Lousy house, grouchy mouse: Assessing the effects of standard barren housing on laboratory mouse sociability and attractiveness

by

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ABSTRACT

LOUSY HOUSE, GROUCHY MOUSE: ASSESSING THE EFFECTS OF STANDARD BARREN HOUSING ON LABORATORY MOUSE SOCIABILITY AND ATTRACTIVENESS

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University of Guelph, 2019 — Georgia Mason

Compared to mice from environmentally enriched (EE) enclosures, mice housed in the small, relatively barren cages ubiquitous in research facilities (standard housing) show signs of poor welfare such as increased stereotypic and depressive behaviours and agonism. Previous research in humans and rats suggests that these behavioural changes can lead to reduced sociability, attractiveness as a social partner, or both. I thus sought to determine the effects of housing on sociability and attractiveness in female mice using a modified sociability and social preference test and a novel familiarisation paradigm. My results indicated that standard housed (SH) mice were not generally less sociable or attractive than those from enriched housing. However, mice who received more agonism in their home cages strongly preferred to spend their time near EE mice (the less agonistic housing type), suggesting that agonism between female cagemates, while not usually injurious, is aversive to the recipient.

DEDICATION

This thesis is first and foremost dedicated to the 188 mice used in this project and the millions of mice exploited and killed for research worldwide each year.

But I would also like to dedicate this work to the person I became throughout my master's degree.

I started this for the mice, but I finished it for me.

ACKNOWLEDGEMENTS

First, a big thank you to my advisor Georgia Mason for her insightful guidance and for always going the extra mile (or two!) to make sure her students are successful. I would also like to thank my advisory committee members Lee Niel, Lena Levison, and Elena Choleris for their advice in the planning stage, and their many helpful comments towards my finished thesis, as well as Michelle Cieplak and everyone at the Campus Animal Facility who took great care of our mice.

To Emma Nip and Aileen MacLellan, thank you for your emotional support and hands-on help with my project; I couldn't have done it without you! I am eternally grateful for all the friends I made in the past three years who have provided endless comfort and encouragement throughout all the ups and downs.

Finally, to my husband Robert who was there for me in the toughest times and believed in me when I didn't believe in myself (sappy, I know, but it's true!). Thank you for never giving up on me.

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1.1 Introduction

Mice are one of the most commonly used species in research. For example, they consistently make up between 30 and 40% of all research animals used in facilities accredited by the Canadian Council on Animal Care (CCAC) which amounts to over 1.2 million used each year (Canadian Council on Animal Care 2012 – 2017). In 2017, more mice were used in CCAC-accredited research institutions than any other animal (CCAC 2017). These mice are typically housed in groups of at least two in relatively barren shoebox-sized cages which contain food, water, corncob or wood chip bedding, and sometimes nesting materials such as cotton pads or crinkled paper strips. Although this type of housing is considered "standard" and provides the most basic necessities for mice to live, evidence strongly suggests it promotes poor welfare (i.e. a negative emotional state; e.g. Gross et al. 2012), and mice prefer more environmentally enriched (EE) cages (containing e.g. multiple levels, running wheels, shelters, tunnels, extra nesting materials, hammocks, and items to climb and chew; Tilly et al. 2010). Furthermore, even in laboratories, mice are highly sociable and can develop preferences for certain individuals over others (e.g. Benson et al. 2013), aspects of their behaviour which have been little studied with respect to housing. Thus, while mice are interesting and important subjects in their own right, they can also serve as a model for other social species in laboratories, on farms, and in zoos, as well as models of human diseases (i.e. organisms which are physiologically homologous to humans in ways relevant to the disease of interest, and which can be manipulated in various ways to induce disease symptoms which can then be treated experimentally). As such, in this first chapter of my thesis I will briefly summarize the effects of standard housing in mice, followed by a description of mouse social behaviour and its importance for various aspects of their lives. Lastly, I will provide an overview of my research questions and the studies I carried out to answer them.

1.2 How standard housing affects mouse welfare and cognition

Compared to EE mice, standard-housed (SH) mice show many signs of poor welfare. Mice in standard housing typically show increased anxiety-related behaviour: spending less time in the open arms of an elevated plus maze (e.g. Bouet et al. 2011); showing longer latencies to enter, and fewer entries into an aversive, brightly lit chamber (e.g. Chourbaji et al. 2005); and having increased startle response to loud auditory stimuli (Hattori et al. 2007). Barren housing also leads to the development of depression-like behaviours such as anhedonia (reduced motivation for a reward; Vega-Rivera et al. 2016) and learned helplessness (rapidly giving up trying to escape when confronted with stressful situations). For example, SH mice spend more time helplessly floating instead of swimming in a forced swim test (Brenes et al. 2008, Fureix et al. 2016, Hattori et al. 2007, Llorens-Martín et al. 2007, Vega-Rivera et al. 2016), and in one case this helplessness correlated with time spent inactive but awake (IBA) in the home cage (Fureix et al. 2016), a behaviour recently found to be more prevalent in SH mice than EE mice (Harper et al. 2015, Tilly et al. 2010, Fureix et al. 2016). When placed in a two-compartment box where electric shocks are delivered on one side, SH mice also helplessly endured the shocks instead of moving to the other compartment (Chourbaji et al. 2005). Furthermore, standard housing conditions increase the prevalence and severity of stereotypic behaviours (SB): repetitive, seemingly functionless movements caused by frustration, need to cope, or brain dysfunction (Gross et al. 2012, Harper et al. 2015, Howerton et al. 2008, Olsson and Sherwin 2006, Tilly et al. 2010) which are often used as indicators of suboptimal housing and poor welfare (e.g. Mason and Latham 2004). In mice, SBs include patterned running and climbing, bar-mouthing, back-flipping, and jumping as well as auto-barbering (excessive self-grooming causing baldness). Some studies also found an increase in another form of repetitive behaviour in SH: perseveration (i.e. *ad hoc /* elicited tendencies to form routines and repeat inappropriate responses in test situations). Although researchers do not always find housing effects on perseveration (e.g. Gross et al. 2012, Bailoo et al. 2018), where there are housing differences SH mice are always the more perseverative (Zelenikow-Johnston et al. 2017, Whitehouse et al. 2017, Rountree-Harrison et al. 2018).

Standard housing can also affect cognitive ability in mice by affecting learning and memory. A common method of assessing spatial learning and memory is the Morris water maze which takes advantage of mice's aversion to cool water (20-22°C, e.g. Vorhees and Williams 2006). Here, mice must locate a fixed submerged platform, starting from different locations within the test pool on subsequent trials (Morris 1981). Compared to EE mice, SH mice take less direct paths and take longer to find the platform (Martínez-Cué et al. 2002, Frick et al. 2003, Goshen et al. 2009, Bouet et al. 2011, Hendershott et al. 2016). Other types of learning and memory are also affected. For example, SH mice have a reduced ability to discriminate between novel and familiar objects (thus while EE mice spent more time sniffing the novel object compared to the familiar object, this difference was less pronounced in SH mice; Bouet et al. 2011). These changes in cognition are paralleled by anatomical changes in the brain such as decreased brain weight in SH mice compared to their EE counterparts (La Torre 1968). Environmental enrichment also promotes neurogenesis in the dentate gyrus (a part of the hippocampus which contributes to the formation of memories among other functions; Kempermann et al. 1997) of adult mice and these new neurons have better survival rates than the neurons of SH mice (Kempermann et al. 1998, van Praag et al. 2000).

1.3 Mouse social behaviour and its importance in their lives

Mice are social animals: they exhibit social learning (e.g. Valsecchi et al. 1996), females live in groups and nest communally in the wild (Latham and Mason 2004), and when housed individually in laboratories both sexes experience behavioural changes associated with poor welfare such as SB and numerous physiological signs of prolonged stress such as elevated corticosterone and decreased immune function (see Van Loo et al. 2001 for a brief review). Indeed, members of gregarious species like mice form social groups to regulate stress (especially females, e.g. Taylor et al. 2000, Anacker et al. 2016): in addition to showing reduced signs of stress and poor welfare, mice who are housed with conspecifics recover faster (Van Loo et al. 2007) and self-administer less analgesic after an operation (Pham et al. 2010) compared to those housed individually (see also Appendix III). They also rely on their cagemates for

thermoregulation which requires collaborative nest building and maintenance, as well as huddling to conserve heat (Latham and Mason 2004). Thus, it is clear that sociability is essential for many aspects of a mouse's life and housing them in a way that encourages normal social interaction is important for their wellbeing.

It is no surprise then that mice are highly socially motivated. Most strains engage in high rates of social behaviour and prefer social contact to interaction with an inanimate object (e.g. Van Loo et al. 2004, Brodkin et al. 2004). The most common way of measuring sociability in mice is using a three-chamber apparatus which has a confined stimulus mouse (usually an unfamiliar age-, weight-, and sex-matched conspecific) in one side chamber, an inanimate object (usually the same object used to confine the stimulus mouse) in the opposite side chamber, and an empty chamber in the middle (e.g. Brodkin et al. 2004; see Figure 1.1). A test or focal mouse is then placed in the middle chamber and, after a habituation period, she is allowed to roam freely between the three chambers. Researchers can then compare the number of entries into and/or time spent in the chamber with the stimulus mouse to entries into and/or time spent in the other two chambers. Another test of sociability involves allowing two mice to interact freely in a neutral arena (e.g. Mesa-Gresa et al. 2013). An observer then scores all social interactions or a subset thereof (e.g. sniffing as a measure of olfactory investigation, or all "affiliative" social interactions which can include sniffing, approaching, grooming, and following; e.g. Mesa-Gresa et al. 2013), and assumes that a longer duration or greater number of bouts of social behaviour is indicative of a more sociable mouse. The three-chamber apparatus can also be used to test the relative attractiveness (i.e. how preferred an individual is as a social companion) of one stimulus mouse compared to another by placing a stimulus mouse in each of the two side chambers and comparing time spent with each by the focal mouse (e.g. Benson et al. 2013).

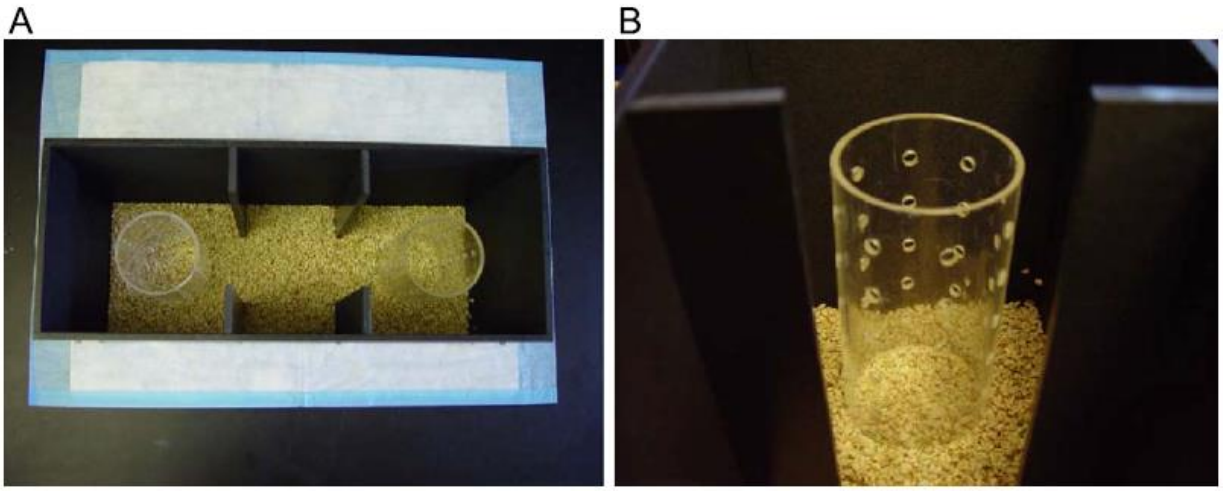

Figure 1.1 The three-chamber apparatus typically used to measure sociability in mice showing A) the whole apparatus with confinement cylinders for stimulus mice in the lateral chambers, and B) an up-close view of the perforated cylinder through which the focal mouse can smell olfactory cues from the stimulus mouse. Photo from Brodkin et al. 2004.

Unlike sociability, which is measured as an attribute of the focal mouse, the attractiveness of same-sex stimulus animals has not been well studied in mice (the vast majority of work on attractiveness focussing on mate choice, e.g. Ehman and Scott 2002, Asaba et al. 2014). Furthermore, beyond their preference for related and familiar mice (e.g. in communally nursing females, Weidt et al. 2008, Harrison et al. 2018), relatively little is known about how mice choose same-sex social partners. The few studies which address this question show that attractiveness does vary across individuals: female and male C57BL/6J mice are more attractive to same-sex conspecifics when intoxicated with ethanol (Wood and Rice 2013, Kent et al. 2014); male Balb/cJ mice are more attractive to C57BL/6J (albeit not CD-1) males after being injected with D-cycloserine, a partial NMDA receptor agonist which promotes sociability in the three-chamber sociability test (Benson et al. 2013); and CD-1 mice reared in mixed-sex litters are more attractive to both CD-1 males and females compared to those reared in single-sex litters (Terranova et al. 2000). Thus, not all social companions are created equally in the mouse world. And yet despite this, relatively little is known about social attractiveness, and the impact of housing condition on attractiveness has not yet been investigated.

1.4 Study overview

One of the first things I read as a graduate student was my advisor Georgia's NSERC discovery grant application which suggested that SH might affect the ability of mice to engage in normal social interactions. This led me to my research question: does SH impair social behaviour in mice by making them less sociable and/or less attractive as social companions? This seems like an important question to answer since laboratory mice often spend their whole lives in a confined space with the same restricted social group which they have to rely on to keep warm and regulate stress. I therefore tried to answer this question with female mice using a novel familiarization paradigm to equally expose focal mice (EE and SH C57BL/6 mice) to two stimulus mice (one EE, the other SH) in a modified sociability apparatus. Chapter 2 of my thesis will describe this process and explore the differences in sociability and attractiveness between EE and SH mice. Then, in Chapter 3 I will investigate how three SH-induced behaviours (SB, depressive IBA, and agonism [i.e. aggression and milder dominance-related behaviours, see Chapter 2]) influence sociability and attractiveness. Finally, in Chapter 4 I will discuss the implications and limitations of my research and suggest some ideas for future research.

