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ANIMAL WELFARE

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**Stereotypic mice are aggressed by their cagemates, and tend to be poor
demonstrators in social learning tasks**

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Running title:

Social characteristics of stereotypic mice

Abstract

Stereotypic behaviours (SBs) are linked with behavioural inflexibility and resemble symptoms of autism, suggesting that stereotypic animals could have autistic-like social impairments. SBs are also common in caged mice. We therefore hypothesised relationships between stereotypic and social behaviours, predicting that highly stereotypic mice would give/receive more agonism and be less effective in social learning tasks. Experiment One used C57BL/6 and DBA/2 mice in non-enriched or enriched housing (15 cages each); Experiment Two, more cages (6 non-enriched, 44 enriched) plus a third strain (BALB/c). Across both experiments, enrichment reduced SB and agonism (aggression, plus 'displacements' where one mouse supplants another at a resource). These effects appeared related: housing effects on agonism became negligible when SB was statistically controlled for; and, at least in enriched cages, SB covaried with receiving aggression. In Experiment Three, 20 DBAs varying in SB from Experiment Two acted as demonstrators in a 'social transmission of food preferences' task. They were fed a novel flavour (shatavari powder), then each mingled with a familiar but flavour-naïve C57 observer. Observers were subsequently offered two novel flavours: shatavari or marjoram. Those spontaneously choosing more shatavari ($n = 10$) tended to have had less stereotypic demonstrators than the other 10 observer mice. Overall, highly stereotypic mice thus received more agonism -- an effect with obvious welfare implications that can be reduced with enrichment -- and seemed potentially less effective at inducing flavour preferences in conspecifics. Such effects are consistent with social impairment, suggesting that reducing SB may perhaps enhance interactions between conspecifics.

Keywords: *aggression, animal welfare, environmental enrichment, mice, stereotypic behaviour, social learning*

Introduction

Most laboratory rodents are housed in small, barren environments. These conditions are associated with poor welfare, as well as impaired learning and memory (Mason & Latham 2004; Simpson & Kelly 2011), and reduced levels of normal activity that are replaced by spending more time standing still, doing nothing (Tilly *et al* 2010) and/or performing stereotypic behaviours, SBs (*eg* Sørensen 1987, Mason & Latham, 2004, Tilly *et al* 2010): repetitive activities arising from frustration, needs to cope, or central nervous system dysfunction (*eg* Campbell *et al* 2013). Whether environments are enriched or impoverished can also affect laboratory rodents' interactions with cagemates. For example, male bank voles (*Clethrionomys glareolus*) housed in enriched rather than barren environments were less aggressive (Sørensen 1987); social play that had been reduced by prenatal stress in rats (*Rattus norvegicus*), was restored to normal levels through enrichment (Morley-Fletcher *et al* 2003); and in laboratory mice (*Mus musculus*), enriched caging can also improve maternal care (Whitaker *et al* 2009). Enrichment can also affect aggression: in male mice, adding a shelter increased levels of aggression (Van Loo *et al* 2002), perhaps by increasing resource competition, but providing nesting material significantly decreased it, and more recently, providing diverse enrichments has been shown to reduce aggression in mice of both sexes (Turner *et al* under revision).

Our aims were therefore to study how providing enrichments affects interactions between mice within their cages, and to investigate how any changes relate to alterations in SB. This is because SBs in captive animals share similarities with the repetitive behaviours seen in certain socially disabling human disorders, especially schizophrenia and autism

(Lam *et al* 2008; Dallaire *et al* 2011): both have been linked to changes in basal ganglia function (*eg* Tanimura *et al* 2008; 2011) that help cause behavioural inflexibility and perseveration (the repetition of responses when no longer appropriate) (*eg* Lopez *et al* 2005; Campbell *et al* 2013). Thus to give examples from caged rodents, high levels of SB correlate with elevated perseveration in extinction learning, reversal learning and other tasks in bank voles, African striped mice (*Rhabdomys*), deer mice (*Peromyscus maniculatus*) and one strain of laboratory mouse, C57BL/6 (Garner and Mason, 2002; Tanimura *et al* 2008; Jones *et al* 2011, Garner *et al* 2011), and rearing conditions that reduce SB may also reduce perseveration (Tanimura *et al* 2008 and Jones *et al* 2011). Such effects seem likely to be relevant for social interactions, since in autistic humans, perseveration and SB predict reduced social competence. Autistic humans thus often show social impairments if prone to repetitive behaviour (McEvoy *et al* 1993; Lam *et al* 2008); furthermore, in autistic children, tendencies to perseverate correlate with poorer social skills (McEvoy *et al* 1993) and reduced social interaction (Memari *et al* 2013). Do barren-housed stereotypic animals also show such social impairments? This topic has been little studied. However, consistent with this hypothesis, ‘knockout’ mice lacking a dopamine transporter gene show both elevated SB and aggressive, unstable social hierarchies (Rodríguez *et al* 2005); while in mate choice tasks, non-stereotypic enriched male mink gained more copulations with females than stereotypic non-enriched males (Diez-Leon *et al* 2013).

