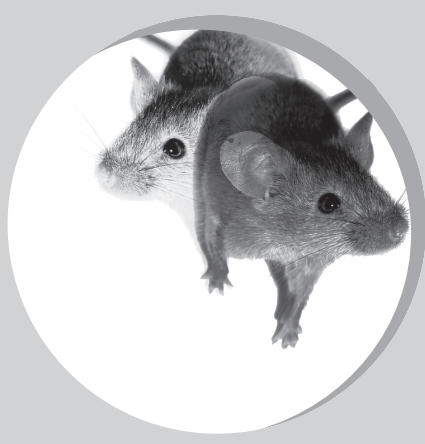
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ABOUT THE AUTHOR

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Hetty Boleij was born on the 19th of March,1985 in Boxtel. She followed her secondary education at the "Jacob-Roelands lyceum" in Boxtel, with a Nature and Health profile (including Mathematics, Physics, Biology and Chemistry). From September 2003 until June 2006 she studied Biology at Utrecht University, resulting in a Bachelors degree. After that she continued with the special track Ethology and Welfare within the Master Programme Bioveterinary Sciences, from the Biomedical Sciences Master. Part of this Master was a minor research project of 6 months; this internship was performed under the supervision of dr. Francien de Jonge at the Department of Animals in Science and Society of the Veterinary Faculty, Utrecht University and was focused on a context-conditioning method to improve welfare of piglets around the time of weaning. Her major research project of 9 months was performed at the Rudolph Magnus Institute of Neuroscience, Univerisity Medical Centre Utrecht. This internship was done under the supervision of Prof. dr. Louk Vanderschuren and dr. Maartje Veeneman en focussed on the rewarding and sensitizing properties of morphine in rats. After obtaining her Masters degree in August 2008 she started with her PhD research on October 1st 2008 under the supervision of Prof. dr. Frauke Ohl and dr. Saskia Arndt at the department of Animals in Science and Society at the Faculty of Veterinary Medicine, Utrecht University, from which the results are presented in this thesis. At the moment she is appointed as a Junior Teacher within the same department.

LIST OF PUBLICATIONS

Boleij, H., Salomons, A.R., van Sprundel, M., Arndt, S.S., Ohl, F. "Not all mice are equal: welfare implications of behavioural habituation profiles in four 129 mouse substrains" (2012) PLoS ONE, 7(8), e42544

Boleij, H., van 't Klooster, J., Kirchhoff, S., Lavrijsen, M., Arndt, S.S., Ohl, F. "A test to identify judgement bias in mice" (2012) Behavioural Brain Research, 233, 45-54

Salomons, A.R., Pinzon, N.E., Boleij, H., Kirchhoff, S., Arndt, S.S., Nordquist, R.E., Lindemann, L., Jaeschke, G., Spooren, W., Ohl, F. "Differential effects of diazepam and MPEP on habituation and neuro-behavioural processes in inbred mice" (2012) Behavioral and Brain Functions, 8, art. no. 30

Veeneman, M.M.J., Boleij, H., Broekhoven, M.H., Snoeren, E.M.S., Guitart Masip, M., Cousijn, J., Spooren, W., Vanderschuren, L.J.M.J. "Dissociable roles of mGlu5 and dopamine receptors in the rewarding and sensitizing properties of morphine and cocaine" (2011) Psychopharmacology, 214 pp. 863-876

de Jonge, F.H., Boleij, H., Baars, A.M., Dudink, S., Spruijt, B.M. "Music during play-time: Using context conditioning as a tool to improve welfare in piglets" (2008) Applied Animal Behaviour Science, 115 (3-4), pp. 138-148

CONFERENCE ABSTRACTS

Boleij H, Arndt SS and Ohl F "Judgement bias in a mouse model for pathological anxiety" 16th annual PhD meeting ONWAR19-20, November 2009, Zeist The Netherlands

Boleij H, Arndt S.S., Salomons AR, Baars JM, Ohl F "Judgement bias in a mouse model for pathological anxiety" FENS Forum of European Neuroscience, 3-7 July 2010 Amsterdam The Netherlands

Boleij H, Salomons A.R., Arndt S.S. and Ohl F "Implications for animal welfare: habituation profiles of 129S2, 129P2 and 129X1 mouse strains" 45th ISAE conference, July 31 – August 4 2011, Indianapolis USA

Boleij H, Arndt S.S., Salomons A.R., van 't Klooster J, Baars J.M. and Ohl, F "A test for judgmentbias - possibly indicating (trait) anxiety in mice" 43th EBBS meeting 9-12 September 2011 Sevilla, Spain

Hetty Boleij, Tim van der Liet, José van 't Klooster, Saskia S. Arndt, Frauke Ohl "Judgement bias as tool to assess internal emotional states in mice" Minding Animals conference 4-6 July 2012 Utrecht, The Netherlands

Hetty Boleij, Tim van der Liet, Saskia S. Arndt, José van't Klooster, Annemarie Baars, Frauke Ohl "A tool to assess judgement bias in mice" FENS Forum of European Neuroscience, 14-18 July 2012, Barcelona, Spain