2 Chapter 2: The effects of standard housing on mouse sociability and attractiveness in a social preference test

2.1 Introduction

Since social behaviour is crucial for many aspects of a mouse's life (see Chapter 1), and small barren cages are the norm in research facilities, it is important to know whether standard housing (SH) impairs the ability of mice to interact normally with other mice. This introduction will review what we know and what we do not know about the effects of relatively enriched versus relatively barren housing on social interactions, taking a comparative approach but always ending with the evidence for mice. I first cover housing effects on playfulness and agonism, which I will propose influence social attractiveness. I then review the few studies investigating housing effects on sociability, before outlining the hypotheses that will be tested in this chapter.

Across a range of species, standard barren housing can affect the social behaviour of captive species by altering levels of aggression and play compared to the levels seen in more enriched housing. Of course these behaviours vary across species but aggression usually includes biting or pecking, chasing, and pushing conspecifics away from a resource, while play is usually measured in juveniles and often includes "toned-down" or inhibited versions of aggressive behaviours (e.g. gentle biting and chasing that does not elicit escape attempts) which are sometimes reciprocal or accompanied by signals of non-aggression such as the open-mouth "play face" (e.g. in ferrets, *Mustela putorius furo*, Reijgwart et al. 2018). Rats (*Rattus norvegicus domestica;* Abou-Ismail et al. 2010), ferrets (Reijgwart et al. 2018), mink (*Neovison vison;* Meagher et al. 2014), pigs (*Sus scrofa domesticus;* e.g. Schaefer et al. 1990), horses (*Equus caballus*; Jørgensen et al. 2011), chickens (*Gallus gallus domesticus*; Gvaryahu et al. 1994), and several species of primate (Honess and Marin 2006) are more aggressive towards intruders and cagemates when kept in barren cages than when kept in environmentally enriched (EE) enclosures. Compared to their EE counterparts, standard-housed (SH) rats (Morley-Fletcher et al. 2003, Schneider et al. 2006), ferrets (Reijgwart et al 2018), mink (Meagher et al. 2014), and pigs (Chaloupková et al. 2007) are also less playful as juveniles. Similar housing-induced changes in

social behaviour have been found in mice (*Mus musculus domesticus*), my subject species: SH juveniles exhibit less socially stimulated playful hopping (Marashi et al. 2003, Laviola and Alleva 1995), and SH adult females are more agonistic than EE females (i.e. prone to bouts of chasing, ano-genital sniffing and displacing cagemates from resources such as food; e.g. Harper et al. 2015, Nip et al. 2019). In contrast, EE often increases aggression in male mice due to resource defence, and here aggression can be much more severe and injurious (e.g. Howerton et al. 2008). One study, however, found that compared to SH males, EE male mice employ a "less aggressive and more affiliative social interaction strategy" (Pietropaolo et al. 2004). Like aggression, rough grooming of cagemates is more prevalent and severe in SH female mice and can lead to balding and whisker loss (Bechard et al. 2011). Thus, housing in barren standard cages affects the quality of social interactions between cagemate mice, especially females, reducing playful behaviour and promoting negative social interactions such as aggression and over-grooming.

Could these behavioural changes, especially in aggression, make SH animals less attractive as social companions? One study of play in young rats suggests that playfulness is attractive: when given the choice to interact with a normal, playful conspecific or one rendered non-playful, but still socially responsive, by physical confinement or treatment with amphetamine or chlorpromazine, juvenile females consistently chose the arm of the T-maze containing the playful conspecific (Humphreys and Einon 1981, although these treatments may also affect other behaviours). Research in humans and rats also suggests that agonistic subjects are less attractive to their peers: more aggressive male and female teenagers are less liked by their classmates (like female mice, teenage girls tend toward non-injurious forms of aggression, and these predict low 'likeability'; Prinstein and Cillessen 2003, Cillessen and Mayeux 2004), and, when given the choice between a more aggressive male and a less aggressive male in a T-maze, male rats prefer to affiliate with the less aggressive conspecific (Taylor 1976). However, the direct role of physical housing on social attractiveness has not been looked at for same sex affiliates: to date it has only been assessed in the context of mate choice experiments. Male fruit flies (*Drosophila melanogaster,* Dukas & Mooers 2003) and mink (Díez-León et al. 2013) thus gained fewer copulations if SH, while SH male rats received less attention from females than did

EE males (Cao et al. 2017). In mice, female preference for EE over SH males has been shown only indirectly: females invest less maternal care (i.e. licking and nursing) in pups sired by SH males than those sired by EE males (Mashoodh et al. 2012). Thus, although the effect of SH on attractiveness of same-sex social partners has not been investigated, housing-induced changes in social behaviour may make SH mice less socially attractive than EE mice.

There is also evidence that SH reduces sociability in rats and mice, although their reaction to EE conspecifics has not been assessed. Juvenile male rats housed pre- and post-natally in EE cages spent more time sniffing an unfamiliar male SH conspecific in a novel arena than did juveniles housed in SH (Sparling et al. 2018). Likewise, in adulthood male and female rats housed in large multi-level cages with toys, tunnels, and ramps initiated more social contact (e.g. approach/follow, mounting, and allo-grooming) than SH rats in a 10-minute interaction with a novel same-sex SH stimulus rat in an arena to which they were previously habituated (Kentner et al. 2018). Finally, in rats modified to model schizophrenia (by embryonic treatment with the mitotoxin methylazoxymethanol), just seven days of EE during adolescence (postnatal days 23-29) prevented the reduction of social interaction between two unfamiliar rats seen in the SH group (Bator et al. 2018). Turning to mice, compared to those kept in standard cages, male CD-1 and NMRI mice housed in EE cages (i.e. a larger cage with running wheels, shelters, tunnels, and extra nesting material) starting at weaning spent more time near or investigating an unfamiliar, same-sex conspecific in a novel arena (McQuaid et al. 2018, Mesa-Gresa et al. 2013). Male mice prenatally exposed to valproic acid (which, when administered embryonically, induces autism-like behaviour including anxiety and reduced social interaction) also sniffed an unfamiliar same-sex SH conspecific less if housed in SH rather than EE cages (Yamaguchi et al. 2017). Finally, in a study comparing mice enriched with a nest, shelter, and running wheel, compared with being housed with only a nest (Whitaker et al. 2016), no housing effects were seen on male Balb/c mice or 129/SV mice of either sex. However, EE female Balb/c mice (a strain known to engage in low levels of social interaction, at least when SH; e.g. Sankoorikal et al. 2006) spent significantly more time in the social chamber of a social interaction test. While the mechanisms underlying this housing effect are unknown, it seems that SH is associated with reduced social interaction directed to unfamiliar conspecifics.

Overall it thus seems that SH reliably reduces the quantity of social interaction (at least to novel conspecifics), and that it also promotes aggression in female mice, which perhaps has the potential to make individuals less attractive as social partners. Based on these past findings, I hypothesized that SH would impair sociability and attractiveness in female mice. Thus, in a modified social preference test where a focal mouse can choose to spend time near an EE stimulus mouse, a SH stimulus mouse, or inanimate objects, I predicted that 1) SH focal mice would be less sociable, i.e. they would choose to spend less time near stimulus mice than would EE focal mice, and 2) SH stimulus mice would be less attractive than EE mice as social partners, i.e. both types of focal mice would spend less time near SH stimulus mice compared to EE stimulus mice. If my predicted effects were found, I then planned to investigate the role of SH-induced behavioural changes including agonism (given and received) in regulating sociability and social attractiveness: these results can be found in Chapter 3.

2.2 Methods

2.2.1 Ethics statement

All procedures and husbandry techniques were approved by the University of Guelph Animal Care Committee (AUP #3700) and comply with Canadian Council on Animal Care guidelines.

2.2.2 Animals and housing

Unrelated female mice of three different strains (N=55 each of BALB/c [‘BALB’], C57BL/6 [‘C57’], and DBA/2 [‘DBA’]) arrived at our facility from the supplier (Charles River Laboratories, Raleigh, North Carolina, USA) in two batches at three to four weeks old (all weaned at the same age to ensure the same early socialisation). They were randomly assigned to either EE or SH housing, where they lived in mixed-strain trios: a system that enhances statistical

power and allows individual identification without the use of painful or aversive individual marking methods (BALBs being naturally white, C57s black, and DBAs grey-brown; Walker et al. 2013, 2016). Other benefits to this combination of strains are their different reactions to SH: while C57s typically spend a large proportion of their waking hours (i.e. the dark phase) inactive-but-awake (IBA; i.e. standing still with eyes open), DBAs perform high levels of route tracing, and BALBs high levels of bar-mouthing (e.g. Nip 2018). C57s were chosen as the focal strain (see *Test subject selection*) because of their high sociability compared to BALBs (Sankoorikal et al. 2006) and because they exhibit a depressive response to SH (showing increased IBA, e.g. Nip 2018) which may affect sociability. Standard housed mice were kept in transparent plastic shoebox cages (27cm l X 16cm w X 12cm h, Allentown Inc.) with corn cob bedding and two types of nesting material (crinkled paper strips and cotton pads). EE cages were made of opaque plastic, measured 60cm l X 60cm w X 30cm h and were provisioned with enrichment items which mice are motivated to access (Walker 2016, Tilly et al. 2010) and which reduced SB, depressive-like behaviours, anxiety and aggression in past studies (e.g. Llorens-Martín et al. 2007, Bouet et al. 2011, Gross et al. 2012, Nip et al. 2019). These included a large plastic nest box, one cardboard box, one metal upright running wheel, one plastic saucer-shaped running wheel with igloo shelter base, one plastic shelter, two plastic tubes (two to four inches long), two plastic balls, two tissues stuck in the cage bars, one pinecone (various species), one small sock, one large sock hammock, one paper cup and one half egg carton suspended from the cage lid, one climbing item (wooden arch or ladder), and one chewable item (three small wooden blocks or one large wooden popsicle stick) in addition to the bedding and nesting materials provided in SH cages (Fig. 2.1). Climbing and chewable enrichment items were each swapped monthly. All mice were given food (Harlan Teklad 14% protein rodent chow) and tap water *ad libitum* and were kept on a reverse 12:12 hour light cycle with lights off at 7:00 and lights on at 19:00 in a temperature- and humidity-controlled room (21±1ºC and 35-55% relative humidity). SH cages were cleaned weekly, while the larger EE cages were cleaned monthly because we did not have enough of them to clean all EE cages at once so five or six were cleaned each week on a rotational basis.

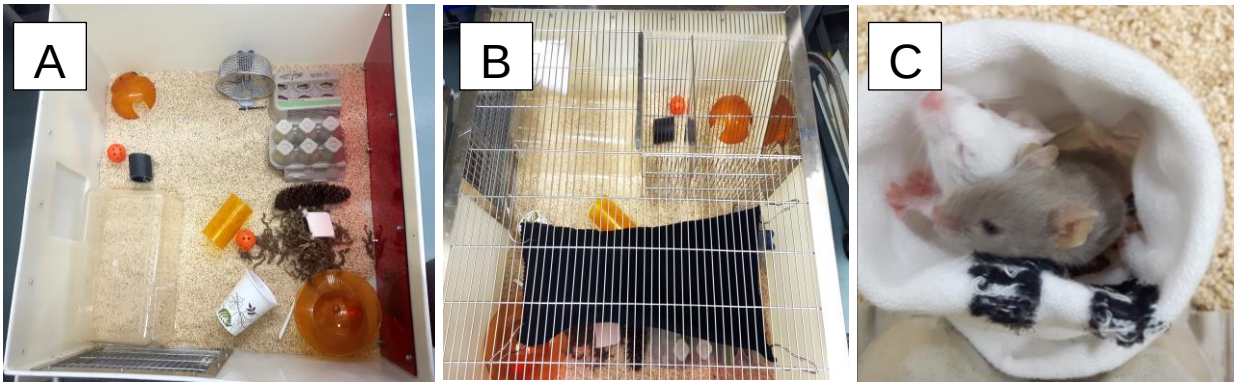

Figure 2.1 Environmentally enriched (EE) cage set-up viewed from above without (A) and with lid (B). Photo (C) depicts DBA and Balb/c mice snuggling together in a sock added as enrichment. Enrichment items not pictured are the wooden arch, wooden ladder, and half egg carton suspended from the cage lid.

2.2.3 Test subject selection

At five (N=16) or eight (N=24) months old (ages pragmatically chosen to schedule tests around other projects going on in the lab), 40 focal mice (all C57s; 20 EE and 20 SH) and 80 stimulus mice (40 BALB and 40 DBA, again evenly split between EE and SH) were chosen from the population. All barbered mice were excluded from the study to maintain observer blindness (since at the beginning of the study barbering was only seen in standard cages). Unbarbered C57s were then ranked by their levels of IBA (see Chapter 1; the most prevalent SH-induced behaviour in this strain) in the most recent data collection period. These rankings were used to split C57s into tertiles of high, medium and low IBA performance within each housing type, relative to other mice in the same housing condition, with six or seven mice from each tertile in each housing type being randomly selected (to ensure mice with a range of IBA from both housing types would be included in the social behaviour tests). Turning to the BALB and DBA subjects who would act as stimulus mice, we first excluded mice in cages where the C57 had been removed due to barbering, to ensure that all stimulus mice had had equal exposure to a mouse of the focal strain before testing. We ranked remaining BALBs by home cage SB level (see benefits of selected strains above; also see Chapter 1), split them into tertiles of relatively high, medium and low SB performance within each housing type, and six or seven mice of from

each tertile in each housing type were randomly selected. The same process was followed for the DBAs.

The hypothesis that EE enhances social interaction in female mice predicts that compared to SH mice, EE mice will 1) as focal mice, choose to spend more time in proximity to other (familiar, but non-cagemate) mice than to non-social stimuli, and 2) as stimulus mice, be more attractive to other mice. Each focal mouse was therefore allocated two stimulus mice of the same strain but differentially housed to ensure that differences between stimulus mice were only phenotypic and not genetic. Thus, for each focal mouse, stimulus mice were either two BALBs or two DBAs, one of whom was EE and one of whom was SH. To ensure all mice within a group were of similar size, this allocation was based on bodyweight (since bodyweight can affect dominance ranking and thus potentially social behaviour [e.g. Kim et al. 2015]), with the heaviest C57 being grouped with the heaviest stimulus mice (whether BALB or DBA), alternating between stimulus strains so that average C57 size was similar for each stimulus strain (mean C57 weight ± SD = 27.06 ± 5.00g for Balbs and 26.22 ± 4.29g for DBAs). All stimulus mice were then shaved (a 10 mm X 5 mm rectangle), on either the upper or lower back, as a method of individual identification during live observation during the tests. Shave patterns were counterbalanced across stimulus mouse housing in each batch, and were always unknown to the observer to maintain blinding. All subjects were housed in their original mixed-strain trios and housing treatments throughout the study, only interacting with stimulus mice during testing. For this testing (details below), the 40 focal mice and their allocated stimulus mice were exposed to each other, and tested, in four batches ranging in size from 18-36 mice (6-12 focals and their respective stimulus mice). This was to make the work practically feasible, and to accommodate other research going on in the same room.

2.2.4 Familiarisation sessions

For focal mice to make an informed choice between their stimulus mice, they first had to become equally familiar with them and learn the differences in their behaviour. The exposure of

focal mice to conspecifics from EE vs. SH cages was thus achieved via a novel paradigm of 'familiarisation sessions' in which SH and EE focal animals were familiarised to stimulus mice of both housing types in a neutral environment to avoid creating a resident-intruder relationship. All sessions occurred under red light, starting at two hours after the beginning of the dark phase and all tests were recorded with a Sony DCR-DVD103 DVD Handycam. Sessions took place in a modified social preference apparatus (cf. e.g. Moy et al. 2004; Fig. 2.2): a 60cm X 60cm square with 15cm tall corrugated plastic walls with wire mesh (1x1cm) across each corner. Each corner also had a semi-circle drawn around it 7.5 cm (approximately one mouse body length, tail excluded) from the wire mesh to delineate the area in which the focal mouse would later be scored as "near" the stimulus mice during the sociability and social attractiveness test (see below). We included an overturned transparent Tupperware container (24cm l X 24cm w X 12cm h) with the bottom removed and holes cut in each corner and each side in the centre of the apparatus, to reduce the amount of open space perceived by the mice while still allowing them to see and pass through the container. The apparatus was covered with a clear sheet of perforated plexiglass at all times while mice were inside to prevent them from crawling out.

All three mice per focal-stimuli group were carried into the testing room at the same time through an adjoining door by a research assistant, to maintain observer blindness to mouse identity. The focal mouse was immediately placed in the centre of the apparatus and allowed to habituate for ten minutes (cf. e.g. Whitaker et al. 2016), with stimulus mice remaining in their transport cages for this time. The focal mouse was then guided under a wire mesh dome and enclosed in the centre of the apparatus while experimenters added a novel object (a large foam die for Batch One, or a plastic toy dinosaur for Batches Two-Four) to two opposite corners of the apparatus, and one stimulus mouse to each of the other corners behind the wire mesh (Figure 2.2). Inanimate objects were included in the test to control for potential differences in general exploration by the focal mice (cf. Moy et al 2004). The position of EE and SH stimulus mice was counterbalanced between the two stimulus corners, but held constant for each group of three throughout the experiment. For the "enclosed" phase, the focal mouse was released and, while the stimulus mice were enclosed in their corners, an observer (blind to the identities of all mice) live recorded the amount of sniffing directed at each stimulus mouse by the focal mouse for five

minutes using a stopwatch in each hand. This included all instances of the focal mouse sticking her nose through the lower half of the wire mesh divider (cf. Choleris et al. 2006) with or without obvious sniffing, and any sniffing through the upper part of the wire mesh if the focal mouse made direct contact with the stimulus mouse. This investigatory sniffing did not include biting the bars of the wire mesh.

After five minutes, the focal mouse was again trapped under a wire mesh dome in the centre of the apparatus while the wire mesh dividers were removed from the novel object corners. The focal mouse's dome and the stimulus mice's dividers were then removed simultaneously, and the observer again scored investigatory sniffing by the focal mouse towards each stimulus mouse for five minutes. In this "free interaction" portion of the encounter (*cf.* Thor and Holloway 1982), investigatory sniffing was defined as sniffing or making nasal contact with any part of the stimulus mouse's body or closely following the stimulus mouse at a walking pace (within about 1 cm; Thor and Holloway 1982). This kind of sniffing is a good indicator of familiarity because it occurs at high rates between unfamiliar mice and much lower rates when mice are familiar (Phan et al. 2011). All instances where the stimulus mouse squeaked while being sniffed, usually in the ano-genital region, were considered agonism and excluded from investigatory sniffing. Agonistic and stereotypic behaviours of the focal and stimulus mice were later scored from video by an observer blind to mouse identity using JWatcher V1.0 video analysis software (Blumstein et al. 2006): see Chapter 3.

Two identical apparatuses were used during testing. To remove odours, each was thoroughly sprayed and wiped down with 70% ethanol (cf. Leo and Pamplona 2014) after each use and left to dry while the other apparatus was in use. Sessions were repeated daily until paired t-tests showed that investigatory sniffing had reached a lower plateau for all combinations of focal housing and stimulus housing (i.e. until both EE and SH focal mice were familiar with both their EE and SH stimulus mice). Once sniffing was significantly decreased for three successive days compared to Day One, and was also not significantly different from day to day (see Figure 2.3), mice were deemed familiar to each other, and thus able to make an informed decision between stimulus mice. This took five sessions for Batches One-Three, and four sessions for Batch Four. The sociability and social attractiveness tests were then run the next day.

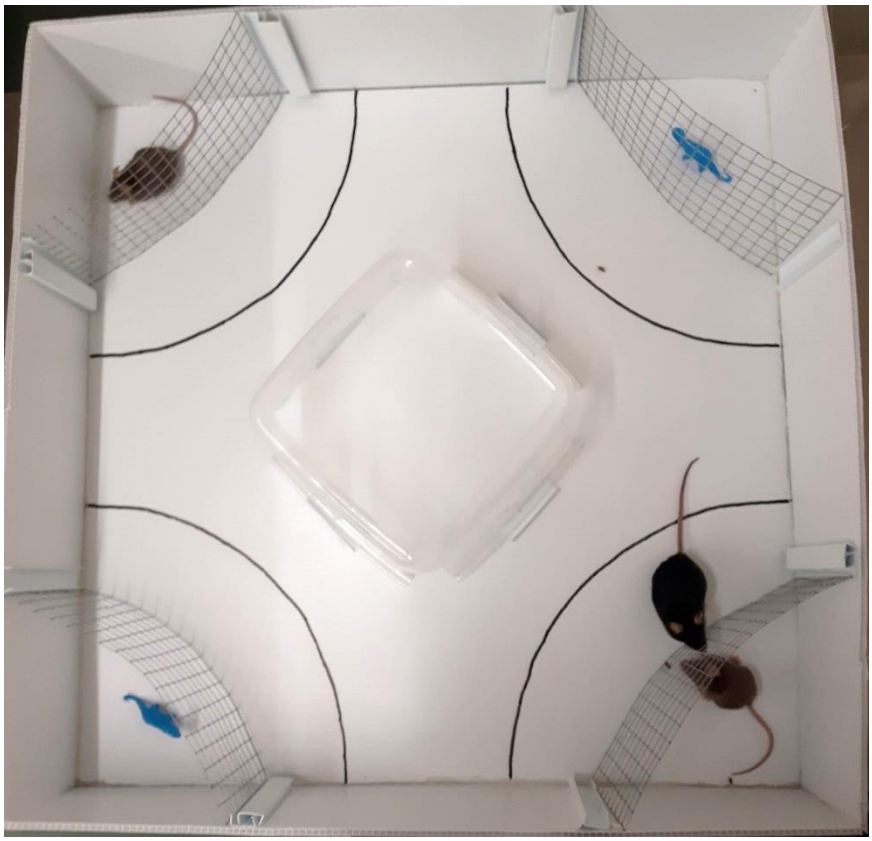

Figure 2.2 Sociability and attractiveness apparatus during the 'enclosed' phase of testing. The black C57BL/6 mouse is the focal animal and can move around the apparatus freely, while the brown DBA/2 stimulus mice are confined behind wire mesh dividers. During the 'free interaction' phase, wire mesh dividers were removed from all corners and all mice could move freely, interact, and access the inanimate objects (here, blue plastic dinosaur toys).

2.2.5 Sociability and social attractiveness test

On the day following the final familiarisation session (and in the same order as in the familiarisation sessions), the focal mouse was placed in the apparatus for ten minutes to habituate, before being enclosed under a wire mesh dome while the novel objects and stimulus mice were added to their respective corners. The focal mouse was then released and filmed for 30 minutes. From these videos we scored the amount of time the focal mice spent in the delineated area near each corner (i.e. with at least two feet in the area between the semi-circle

and the wire mesh divider) using JWatcher V1.0 video analysis software (Blumstein et al. 2006). These data were used to infer each mouse's interest in being near a social stimulus vs. an inanimate object. They were also used to assess the relative social attractiveness of each stimulus mouse over the other (as used in e.g. preference tests for mates, or for conspecifics dosed with drugs like alcohol: Kavaliers et al. 1999, Kent et al. 2014). Shave patterns were then used to decode mouse ID (all video observations again being conducted blind to housing).

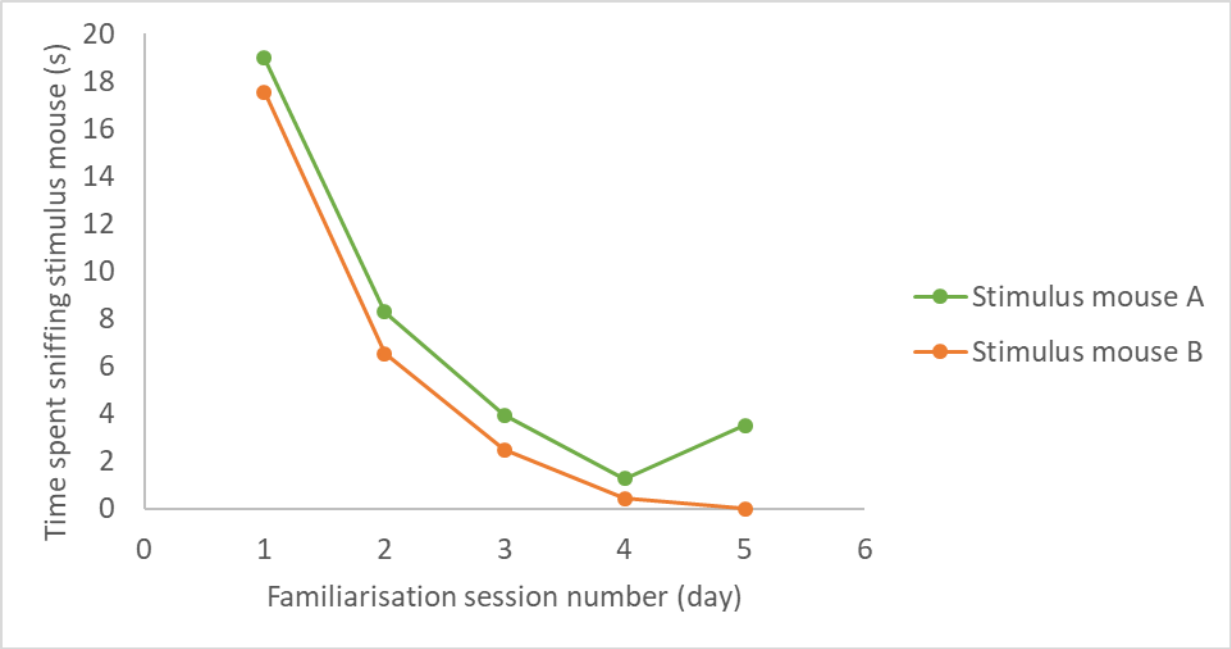

Figure 2.3 A typical graph of investigatory sniffing by a focal mouse towards each of her stimulus mice over the course of five familiarisation sessions. Stimulus mice are labelled A and B to ensure blinding.

2.2.6 Statistical analyses

Analyses of preference and sociability data were conducted using general linear mixed models in JMP 14 (SAS Institute, 2018). Data transformations (e.g. Box-Cox, Log, and square root) were performed as needed to meet the assumptions of parametric models. First, a model analysed the fixed effects of focal mouse housing, stimulus mouse strain and their interaction on sociability, with total time near both stimulus mice combined as the dependent variable, and batch and focal mouse cage nested within focal mouse housing and stimulus mouse strain as random effects. Object exploration was then assessed with the same fixed and random effects, and total time spent near inanimate objects as the dependent variable. When interactions were

significant, planned contrasts were used to compare SH and EE mice within each relevant sub-group (to avoid the problems of multiple testing inherent in the Tukey's tests; Lazic 2016). A repeated measures model was then used to assess effects of stimulus mouse housing on social attractiveness (i.e. time spent by the focal mouse near each stimulus mouse), with stimulus mouse housing, focal mouse housing, stimulus mouse strain, and all interactions as fixed effects, and with batch and focal mouse cage as random effects. Again, whenever interactions were significant, planned contrasts were used to compare SH and EE mice.

2.3 Results

Both types of focal mice spent more time near stimulus mice than inanimate objects (EE focal mice: $F_{1,34}$=58.199, P<0.0001; SH focal mice: $F_{1,34}$=9.759, P=0.004). However, compared to SH focal mice, EE focal mice spent significantly more time near stimulus mice ($F_{1,37.6}$=16.419, P=0.0002; Fig. 3), and this was not because they were more exploratory overall: compared to SH focal mice, EE focal mice did not spend more time near the inanimate objects, and indeed their average values were lower ($F_{1,37.6}$=2.593, P=0.116; Fig. 2.4).

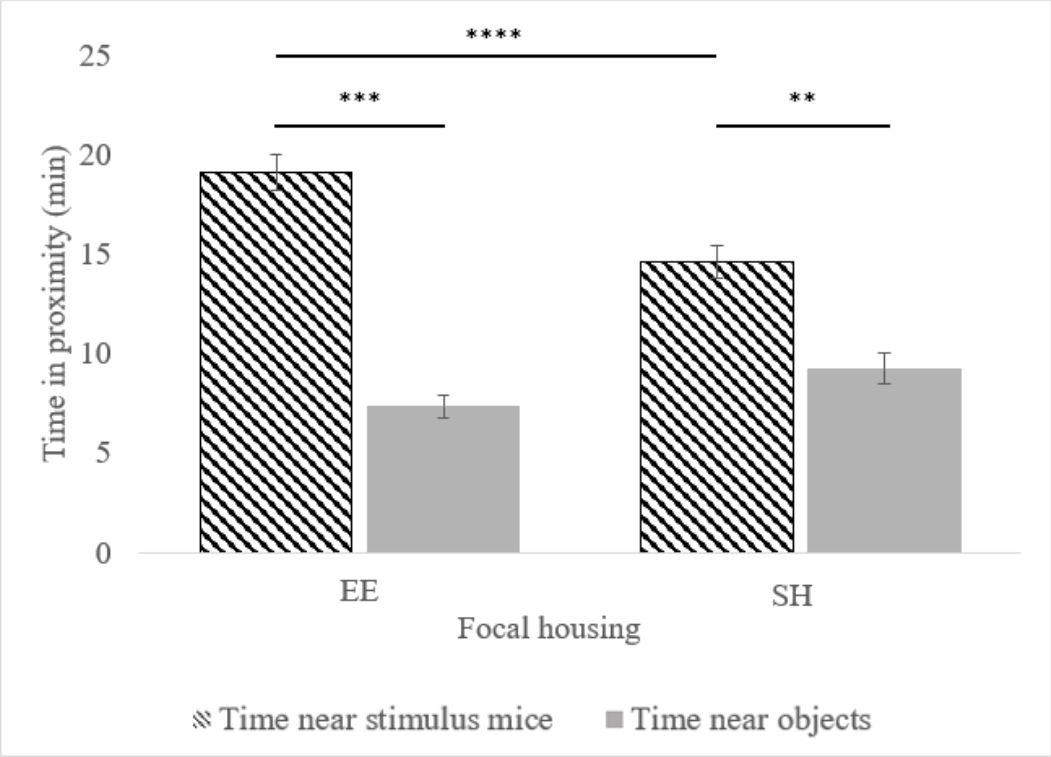

Figure 2.4 Time (min) spent by environmentally enriched (EE) and standard-housed (SH) focal mice in proximity to stimulus mice and objects. Error bars represent standard error of the mean. ** denotes statistical significance at *P*<0.01. * denotes statistical significance at *P*<0.001. **** denotes statistical significance at *P*<0.0001.**

However, the repeated measures model (i.e., including 'stimulus mouse housing' as a factor), revealed a significant interaction between focal mouse housing and stimulus mouse housing ($F_{1,33.14}$=8.521, P=0.006). Planned contrasts showed that this interaction reflected two effects. First, the housing effect on focal mouse behaviour varied with the nature of the stimulus mouse: EE focal mice spent more time near SH stimulus mice than did SH focal mice ($F_{1,54.59}$=18.734, P<0.0001) but the two types of focal mouse did not differ in their responses to EE stimulus mice ($F_{1,54.66}$=0.434, P=0.513; Fig. 4). Second, the relative attractiveness of the stimulus mice depended on focal mouse housing: EE focal mice spent more time near SH stimulus mice ($F_{1,32.39}$=4.566, P=0.040), while SH focal mice spent more time near EE stimulus mice ($F_{1,33.17}$=4.191, P=0.049; Fig. 2.5). There was no effect of stimulus mouse strain in any of these analyses.

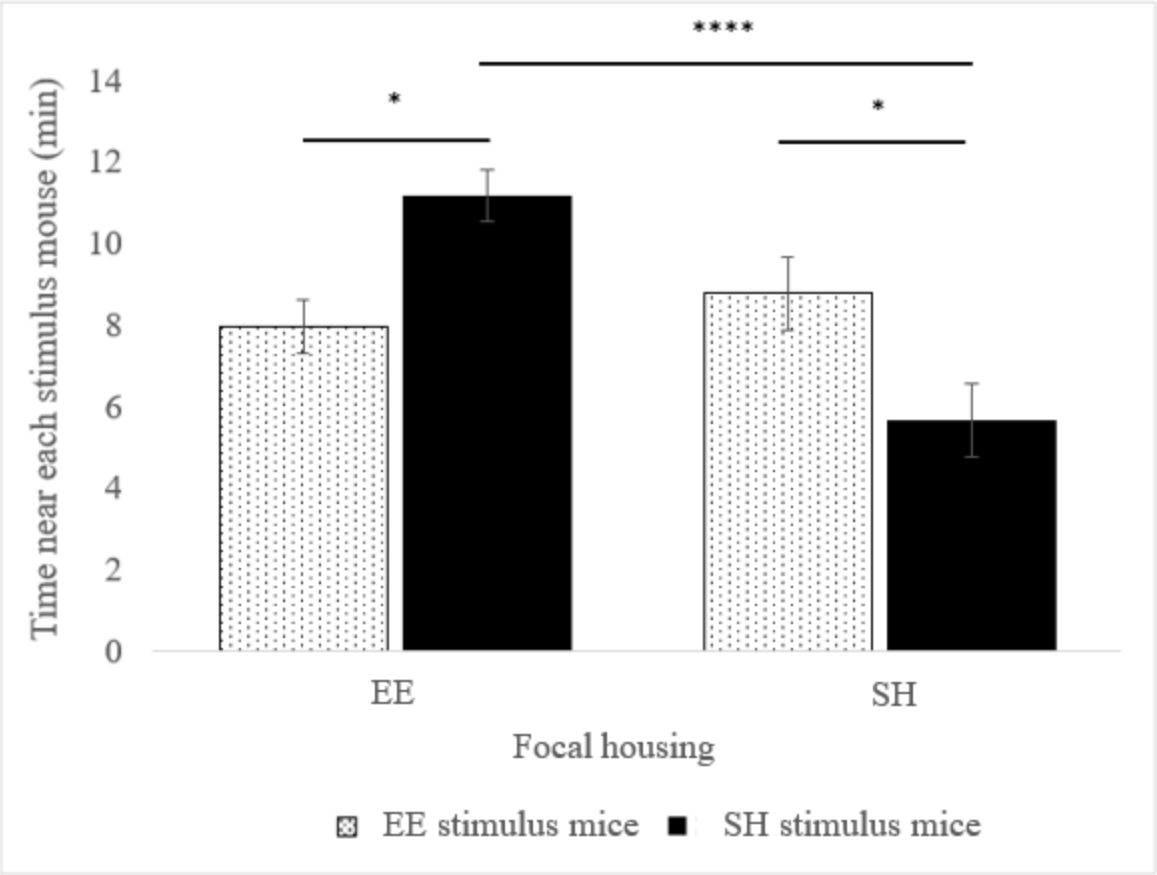

Figure 2.5 Time (min) spent by environmentally enriched (EE) and standard-housed (SH) focal mice near EE and SH stimulus mice. Error bars represent standard error of the mean. * indicates statistical significance at *P*<0.05. ** indicates statistical significance at *P*<0.0001.**

2.4 Discussion

Our first hypothesis, that EE mice would be more sociable than SH mice, was only partially supported, but for interesting reasons: our focal mice, SH and EE C57s, showed the predicted differences in proximity to other mice, EE being more sociable, but only if stimulus animals came from standard cages.

Thus, although SH focal mice spent less time near both their stimulus mice combined, this apparent reduction in sociability was due to avoidance of SH stimulus mice. Housing female C57 mice in typical, barren laboratory cages therefore did not reduce their overall sociability (since

interest in EE conspecifics was unaltered compared to SH focal mice). Nor did it reduce their propensity for exploration: SH and EE focal mice did not differ in proximity to inanimate objects. But in an apparatus where both EE and SH stimulus mice were present, compared to EE C57s, SH C57s showed an apparent selective reduction of interest in animals raised like themselves. This effect occurred regardless of stimulus mouse strain, applying equally to BALBs and DBAs. We thus replicated past work showing that SH decreases sociability towards SH stimulus mice (Mesa-Gresa et al. 2013, Whitaker et al. 2016, McQuaid et al. 2018), but in a new strain, C57s. As the first study to use stimulus mice from different housing systems (not just SH), we also revealed this effect to be limited to SH stimulus mice.

Our second hypothesized housing effect was that SH stimulus mice would be less socially attractive than EE animals. Proximity data showed that SH stimulus mice were less attractive to SH focal mice, as predicted. However, EE focal mice showed the opposite pattern, finding SH stimulus mice *more* attractive than those housed in EE. Thus, instead of supporting our hypothesis, the data suggested that each group of differentially housed C57s was most interested in mice from housing unlike their own.

Together, our results cautiously suggest that, compared to EE female mice, SH females are less sociable, at least towards SH stimulus mice; and that they are also less attractive, albeit only to other SH mice. As such, the most social interest occurred in EE focal mice approaching SH stimulus mice, and the least, in SH focals faced with SH stimulus mice. Results were therefore more complex than we had anticipated, but are exciting nonetheless: they partially support hypotheses 1) and 2), and show for only the second time (cf. Mashoodh et al. 2012), and the first time for females faced with other females, that laboratory mice can distinguish between conspecifics raised in physically different environments. Chapter 3 will now discuss and investigate the potential reasons why.

3 Chapter 3: Why EE and SH mice differ in sociability and attractiveness

3.1 Introduction

In Chapter 2 I outlined the way in which SH-induced agonism could potentially make female mice less attractive to each other. But in Chapter 1 I also described other behavioural effects of SH conditions, namely depressive behaviour and SB. These SH effects on other behaviours seem likely to relate to social interaction, as I will review in this chapter, and are also relevant to my own subject mice. The mice used in this study were also part of a project investigating the effects of standard housing (SH) on agonism, stereotypic behaviour (SB), and depressive behaviours (Nip 2018, Nip et al. 2019). That project revealed that, in our mice, agonism and SB were higher in SH than in enriched housing for all strains (C57BL/6, DBA/2, and Balb/c; Nip 2018), as expected (see Chapter 1). Depressive inactive-but-awake (IBA) behaviour in the home cage and immobility in the Forced Swim Test (FST) were also consistently higher in SH, as expected, for C57s and Balbs, but not for DBAs (Nip et al. 2019). Because all these behaviours can have negative effects on social interaction (which I will review next), I decided to examine these behaviours as potential mechanisms regulating the sociability and attractiveness results found in Chapter 2. Thus, in this introduction I will first describe the known effects of depressive symptoms on sociability and attractiveness, first in humans, then in mice. Similarly, I will then explain how SB and perseveration can affect social behaviour, again first in humans, then in mice. Then I will finish by outlining the hypotheses under test in this chapter.

In humans, depression is associated with a slew of psychological and behavioural changes which can cause reduced interest and participation in social activities (i.e. low sociability). These include learned helplessness, anhedonia, negative judgement bias, apathy (i.e. reduced goal-oriented behaviour), and increased aggression. Thus for example, children who exhibit learned helplessness (in this case those who attributed their rejection by a potential pen pal to their own social incompetence) were more likely to socially withdraw (in this study, by

refusing to write their pen pal another letter) and to repeat previously unsuccessful social strategies (here, by writing their pen pal a second letter which contained relatively little new information compared to the first; Goetz and Dweck 1980). These children were also rated as least likeable by their same-sex peers (compared to those who did not attribute their rejection to their own social incompetence; Goetz and Dweck 1980), suggesting that 'helpless' children are both less sociable and less attractive than non-helpless children. Depressed children also tend to interpret ambiguous peer interactions as threatening (Reid et al. 2006), likely because of negative judgment bias (i.e. the tendency to interpret neutral stimuli as aversive, see Chapter 4).

Anhedonia and apathy are two other common features of depression which can pertain to social contact, leading to social withdrawal (American Psychiatric Association 2013). In schizophrenia patients, for example, anhedonia is associated with less participation in social interactions such as meeting up with and speaking with friends on the phone (Blanchard et al. 1998); and apathy can similarly reduce an individual's motivation to socialise with same- and opposite-sex social partners (Cathomas et al. 2015). Apathy has also been argued to have similar effects in rodents (Cathomas et al. 2015). So, turning now to social correlates of other aspects of depressive behaviour in rodents, examples are few, but all come from mouse and rat models of depression. Social defeat is often used to create rodent models of depression which, unsurprisingly, then exhibit social avoidance (e.g. Chaudhury et al. 2013, Covington et al. 2011). However, using unpredictable chronic mild stress (e.g. long-term exposure to different stressors, some housing-related such as cage tilting and damp bedding) can also induce both depression-like states in rodents and low sociability (e.g. fewer entries into and time spent in a compartment next to a confined same sex conspecific, compared to an empty compartment out of view of the conspecific): an effect correlated with anhedonic reduction of sucrose consumption (Kompagne et al. 2008, though c.f. D'Aquila et al. 1994). Lastly, depressive behaviour is associated with increased aggression in humans (American Psychiatric Association 2013), mouse models of depression (Mineur et al. 2003), and female mice in SH (those who do more helpless inactive-but-awake behaviour [see Chapter 1] are more aggressive towards their cagemates, Nip et al. 2019). In humans, depressed children often direct this aggression towards their peers (Dodge and Crick 1990, Pornari and Wood 2010) which, as previously discussed (see above), has a negative impact on peer-rated social attractiveness (Prinstein and Cillessen 2003, Cillessen and Mayeux

2004). Thus, anhedonic and apathetic individuals are generally less sociable, and one can imagine that individuals who are aggressive or who do not find social interaction pleasurable might also be less attractive social partners. For all these reasons, the depression-like states induced in mice by SH might predict both low sociability and low social attractiveness.

Stereotypic behaviour (SB) has also been linked to low sociability and attractiveness in mice and other species. In humans, SB is a core symptom of autism spectrum disorder which is typified by impaired social communication (American Psychiatric Association 2013). Although some researchers have found no relationship between social and stereotypic behaviours in autistic individuals (Benning et al. 2016, Shuster et al. 2014, Joseph et al. 2013), others dispute this. For example, there is a negative correlation between levels of SB and social behaviour in autistic toddlers (Schertz et al. 2016). Autistic children with higher levels of SB also have greater social and communication deficits, including low social initiation indicating low sociability (Lam et al. 2008). Various interventions to reduce SB in autistic humans also increase social interaction rates (reviewed by Lanovaz et al. 2013). Regarding social attractiveness, the odd nature of some SBs can make others less likely to interact with autistic people (Durand and Carr 1987). Indeed, autistic children are also bullied more than their neurotypical classmates (Cappadocia et al. 2012, Schroeder et al. 2014). Stereotypic behaviour thus influences both sociability and attractiveness in humans. In mice, Nip (2018) found that the most stereotypic DBA/2 females engaged in less social behaviour even after accounting for their large amount of time spent stereotyping. In another study, highly stereotypic female mice tended to be poor at passing on information in a social learning task suggesting that they are perceived as unreliable by their peers and, for those in EE cages, performance of SB also covaried with receiving aggression from cagemates (Harper et al. 2015, though c.f. Pond et al. unpublished manuscript). Mouse models of autism such as the BTBR T+tf/J and C58/J strains exhibit increased SB as well as low sociability (e.g. Moy et al. 2007, Ryan et al. 2010). Furthermore, many treatments which promote SB concurrently induce poor social skills or increased aggression in rodents such as exposure to exhaust (Chang et al. 2018), serotonin depletion (Kane et al. 2012), dopamine transporter knockout (Rodriguiz et al. 2004), *shank*3 gene deletion (Peça et al. 2011), and treatment with valproic acid (Schneider and Przewlocki 2005). The reverse is also true: in mice,

treatment with oxytocin (Teng et al. 2016) and housing in EE cages (Schneider et al. 2006) reduce SB while promoting social exploration.

SB is also often associated with perseveration (see Chapter 1) which, in autistic children, predicts reduced frequency of initiation of social contact (McEvoy et al. 1993) and a reduced participation in social activities (Memari et al. 2013). In C57BL/6 lab mice, SB severity correlates positively with levels of perseveration (Garner et al. 2011; Novak et al. 2016). Specifically, C57BL/6 mice who spent more time performing stereotypies of all kinds (bar-mouthing, route tracing, twirling, and jumping) produced more non-random sequences of guesses in a two-choice guessing task (Garner et al. 2011). Likewise, C57BL/6 mice with higher levels of stereotypic bar-mouthing, although not route-tracing, tended to choose the same option multiple times in a row in a two-choice guessing task (Novak et al. 2016). This corroborates similar results in dogs (Protopopova et al. 2014), parrots (Garner et al. 2003a), songbirds (Garner et al. 2003b), zoo bears (Vickery and Mason 2005), caged mink (Dallaire et al. 2011), rhesus macaques (Pomerantz et al. 2012), and bank voles (Garner and Mason 2002). To my knowledge, only two studies have investigated the relationship between perseveration and social behaviour in non-humans (both in mice). Bortolato and his colleagues (2011) developed a line of mice with reduced expression of the enzyme monoamine oxidase-A (MAO-A) which showed perseverative marble burying and mist-induced grooming as well as significantly reduced number and duration of social approaches under test. They additionally noted increased resident-intruder aggression in MAO-A knockout mice (Bortolato et al. 2011). Similarly, Yang and colleagues (2012) found that mice of the BTBR T + tf/J strain display poor reversal learning and low sociability in the three-chamber social approach task. Given these findings, there is potential for SB to compromise social behaviour in mice, either through the behaviour itself or through its association with perseveration. Thus, both the SB *and* the associated perseveration induced in mice by SH might predict low sociability and/or low social attractiveness.

Since focal mice from different housing conditions had different reactions to the stimulus mice (see Chapter 2), I investigated the role of SH-induced behavioural changes on mouse sociability and attractiveness. I thus hypothesized that low sociability and attractiveness are due to increased SB (in which case sociability and attractiveness would covary with individual SB

levels) and/or depressive behaviour (in which case sociability and attractiveness would covary with time spent IBA). While I did not expect more agonistic mice to be less sociable, I did predict that more agonistic mice would be less attractive social partners and thus that mice from both housing conditions would spend more time near less agonistic stimulus mice. Based on studies of social defeat-induced depression, I additionally predicted that mice who received more agonism in their home cages would be less sociable and thus spend less time near stimulus mice from both housing conditions. Two additional physical characteristics of the stimulus mice were also analyzed to ensure they were not confounds: bodyweight, which can affect dominance ranking and thus potentially social behaviour (e.g. Kim et al. 2015), and odour, which may be affected by housing if the enrichment items we used (e.g. cardboard, pinecones, wood blocks, etc.) imparted a smell on the EE mice, or if EE conditions somehow alter the olfactory social cues given off by EE mice. I further hypothesized that differences in agonism, SB, and IBA would explain, i.e. statistically account for, the housing effect seen in Chapter 2. I therefore predicted that the significant focal housing*stimulus housing interaction would become non-significant when individual agonism, SB, and IBA levels were added to the statistical model. To do this, I used general linear mixed models (GLMM) to examine the relationships between housing-induced behaviour and social interaction: a more sophisticated statistical approach than the typical method in mouse sociability research of using a series of ANOVAs or t-tests (e.g. Moy et al. 2007). Compared to ANOVAs and t-tests, which only determine group differences, GLMMs can analyze covariation between fixed and independent continuous variables (cf. e.g. Lazic 2016). GLMMs can also be used to identify elimination or reduction of significant effects when other variables are added to the model, so indicating that they are statistically related or even redundant (cf. e.g. Nip et al. 2019).

3.2 Methods

3.2.1 Behavioural observations

For four hours starting two hours after lights off (cf. Harper et al. 2015), two observers alternated scan sampling behaviour every 10 minutes, recording the first behaviour observed for each mouse according to the ethogram in Table 3.1 (Nip 2018, Nip et al. 2019). Data were collected for five or six days within a four-week span, totalling 20-24 hours and 120-144 scans per cage. This unit was dubbed one 'observation period', and this process was conducted four times (opportunistically to accommodate other research going on in the lab) when mice were three, five, eight, and ten months old. Behavioural data collected at five months was used for Batches One and Two, and data collected at eight months was used for Batches Three and Four because they were closest in time to the sociability and attractiveness test for each batch. Because EE mice had more opportunities than SH mice to be out of sight, we calculated the *proportion of visible scans* in which each mouse performed SB, IBA, or gave/received agonism. Agonism encompasses both aggressive behaviours (pinning, chasing, mounting and rough allo-grooming) and milder agonistic behaviours (displacement and genital sniffing), and was used instead of overt aggression (e.g. fighting and biting) which is very rare in female mice (see Chapter 2).

Behavioural data for the stimulus mice was also collected during the final familiarisation session (see Chapter 2), using video scored by a blind observer who recorded the duration of each behaviour included in the ethogram below (Table 3.1). JWatcher was not used for this data collection: social interactions between mice can be very fast and short, and often require rewinding the video and watching interactions multiple times, which is not amenable to video scoring software. I did collect behavioural data for the focal mice in the familiarization sessions, to assess SB or IBA, but they did not perform either behaviour in these sessions.

All mice in each batch were weighed as part of the Test Subject Selection procedure (see Chapter 2) which occurred on the Friday before testing began for that batch on Monday. This generated the weight data used to check weight was not a confound or influential factor.

3.2.2 Odour preference test

One at a time, SH C57 focal mice [N=6 previously used in the sociability and attractiveness test, N=6 naïve mice] were placed in the sociability and attractiveness apparatus (see Chapter 2) with wire mesh dividers in place, but empty corners, and allowed to habituate for 10 minutes. Only SH focal mice were used for this test because they showed the biggest difference in time spent near each of their stimulus mice. The sample size is small, and both naïve and previously tested C57s were used for the odour preference test, because this was the last test I ran, and it occurred after some of the mice, now aged 17 months, had already been killed for another project. After habituation, the focal mouse was contained under a metal running wheel while dirty bedding and nesting materials were added to the corners behind the wire mesh. Both bedding and nesting materials were used because they have been found to contain different types of odour cues in mice (Van Loo et al. 2000), and because these materials likely smell of the enrichment items present in EE cages. Approximately 2 tbsp of bedding and an approximately 6 cm diameter ball of nesting material from EE and standard home cages were placed in opposing corners of the apparatus (bedding across from bedding, nesting material across from nesting material). While bedding was always placed in the same two opposing corners, and nesting material in the other two opposing corners, the location of EE vs. SH materials was alternated between trios of mice. Donor cages were chosen such that the focal mouse was not previously exposed to any mouse from that cage so that all scents would be equally novel. Furthermore, all selected donor cages contained a complete trio (ie. BALB/c, DBA, and C57) to ensure that the EE and SH stimuli each contained smells from the same number, and strain of mice. Once odour stimuli were added to the apparatus, the focal mouse was released, the plexiglass lid placed on the apparatus, and the mouse was filmed from above for 30 minutes. From video, the amount of time the focal mice spent in the delineated area of each corner was scored using Jwatcher V1.0 video analysis software (Blumstein et al. 2006) as in the sociability and attractiveness test (see chapter 2). One mouse gained access to the nesting material in one of the corners so was excluded from the analysis, leaving a sample size of 11 focal C57 mice.

3.2.3 Statistical analyses

Analyses were conducted using GLMMs in JMP 14 (SAS Institute, 2018). Data transformations (e.g. Box-Cox, Log, and square root) were performed as needed to meet the assumptions of parametric models.

To identify why, compared to EE mice, SH mice spent less time near SH stimulus mice (see Chapter 2), we added each of our chosen measures of focal and stimulus mouse home cage behaviour to the repeated measures model used in Chapter 2: IBA to test ideas about the effects of depression-like states, SB to test ideas about autism-like effects, and agonism given and received in the home cage. Thus variables pertaining to focal and stimulus mouse SB, IBA and agonism (given and received), both in their home cage (in the most recent observation period) and in the familiarisation sessions (for stimulus mice), as well as stimulus mouse bodyweights, were then individually added to the repeated measures model to determine factors that predicted focal mouse behaviour, and /or reduced the housing effect (see Nip et al. 2019 for a similar example). Non-significant interactions with continuous variables were sequentially removed, starting with the largest, to avoid over-fitting (e.g. Enqvist, 2005). Upon adding such continuous variables, their impact on the housing effects evident in the model used in Chapter 2 was also assessed by seeing whether or not these housing effects became less significant. As in the analyses for Chapter 2, when interactions were significant ($P<0.05$) or trending ($0.05<P<0.1$), planned contrasts were used to compare SH and EE mice within each relevant sub-group to avoid the problems of multiple testing inherent in the Tukey's tests (Lazic 2016).

The odour preference test was analyzed separately using a GLMM in JMP 14 (SAS Institute, 2018) with housing type of odour cage as a fixed variable and focal mouse ID as a random variable. Because of the small sample size, I conducted a power test for this analysis by calculating the effect size of the sociability and attractiveness test (Cohen's $d=0.8229$), then inputting this value, along with the sample size of the odour preference test ($N=11$) and the desired significance level ($\alpha=0.05$), into an online power calculator (Australia and New Zealand Melanoma Trials Group 2019) for a paired one-sided t-test.

Table 3.1 Ethogram used for sampling of home cage and familiarisation session behaviour adapted from Tilly et al. (2010), Harper et al. (2015), and Nip (2018).

Category	Behaviour	Description
Stereotypic behaviour	Bar-mouthing	Mouse holds bar in mouth with or without biting for 1 second or more
	Route tracing	Mouse runs around in the same pattern for 3 or more consecutive repetitions on floor or cage lid
	Jumping	Mouse jumps up and down in the same spot 3 or more times in a row
	Backflipping	Mouse does 3 or more backflips in a row
Agonism	Pinning	Mouse stands atop another mouse who is on her back
	Chasing	Mouse follows another mouse closely at a running pace
	Rough grooming	Mouse vigorously grooms another mouse, particularly in the shoulder regions; mouse is pulling the hair and pushing her paws down on the other mouse
	Mounting	Mouse mounts another mouse from the front or back
	Displacement	Mouse treads over or under another mouse (except when resting together); or pushes her aside or away from a resource
	Genital sniffing	Mouse sniffs the ano-genital region of another mouse
Other	Borderline bar-mouthing	Mouse holds bar in mouth with or without biting for less than 1 second
	Borderline route tracing	Mouse runs around in the same pattern for less than 3 consecutive repetitions (includes climbing on cage lid)
	Borderline jumping	Mouse jumps straight up and down 1 or 2 times
	Borderline backflipping	Mouse does 1 or 2 consecutive backflips
	Other	Any other activity performed on cage floor or lid (includes eating, drinking, non-repetitive locomotion, social interaction, active, but out of sight, etc.)
Inactive	Sleep	Mouse is still with eyes closed
	Inactive but awake	Mouse is still with eyes open
	Out of sight	Mouse is still, but observer cannot tell if eyes are open or closed

3.3 Results

The focal mice's home cage SB ($F_{1,29.75}$=0.642, P=0.429) and IBA ($F_{1,30.14}$=1.174, P=0.287) levels had no significant effect on their sociability towards stimulus mice, and when they were included, the focal housing*stimulus housing effect remained significant, with the same pattern evident as before (see Chapter 2). In contrast, agonism received by the focal mouse in her home cage interacted significantly with stimulus mouse housing to predict proximity ($F_{1,33}$=4.563, P=0.040). Including agonism received in the model also reduced the interaction between focal and stimulus housing to a trend ($F_{1,33}$=3.647, P=0.065); and in planned contrasts, the effect of stimulus mouse housing on social proximity was no longer significant for EE ($F_{1,33}$=2.310, P=0.138) or SH ($F_{1,33}$=1.759, P=0.194) focal mice.

Split by stimulus mouse housing type, the interaction between focal housing and stimulus housing was explained by agonism received having no significant effect on the time focal mice spent near SH stimulus mice ($F_{1,29.7}$=0.852, P=0.363; Fig. 3.1), but strongly predicting the time they spent near EE stimulus mice ($F_{1,29.43}$=6.414, P=0.017; Fig. 3.2): an effect that applied to both types of focal mouse.

Next I sought to determine what focal mice might be detecting in stimulus mice, and finding attractive or aversive. I first tested bodyweight and odour. Stimulus mouse weight did not predict how focal mice reacted to them ($F_{1,8.136}$=1.150, P=0.314); furthermore, the interaction between focal and stimulus mouse housing remained significant showing the same patterns previously reported in Chapter 2. Furthermore, odour preference results for SH mice did not show the same pattern of preference as in Chapter 2's sociability / attractiveness test: for odour, there was no significant effect of cage type on preference for nesting material ($F_{1,10}$=0.328, P=0.579) or bedding odours ($F_{1,10}$=2.368, P=0.155), and although not significant, SH focal mice actually spent more time with SH stimulus bedding compared to EE stimulus bedding (the opposite direction to the pattern seen for stimulus mice, where SH mice found EE stimuli more attractive: see Chapter 2).

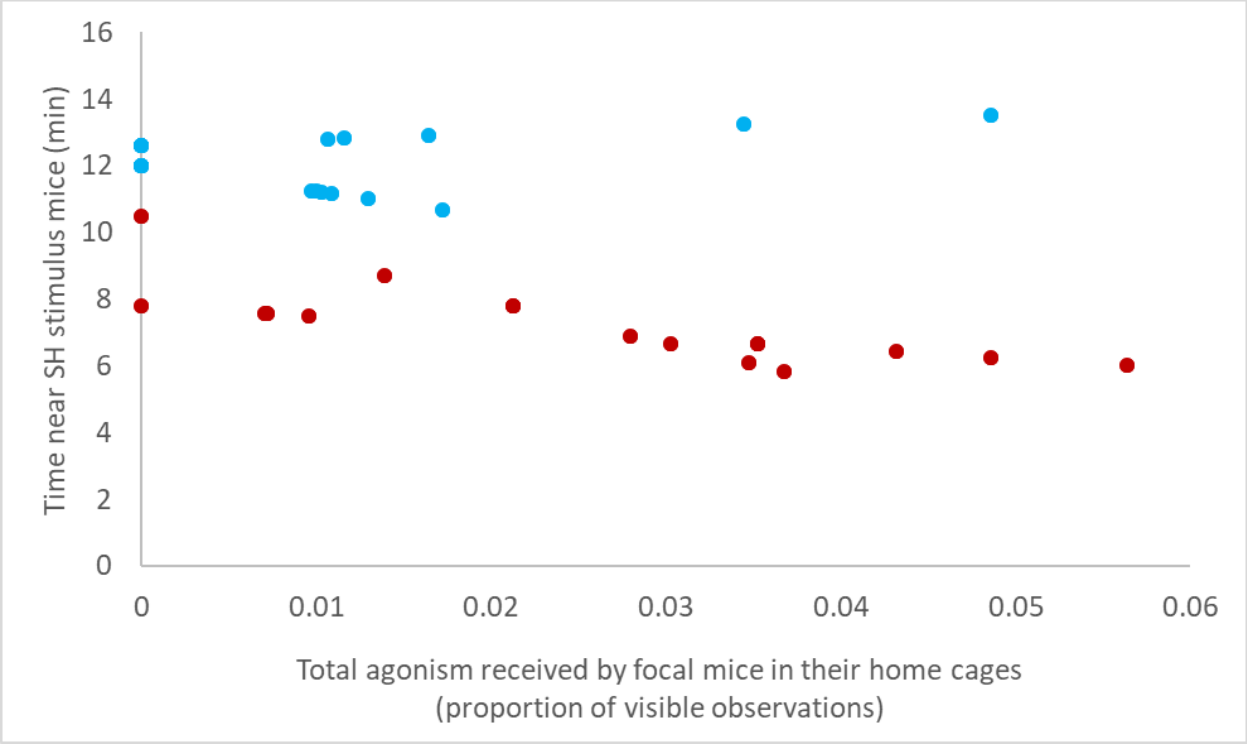

Figure 3.1 Total agonism received by standard housed (SH; red) and environmentally enriched (EE; blue) focal mice in their home cages does not predict time spent near SH stimulus mice in the sociability and attractiveness test.

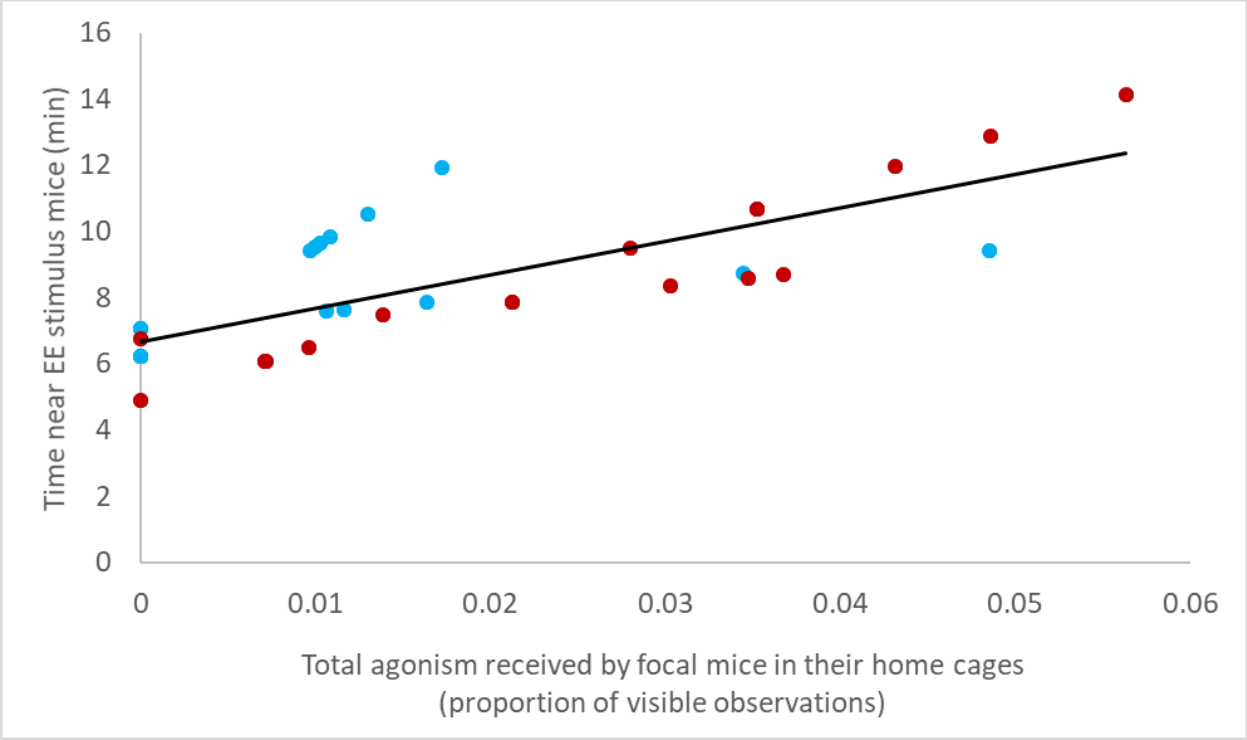

Figure 3.2 Standard housed (SH; red) and environmentally enriched (EE; blue) focal mice spent more time near EE stimulus mice if their home cagemates were more agonistic toward them. Solid line denotes statistical significance at *P*<0.05.

Turning to stimulus mouse behaviour, data from the final familiarisation session was first analyzed to assess the effects of whether or not the stimulus mouse performed SB, and the duration of agonism directed at the focal mouse. Only SH DBAs performed SB (route-tracing) during the final familiarisation session. Neither SB (yes/no) nor stimulus mouse agonism towards the focal mouse predicted the amount of time spent near stimulus mice by focal mice (SB: $F_{1,51.04}$=0.123, P=0.727; agonism: $F_{1,41.38}$=0.002, P=0.969), and the previously detected pattern of housing effects also remained significant. Next, the effect of stimulus mouse home cage behaviour was analysed. Home cage agonism by stimulus mice did not significantly affect how much time focal mice spent near them ($F_{1,48.37}$=0.226, P=0.655); and the same held for their home cage SB ($F_{1,55.52}$=0.146, P=0.704) and IBA ($F_{1,48.08}$=6.678, P=0.4032). Again, the previously detected focal housing*stimulus housing interaction remained significant in these models, further emphasising these behaviours' lack of effect.

The power test for the odour preference test revealed that for the expected Cohen's d effect size of 0.8229 in a one-sided paired t-test, a sample size of 11 yields a statistical power of 0.8141 which is above the generally accepted minimum value of 0.8 (Lazic 2016).

3.4 Discussion

In this study we tried to determine which housing-induced behavioural changes explained the increased sociability of EE female mice compared to SH mice towards SH stimulus mice, and the low attractiveness of SH stimulus compared to EE stimulus mice for SH focal mice (see Chapter 2). Our hypotheses regarding SB and IBA were not supported: SB and IBA levels did not predict sociability of focal mice or the attractiveness of stimulus mice. Thus, even though some stimulus DBAs performed high levels of SB during the familiarisation sessions, surprisingly this did not affect their attractiveness as a social partner. Likewise, stimulus mouse agonism levels in the home cage and in the tests also failed to predict their attractiveness. However, the focal mice's past experience of being aggressed by their home cagemates did affect their choice of social partner. Specifically, receiving agonism in the home cage was positively correlated with spending time near the EE stimulus mouse. Furthermore, this effect largely accounted for the stimulus mouse housing effect seen in Chapter 2.

Thus, mice who received more agonism from their cagemates spent more time near their EE stimulus mouse. This finding adds to the literature showing that the behaviour of cagemates can affect social behaviour in mice. For example, Kalbassi et al. (2017) found that wild-type littermates of mice lacking the neuroligin-3 gene (a gene deletion associated with autism in humans which induces low sociability) *themselves* exhibited low sociability levels similar to the mutants (an effect reversed in both wild-type and mutant littermates neuroligin-3 was re-expressed in the mutants). Furthermore, that SH focal mice would seek out EE stimulus mice also makes sense for these animals: they received more agonism in their home cages than EE mice (Nip et al. 2019) and might thus have sought out stimulus mice who were dissimilar from their cagemates to avoid being harassed. Strangely, they did not avoid stimulus mice who were

more agonistic toward them in the familiarisation sessions and I propose two potential reasons for this. Firstly, agonism towards the focal mouse was generally low in the familiarisation sessions and mostly consisted of pushing under and over other mice (personal observation), a milder form of agonism (Nip 2018), and thus may have been "tamer" than homecage agonism and not have elicited aversion from the focal mice. Alternatively, the focal mice may not have avoided more agonistic stimulus mice because, unlike in the "free interaction" phase of the familiarisation sessions from which agonism was scored, the ability of the stimulus mice to perform agonism during the actual test was restricted due to the wire mesh divider, allowing the focal mouse to approach agonistic stimulus mice without being aggressed. However, this result does not explain why agonism received by focal mice in their home cages did not predict time spent *away* from SH stimulus mice as one would expect if focal mice were trying to avoid agonistic stimulus mice. One possible explanation for this surprising lack of effect is that female mice, especially C57s (Brodkin et al. 2004, Sankoorikal et al. 2006), are such social creatures that, instead of being motivated by avoidance, they actively seek out other mice to affiliate with even if they find both stimulus mice aversive to a degree. In other words, maybe some focal mice had two relatively agonistic stimulus mice and had to choose between two evils to avoid being alone. This interpretation is supported by a study by Van Loo and his colleagues (2001) which showed that when the alternative is an empty cage, male mice consistently chose to spend more time in an inhabited cage separated from another male by a partition, even when the other male had repeatedly aggressed him in the past. This result, while unexpected and slightly puzzling, is thus interesting and novel and more research is now needed to fully understand the mechanisms behind it.

However, despite this novel finding, my data left my other questions unanswered. In particular, the greater sociability of EE focal mice, especially to SH stimulus mice, was not explained by any of my variables. It is thus still unclear why EE focal mice preferred SH stimulus mice to EE stimulus mice (see Chapter 2). One hypothesis is that preference for social novelty (see Chapter 2) extends beyond preference for unfamiliar over familiar conspecifics to preference for individuals who are in some way different from oneself compared to those who are similar. Or perhaps EE mice are usually dominant over SH mice and "enjoy" being near

conspecifics who are submissive to them, although this seems unlikely since Cao et al. (2017) found that EE rats were submissive to SH rats in the tube dominance test (where two animals are simultaneously released into opposite ends of a tube and the one who pushes the other out is deemed dominant). Another complementary explanation is that for EE focal mice, agonism-induced preference for other EE mice was not as strong as in focal mice from SH where agonism was two to three times higher (Nip et al. 2019).

Furthermore, I was unable to identify which cues the focal mice used to tell stimulus mice apart. While we now know it was not related to the stimulus mice's SB, IBA, agonism, odour, or bodyweight, studies replicating this work should assess the role of other factors which are affected by housing , such as the anhedonia, learned helplessness and perseveration discussed in the Introduction, and other variables known or likely to be affected by housing such as anxiety, judgment bias and learning abilities (see Chapter 1). In the final chapter I will therefore explore the implications of these results and those in Chapter 2, while acknowledging the limitations of these studies; and propose several avenues for future research related to my findings.

4 Chapter 4: General discussion

4.1 Implications- raising the standards of laboratory mouse housing

The results presented in Chapter 2 showed that although SH mice were not less sociable overall, they avoided other SH mice, strongly preferring to spend their time near EE stimulus mice. Similarly, SH mice were not less attractive as social partners overall as predicted, but they were significantly less attractive than EE stimulus mice to SH focal mice. Chapter 3 then showed that agonism received in the home cage predicts time spent near EE stimulus mice such that the more focal mice were harassed by their cagemates, the more they gravitated towards EE stimulus mice. Taken together, these findings suggest that agonism between female mice, while not usually injurious, is still aversive to the recipient. Since levels of agonism are generally higher in female mice in SH than in EE cages (e.g. Harper et al. 2015, and see Nip 2018 for specific results regarding agonism in our mice), SH mice are at greater risk of experiencing poor welfare. These findings also suggest that the small barren cages used to house millions of mice worldwide yield cagemates who are sub-optimal or somehow incompatible social partners for each other, arguably leading to a stressful social environment in which mice are housed with individuals they would rather avoid. Past research on the effects of so-called "non-enriched" environments on the behaviour of other social species (see Chapter 2) suggests that this might also be the case for many species in laboratories, zoos, and farms. My research thus adds to the ever-growing literature showing that barren environments lead to poor welfare in captive animals (see Chapter 1). Thus, although SH provides the basics of care and is economical in terms of space and cost, it does not promote good welfare in laboratory mice and is not an appropriate "standard" method of housing.

My research is also the first direct evidence that mice can distinguish between conspecifics who come from different physical environments. This is particularly impressive since previous studies indicate that male inbred mice cannot distinguish between cagemates and unfamiliar conspecifics (Nevison et al. 2003), or even between their own scent and the scent of another male of the same strain (Nevison et al. 2000), perhaps because in inbred mouse strains (including

all those used in my study) there is little variation in the major urinary proteins, one mechanism that mice typically use to tell each other apart (Cheetham et al. 2009). Coupled with the results of my odour preference test, which showed that our mice did not show the same pattern of preference for soiled bedding and nesting material from EE and standard cages as they did for mice from the same housing conditions (see Chapter 2), these findings suggest that our mice were not discriminating between stimulus mice based on odour, and that SH induces other changes (e.g. in anxiety, ultrasonic vocalization, cognition, or some other non-odour attribute) which our SH focal mice associated with agonism from their cagemates, although we were unable to determine what these changes might be.

4.2 Limitations and solutions

There were a few limitations which may have contributed to our inability to determine which cues our mice used to distinguished SH from EE conspecifics. Firstly, because of the elaborate set-up of EE cages which included many hiding places and lots of space for the mice to run around, EE mice were more difficult to catch prior to testing than SH mice (Nip 2018). Although focal mice were given a habituation period of ten minutes in the sociability apparatus and stimulus mice were caught and transferred to holding cages in the running room ten minutes before testing began, we believe that the increased stress caused by the longer catching times for EE mice may have affected their behaviour in the familiarisation sessions and the sociability and attractiveness task, making them more fearful of the other mice in their trio for example. Fortunately, other members of the Mason lab have recently developed a solution to this problem: standard cages are attached to the EE cages and mice are trained to enter the standard cage to receive a cheerio. The standard cage is detachable and can then be used to transport EE mice to the testing area without causing them unnecessary stress. However, this does not solve other housing-related problems which led EE mice to be less exposed to humans than SH mice: while our standard cages are transparent and colourless, our EE cages are opaque with a transparent red viewing window, and SH cages were changed four times as often as EE cages because we had access to fewer EE cages, so they had to be cleaned on a rotating schedule. While these

differences were likely good for the welfare of EE mice overall (e.g. by reducing brightness of light entering the cage, preventing disturbance during cage cleaning and from humans passing by), they led to EE mice being less habituated to humans and less used to being handled than SH mice. While using opaque standard cages was not possible for us since we observed the mice through the transparent sides of their home cages, the solution to this problem is to order new EE cages made of clear transparent material, and enough of them so that all EE cages can be changed at once, however this would probably be quite costly!

Secondly, because analyses took so long, the odour preference test had to be conducted after many of the mice had been killed for another project which greatly reduced the sample size. However, a power test revealed that the sample size of 11 was sufficient to test the hypothesis (statistical power = 0.8141, see Chapter 3). However, by using soiled bedding and nesting materials, focal mice were exposed to odour from three different mice from each housing condition. Thus, there may have been a better way to expose focal mice to stimulus mouse scent, for example by shooting a gentle airstream over the stimulus mice while keeping them out of view of the focal mice. However, this method may be stressful for the stimulus mice and therefore introduce stress odours to the test.

Thirdly, I did not assess the mice's behaviour in every familiarisation session (only the final one) and so may have missed important social interactions which affected the sociability of the focal mice and/or attractiveness of the stimulus mice. For example, if some stimulus mice were more aggressive during the first few familiarization sessions because social hierarchies were being established, this could have caused focal mice to avoid these stimulus mice during the sociability and attractiveness test even though they were not more aggressive in the final familiarization session. Furthermore, there were many variables that may affect sociability and attractiveness which I did not measure such as ultrasonic vocalization during the familiarization sessions, the cognitive abilities of the focal and stimulus mice, and many other traits (see below).

Lastly, and perhaps most importantly, I did not measure perseveration or the presence of a depressive state directly, instead using SB and IBA as proxies because they could be easily measured in the home cage. While home cage behaviour is better at capturing the day-to-day

experience of the mice (and can take as little as four hours on two consecutive days to obtain good quality data; see Appendix I) which was important for assessing the effects of agonism (see Chapter 3), tests outside the home cage would be better at pin-pointing specific tendencies and affective states of the mice which may better predict their sociability and attractiveness. I will thus describe these tests, as well as some others, in the next section.

4.3 Future research

Since I was unable to determine what cues our mice used to distinguish between EE and SH stimulus mice, future research should test other variables as predictors of sociability and attractiveness which better test my hypotheses, such as perseveration and depressive states mentioned above. Perseveration can be measured via an extinction learning task where mice are trained to perform a behaviour (e.g. pressing a lever) in exchange for a reward (usually a food reward such as a cheerio). Perseveration is then measured as the number of lever presses after the researcher stops rewarding the behaviour. If SH mice are found to be more perseverative (i.e. they press the lever a significantly greater number of times than EE mice after the response is no longer rewarded), the sociability and attractiveness paradigm could then be used to test the hypothesis that SH mice are less attractive to other mice because they are more perseverative by predicting that both SH and EE focal mice would spend less time near more perseverative conspecifics.

Depressive states can be measured in a variety of ways including judgement bias, anhedonia, and learned helplessness. Judgement bias is an individual's tendency to interpret a neutral stimulus as positive when in a positive affective state, or negative when in a negative affective state. A common anecdotal example in humans is the perception of a glass of water as either half full or half empty; it is believed that a happy person is more likely to perceive the glass as half full, whereas a sad person will likely perceive the glass as half empty. In animals, judgement bias tasks usually involve training an individual to do two different behaviours in response to two extremes of a stimulus where one is rewarded and the other is unrewarded or

punished. The animal is then presented with a series of intermediate stimuli and their behavioural response is compared to the trained responses. Although no judgement bias task for mice has been properly validated, this could involve putting a mouse in an enclosed chamber into which different scents can be pumped (since olfactory cues are very important to mice). The mouse would be trained to respond to the scent of almonds by running to one side of the chamber where she is then rewarded, and to the scent of vanilla by running to the other side of the chamber where she receives no reward. Researchers could then measure the mouse's response to a 50:50 combination of almond and vanilla; running to the almond side (i.e. the rewarded side) would indicate that she is in a positive affective state, whereas running to the vanilla side (i.e. the unrewarded side) would indicate she is in a negative affective state. (A 50:50 ratio of scents is just an example; of course, a range of ratios of the scents should be used in case mice do not perceive the 50:50 mix as equal parts almond and vanilla.) Of course, this test would have to first be validated by showing that mice reliably show a negative judgement bias after being exposed to a treatment that is known to be aversive to them or bad for their evolutionary fitness (i.e. survival and reproduction) such as being injured or forcibly restrained. Conversely, the task should also show that mice experience a positive cognitive bias when exposed to treatments which they are known to prefer, or which increase their survival and reproduction. If SH mice are in a more negative affective state (i.e. they run to the unrewarded side more often than the rewarded side when presented with intermediate scent cues), the sociability and attractiveness paradigm could then be used to test the hypothesis that SH mice are less sociable and attractive because they are in a more negative affective state in which case sociability and attractiveness would covary positively with affective state.

Anhedonia and learned helplessness (see Chapter 3) are comparatively much simpler measures which can be used to infer depressive behaviour in animals. Anhedonia is commonly measured via a sucrose consumption test which, in this case, would involve comparing the amount of sucrose solution drunk by SH and EE mice when both regular water and sucrose solution are available (corrected for total liquid consumed; e.g. Liu et al 2018). Learned helplessness on the other hand is commonly measured using a forced swim test in which mice are placed in a deep container of water and a longer time spent floating immobile is considered

more helpless (Porsolt et al. 1977). If SH mice are more anhedonic and/or helpless (i.e. they drink proportionally less sucrose solution and/or spend more time helplessly floating than EE mice), the sociability and attractiveness paradigm could be used to test the hypothesis that SH mice are less sociable and attractive because they are anhedonic and/or helpless.

In addition to retesting my hypotheses using more precise measures instead of in-cage proxys, future researchers should also consider cognition as the link between housing, sociability and attractiveness since cognitive abilities are often reduced in SH compared to enriched environments (see Chapter 1). An individual's cognition can impact their social behaviour by affecting their ability to recognize and remember social partners, an obviously important skill for developing social preferences. Differences in social recognition and memory could affect sociability in test situations too: mice typically investigate unfamiliar conspecifics more than familiar ones (e.g. Moy et al. 2004), so mice who are unable to recognize or remember previously introduced conspecifics may appear highly sociable when compared to those with normal social recognition and memory. Previous work has already shown that social memory can be impaired by SH over short intervals (e.g. Wang et al. 2018), but my preliminary results (see Appendix II) suggest that mice may retain social memories for an unexpectedly long time. Thus, investigating the role of housing condition on long-term social memory in mice is an exciting opportunity for future research.

Another interesting avenue for future study is using Balb/c mice as the focal strain, instead of C57s, in a replicate of my work. Compared to DBAs and C57s, Balb mice have an intermediate response to SH, displaying moderate levels of both SB and IBA. This would allow researchers to better test hypotheses about agonism because the difference between agonism received in SH and EE cages is much larger for Balbs than C57s (Nip 2018), and the effect of SB on sociability because there is a bigger range of SB performance in SH Balbs than SH C57s (Nip 2018). Moreover, although Balbs typically have low sociability compared to C57s (e.g. Sankoorikal et al. 2006), this may be reversed by environmental enrichment creating a bigger difference in sociability between SH and EE focal mice.

Finally, future researchers should investigate the effect of housing on other social functions which are important to mice such as social buffering (i.e. the reduction of stress response when exposed to an aversive situation with a conspecific compared to alone; see Appendix III). Like sociability and attractiveness, a mouse's effectiveness as a social buffer can impact the welfare of her cagemates; if SH cagemates are unable to buffer each others' stress because they are too stereotypic, perseverative, depressive, or agonistic they will have decreased stress resilience and therefore poor welfare. Moreover, previous research in rats and guinea pigs has shown that social relationships can influence an individual's effectiveness as a social buffer, with unfamiliar and unpreferred individuals being less effective at buffering stress in conspecifics (Terranova et al. 1999, Maken and Henessy 2009).

4.4 Conclusion

The results presented in this thesis show that previous negative social experiences can affect social preferences and sociability in mice later in their lives and that mice can distinguish between conspecifics who come from different housing conditions. While I was unable to determine which cues our mice used to tell SH and EE mice apart, I have suggested many avenues for future research to answer this question. My results also suggest that the small barren cages used ubiquitously to house mice in research facilities do not promote a healthy social environment which may compromise the welfare of the mice. Thus, my work contributes to the growing body of literature showing that "standard" mouse cages are inadequate.

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APPENDIX I: HOW MANY HOURS AND DAYS OF BEHAVIOURAL OBSERVATION DOES IT TAKE TO GET REPRESENTATIVE DATA?

Table AI.1 Comparing one and two days of data (4 hrs/day, 9am – 1pm, lights off at 7am) to data from all five days. Data were collected via live behavioural observations (scan sampling) in November 2017. For descriptions of behaviours, see Ethogram in Table 3.1, Chapter 3.

Behaviour	Housing/strain	First day	First 2 days
All activity	All housing, all strains	R=0.876779, P<0.0001	R=0.925373, P<0.0001
Submissive behaviour	All housing, all strain	R=0.607115, P<0.0001	
	Barren C57s		R=0.757912, P<0.0001
	EE C57s		R=0.479289, P=0.0003
	All BALBs		R=0.698105, P<0.0001
	All DBAs		R=0.564099, P<0.0001
Dominant behaviour	All housing, all strains	R=0.593541, P<0.0001	R=0.734102, P<0.0001
Stereotypic behaviour + borderline stereotypic behaviour	Barren C57s	R=0.5533, p=0.00276	R=0.59596, p=0.00104
	EE C57s	R=0.79009, p<0.0001	R=0.79009, p<0.0001
	Barren BALBs	R=0.635007, P<0.0001	R=0.83151, P<0.0001
	EE BALBs	R=0.62066, p=0.00206	R=0.82477, P<0.0001
	Barren DBAs	R=0.717643, P<0.0001	R=0.940532, P<0.0001
	EE DBAs	R=0.43675, p=0.04212	
Inactive-but-awake (IBA)	EE, all strains	R=0.71792, P<0.0001	R=0.702586, P<0.0001
	Barren C57s	R=0.325937, P=0.0011	R=0.931414, P<0.0001
	Barren BALBs	R=0.345279, P=0.0002	
	Barren DBAs	R=0.3999, p=0.02335	

Legend

RED	NSD or model won't run
YELLOW	non-parametric (Spearman's ρ presented)
NO BCKGRND	parametric test (R^2 presented)
GREEN	ρ or R^2 of 0.8 or higher
ORANGE	ρ or R^2 of 0.7 to 0.79

Table AI.2 Comparing data from one hour (9am – 10am, lights off at 7am) and two hours (9am – 11am) of live behavioural observation for five days to data from all four hours (9am – 1pm) on all five days. Data were collected in November 2017. For descriptions of behaviours, see Ethogram in Table 3.1, Chapter 3.

Behaviour	Housing/strain	First hr	First 2 hrs
All activity	All EE	R=0.818603, P<0.0001	R=0.838729, p<0.0001
	Barren C57s	R=0.559236, P<0.0001	R=0.773801, p<0.0001
	Barren BALBs	R=0.515093, P<0.0001	R=0.627115, p<0.0001
	Barren DBA	R=0.572518, P<0.0001	R=0.711894, p<0.0001
Submissive behaviour	EE C57s	R=0.22356, p=0.32997	R=0.740379, p<0.0001
	EE BALBs	R=0.4572, p=0.03242	
	EE DBAs	R=0.54504, p=0.00871	
	Barren C57s	R=0.573142, P<0.0001	
	Barren BALBs	R=0.263402, P=0.0016	
	Barren DBAs	R=0.108465, P=0.0369	
Dominant behaviour	All housing, all strains	R=0.561753, P=0.0002	R=0.711829, p<0.0001
Inactive-but-awake (IBA)	EE C57s	R=0.735278, P<0.0001	R=0.796926, p<0.0001
	Barren C57s		R=0.797106, p<0.0001
	EE BALBs	R=0.58764, P=0.0001	R=0.4837, p=0.0002
	Barren BALBs		R=0.699135, p<0.0001
	EE DBAs	R=0.66859, p=0.00067	R=0.76971, p=0.00003
	Barren DBAs	R=0.62494, p=0.00013	R=0.84107, p<0.0001
Stereotypic behaviour + borderline stereotypic behaviour	EE C57s	Won't run, all 0s in hr 1	R=0.53857, p=0.01177
	EE BALBs	R=0.81575, p<0.0001	R=0.91747, p<0.0001
	EE DBA	R=0.618886, p=0.00214	R=0.67357, p=0.00059
	Barren C57s	R=0.758678, p<0.0001	R=0.77188, p<0.0001
	Barren BALBs	R=0.783306, P<0.0001	R=0.823805, p<0.0001
	Barren DBAs	R=0.853608, P<0.0001	R=0.90148, p<0.0001

Legend

RED	NSD or model won't run
YELLOW	non-parametric (Spearman's ρ presented)
NO BCKGRND	parametric test (R^2 presented)
GREEN	ρ or R^2 of 0.8 or higher
ORANGE	ρ or R^2 of 0.7 to 0.79

Interpretation

Based on the analyses presented in the above tables, I recommend observing the mice (scan sampling each cage every 10 minutes, see Chapter 3) for four hours per day on two consecutive days.

APPENDIX II: SOCIAL RECOGNITION AND MEMORY IN EE AND SH MICE

INTRODUCTION

Previous research indicates that mice housed in standard conditions (see Chapter 1) have poorer social memories than those housed in environmentally enriched cages, at least when housed individually (Monteiro et al. 2014, Wang et al. 2018). For example, individually housed mice from enriched environments remembered individuals to whom they were exposed ten minutes (Wang et al. 2018) and 24 hours before (Monteiro et al. 2014), while mice from standard housing did not. Group-housed mice seem to have longer social memories, but to my knowledge this has not been tested with retention intervals of more than seven days (Kogan et al. 2007). I thus attempted to test the long-term social memory of my mice and hypothesized that compared to enriched housing, standard cages would impair the ability of the mice to remember previously introduced conspecifics.

METHODS

Focal mice (N=36) were reintroduced to one of the same stimulus mice as in the sociability / attractiveness test, alongside an unfamiliar stimulus mouse, at 16, 19, 26, or 29 weeks after the test for Batches 1– 4 respectively. These long retention intervals were chosen opportunistically to fit around other experiments in the lab. All batches underwent memory testing in the same week. Thus, animals who were older during the sociability and attractiveness test had a shorter retention interval, and retention interval varied with age at first exposure to stimulus mice. Unfamiliar stimulus mice were mice from other batches who had previously participated in the sociability and attractiveness test; thus, all stimulus mice were equally familiar with the testing procedure and apparatus at the time of the social memory test.

This test was conducted using the same methods as the familiarisation sessions (i.e. sniffing was scored in both ‘enclosed’ and ‘free interaction’ phases), with a few modifications. Each focal mouse underwent two tests for social memory: each involved two stimulus mice, who were again both of the same strain (DBA or BALB) but differentially housed (EE or SH).

However, this time one stimulus mouse was also unfamiliar. In one of the two tests, the focal mouse's EE stimulus mouse was unfamiliar; in the other, the focal mouse's SH stimulus mouse was unfamiliar. As in the familiarisation sessions (see Chapter 2), the focal mouse was allowed to acclimate to the apparatus for ten minutes before each test, after which she was placed under a wire mesh dome while inanimate objects and stimulus mice were placed in their respective corners, each in the same location as in the sociability / attractiveness test. Using a stopwatch, an observer blind to the identity of all mice then recorded the amount of time the focal mouse spent sniffing each stimulus mouse in the 'enclosed' and 'free interaction' phases. As in the familiarisation sessions, sniffing in both phases was used here as a measure of investigation and therefore familiarity (e.g. Phan et al. 2011), as opposed to proximity which was used as a measure of social preference in the sociability and social attractiveness test (eg. Moy et al. 2004). By comparing investigatory sniffing towards familiar and unfamiliar stimulus mice, we could infer memory based on the tendency for mice to investigate novel stimuli more than familiar stimuli (e.g. Cavigelli et al. 2011).

Statistical analyses were conducted using general linear mixed models in JMP 14, data being transformed as needed to meet the assumptions of parametric models, and when interactions were significant, planned comparisons being performed to test predetermined hypotheses pertaining to focal and stimulus mouse housing, and the effects of familiarity. The dependent variable in this repeated measured model was the amount of time the focal mouse spent sniffing each stimulus mouse. Fixed effects included focal mouse housing, stimulus mouse housing, stimulus mouse strain, familiarity of the focal mouse with each stimulus mouse (i.e. whether or not they had met in the sociability / attractiveness test) as well as all possible interactions. Batch and focal mouse cage (nested within focal mouse housing, stimulus mouse housing and stimulus mouse strain) were included as random effects. Our hypothesis predicted that focal housing type would interact with familiarity.

RESULTS

When determining whether focal mice remembered their previously introduced stimulus mice, familiarity interacted significantly with stimulus mouse strain ($F_{1,94.76}$=4.186, P=0.044) to

predict the amount of time focal mice spent sniffing (in the enclosed and free phases combined). Planned contrasts showed that this was because focal mice sniffed unfamiliar stimulus mice more than familiar stimulus mice if they had BALB ($F_{1,94.76}$=4.378, P=0.039), but not DBA stimulus mice ($F_{1,94.76}$=0.581, P=0.448; Fig. 6). Furthermore, the interaction between focal and stimulus mouse housing was significant ($F_{1,94.76}$=34.449, P<0.0001), with SH focal mice spending significantly more time sniffing EE than SH stimulus mice ($F_{1,94.76}$=45.198, P<0.0001) and EE focal mice spending more time sniffing SH stimulus mice compared to SH focal mice ($F_{1,38.77}$=4.124, P=0.049; Fig. 7). Housing conditions did not interact with familiarity, however (focal mice: $F_{1,94.76}$=0.0005, P=0.982; stimulus mice: $F_{1,94.76}$=0.013, P=0.910); thus EE and SH focal mice did not differ in their social memory, rejecting our hypothesis.

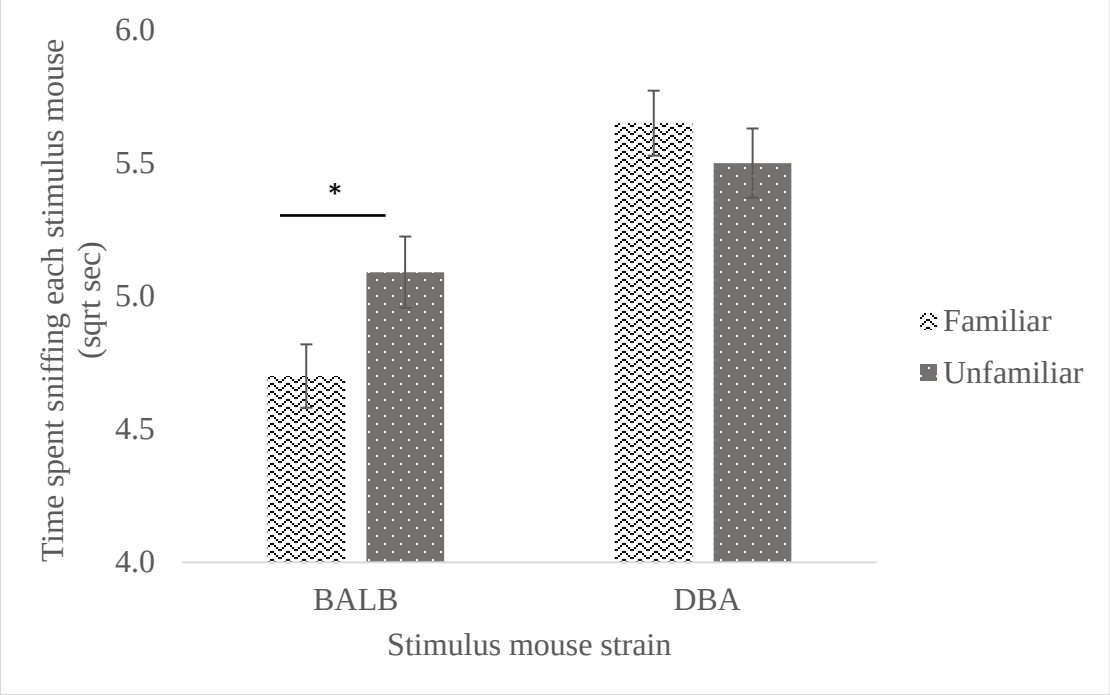

Figure AII.1 Focal mice with BALB stimulus mice spent less time (sec) with their previously familiar stimulus mice compared to unfamiliar stimulus mice. Data are square root transformed. Error bars represent standard error of the mean. * indicates statistical significance at P<0.05.

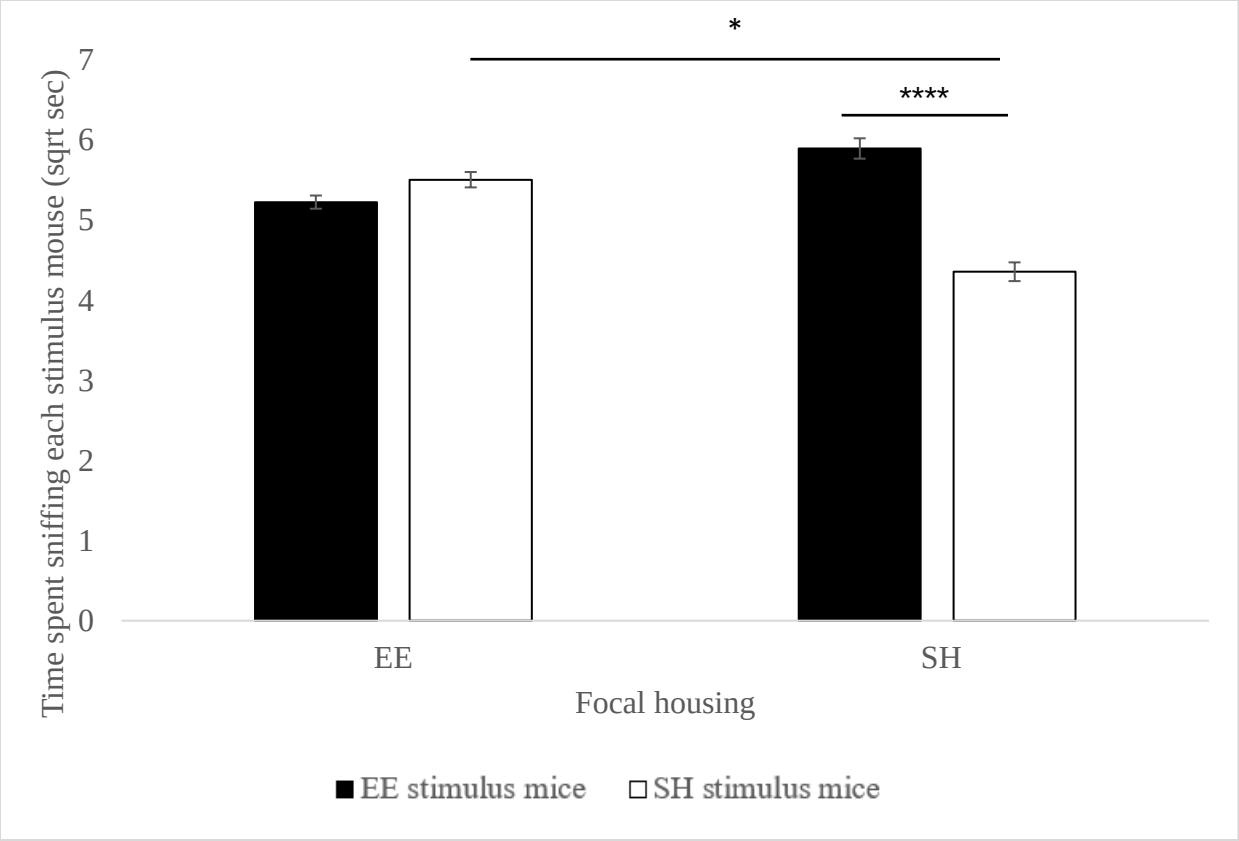

Figure AII.2 Time (sec) spent sniffing environmentally enriched (EE) and standard-housed (SH) stimulus mice by EE and SH focal mice. Data are square root transformed. Error bars represent standard error of the mean. * denotes statistical significance at *P*<0.05. ** denotes statistical significance at *P*<0.0001.**

DISCUSSION

Focal C57s appeared able to remember BALBs they had been exposed to for just ten minutes/day for four to five days, a full four to seven months earlier. This result needs replicating, as it is unclear why it would only manifest for this one strain, but if robust would be the first demonstration of highly prolonged social memory in mice, previous work looking only at short retention intervals of ten minutes (e.g. Wang et al. 2018) to seven days (Kogan et al. 2000). This is extra impressive since our mice came from inbred strains with apparently little individual variation, at least in the urinary proteins mice use as a major cue in social recognition (Nevison et al. 2003, Cheetham et al. 2009). On the other hand, these results are perhaps not surprising given the mice's extremely restricted social experience in this laboratory setting, compared to their naturally complex social lives. Wild female house mice live in 'demes'

comprising other females (often siblings) and their offspring, all of whom must be recognised, and protected from potential intruders (reviewed Latham and Mason 2004). Thus, after being housed with only two other mice all their lives, meeting two more might have been highly salient for these subjects. Our repeated familiarisation sessions also exposed the focal mice to stimulus mice for longer than customary in social memory paradigms (e.g. Kogan et al. 2000, Wanish et al. 2008), which may further have improved their abilities to recognise them. Our main hypothesis that EE mice would have better social memories was not supported, however. The long retention intervals may have been too challenging: familiar DBAs seemed not to be recognised after this many weeks, effectively halving our sample size. And retention interval was confounded with age at first exposure to stimulus mice such that those with shorter retention intervals were eight months old when introduced to their stimulus mice (see Methods); mice in Batches 3 and 4 may therefore have been too old to remember their stimulus mice (e.g. Prediger et al. 2005). Thus, future endeavours to test long-term memory in mice should use retention intervals between seven days and our months.

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APPENDIX III: IS STRESS (ELEVATED HEARTRATE) BUFFERED BY CAGEMATE PRESENCE?

METHODS

I used 21 C57 mice housed in groups of five or six in "half size" enriched cages (60cm L x 30cm W x 30cm H; furnished with two running wheels, two black plastic tunnels, one paper cup, one pinecone, and a sock hammock hung from the cage lid). These mice had previously been housed in standard laboratory mouse cages in groups of two – four and were acquired from other labs which no longer needed them. One by one, mice were placed on the electrode of a modified ECGenie heartrate monitor (Mouse Specifics; Figure AII.1) either with or without a cagemate in the adjacent chamber. The test was conducted on an elevated platform under bright light to induce stress in the mice. Half the mice underwent the test first alone, then with a cagemate present the next day, while the other half were tested with a cagemate on the first day and alone the second day. LabChart (AD Instruments) ECG collection software was then used to record ECGs produced by the mice (minimum 20 peaks), and EzCG analysis software (Mouse Specifics) was used to extract heartrate (HR) and heartrate variability (HRV) values from the recorded ECGs. If no usable ECG was produced within 10 minutes, mice were returned to their home cages and retested at the end of the testing session. Data (collected June 22 and 23, 2017) were analyzed using paired t-tests in JMP 13 (SAS Institute).

RESULTS

HR was slightly, but significantly lower when a cagemate was present (mean±SEM=755.7±5.3bpm) compared to when mice were tested alone (mean±SEM=764.8±4.4bpm; T_{20}=-1.951, P=0.033). HRV tended to be higher when a cagemate was present (mean±SEM=65.6±11.7) than when mice were alone (mean±SEM=42.9±12.3; T_{20}=1.643, P=0.058).

Figure AII.1 A mouse being tested on the ECGenie heartrate monitor with a cagemate on the adjacent platform. The wire mesh divider prevents the cagemate from accessing the electrode so only the focal mouse's heartrate is measured.

DISCUSSION

This result suggests that cagemates may be able to buffer the effects of stress on heartrate in mice, but it needs replicating. Future research should also assess the effects of cagemate presence on other stress responses such as freezing, defecation, hyperthermia, and fecal corticosterone which have been used successfully in rats and guinea pigs (e.g. Kiyokawa et al. 2004, Maken and Hennessy 2009).

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