The first part of this study used behavioural observations to assess in-cage social and stereotypic behaviour in female C57BL/6 (C57), DBA/2 (DBA) and BALB/c mice, housed in both enriched (EE) and non-enriched (NE) cages. All animals were housed in

98 mixed strain trios, enabling the generality of effects across these widely-used strains to be
99 easily assessed, without increasing the animal numbers used (Walker *et al* 2013). We
100 predicted that mice in non-enriched housing would display more SB and more aggression.
101 We also hypothesized that if SB reflects autistic-like impairments that compromise social
102 functioning, then stereotypic individuals should receive more aggression, and fewer
103 affiliative behaviours such as resting/sleeping together.

104
105 The second part of this study aimed to further assess the social normality of stereotypic
106 mice by investigating the effect that SB has on social learning. Social learning involves
107 the transfer of information between individuals (*eg* Kavaliers *et al* 2001), and has been
108 demonstrated in many rodent species, including rats, mice and gerbils (Galef & Wigmore
109 1983; Valsecchi *et al* 1996; Kavaliers *et al* 2001). One commonly used paradigm to
110 assess social learning is the transmission of food preferences, which involves a
111 demonstrator transmitting information about a novel flavour to an observer, as revealed
112 by that observer then preferring diets with this flavour even though they are novel. There
113 has been no research into the role of SB on social learning, despite evidence that the
114 perceived quality of the demonstrator in such tests can be influential. Thus in mice, pups
115 are less effective demonstrators than adults (Choleris *et al* 1997); in gerbils, unfamiliar,
116 unrelated demonstrators are not effective at conferring flavour preferences, while familiar,
117 related demonstrators are (Valsecchi *et al* 1996); in African striped mice, fathers are less
118 effective than mothers at transferring food preferences to their offspring (Rymer *et al*
119 2008); and in deer mice, subordinate demonstrators are less effective than dominant
120 demonstrators (Kavaliers *et al* 2005; see Clipperton *et al* 2008 for potentially similar
121 effects in lab mice). We therefore predicted that if stereotypic animals are perceived as

abnormal, subordinate, or receive less attention from observers in the transmission phase of the task, then they would prove poorer demonstrators.

Methods

Ethics Statement

All procedures and husbandry techniques were approved by the University of Guelph Animal Care Committee, and comply with the Canadian Council on Animal Care guidelines (covered by AUPs #1398 and #2430).

Experiment One

Animals and housing

The subjects were part of another ongoing experiment (a long-term study of enrichment effects on activity levels) and were observed opportunistically for this study in order to reduce the total number of animals used (NC3Rs 2015). Animals were adult female C57BL/6 (henceforth ‘C57’) mice ($n = 60$) and female DBA/2 (‘DBA’) mice ($n = 30$), purchased from Charles River Laboratories (Quebec) at four weeks of age. Mice were housed in groups of three, with two C57 mice and one DBA mouse per cage (see Walker *et al* 2013 for a validation of this mixed strain housing), in either non-enriched ‘NE’ housing (15 cages) or enriched ‘E’ housing (15 cages). NE cages were standard laboratory cages, measuring $12H \times 27L \times 16W$ cm. NE mice were provided with Shepherd Enviro-dri[®] nesting material and a UDEL polysulfone plastic mouse house shelter, but no other enrichments. E cages were each $12H \times 43L \times 21W$ cm in size, and as well as a clear plastic shelter and nesting material (as used in the NE cages), they contained enrichments: a horizontal plastic running wheel, a pinecone, a sock ‘hammock’

measuring roughly 11 L × 7 W cm, 2 paper cups, a piece of PVC pipe roughly 6.5 L cm, a sponge, and two cotton balls. All mice were maintained at 21 °C and were fed a standard laboratory rodent diet (Harlan® Teklad Global Diet [14% protein]). Food and water were given *ad libitum*. Mice were kept on a 12:12 reversed dark/light cycle, with the dark cycle beginning at 1000h.

Observations

Mice were six months old at the time of behavioural observations. They were observed *in situ* in their home cages, using red room lights and headlamps, via live scan sampling. The positions of E and NE cages were randomised across racks and shelves. To assess home cage activity time budgets, scans were taken every 20 minutes, for a period of four hours per session (based on Walker *et al* 2013, and validated using split-half analyses regressing data from odd and even days [Martin & Bateson 1993]). Sampling periods were from 1130h to 1530h, and from 1730h to 2130h. Eight sampling periods were conducted, for a total of 94 scans (one scanning period only had ten scans). Scans were split between two experimenters (LH and KR), whose inter-observer reliability after training was >95%.

Social interactions were assessed by LH using a focal sampling regime, since they were relatively rare occurrences. Each cage was observed for five minutes, with all bouts of social behaviour in the cage recorded; the next cage was then moved on to. Observations commenced at 1130h and again at 1700h. This was repeated 10 times per cage (thus 50 minutes of focal observation over six days, a regime again confirmed as valid using split-half analyses regressing data from odd and even days). An ethogram of behaviours

recorded during scan and focal sampling is provided in Table 1. Note that we chose to assess and analyse being ‘still but awake’ separately, because being inactive despite being awake seems to increase in mice housed in barren rather than enriched cages (Tilly *et al* 2010).

*** Table 1 about here ***

Experiment Two

Animals and Housing

Again, subjects were part of another on-going experiment (this time a long-term study of enrichment effects on life expectancy), being observed opportunistically for this study to maximise their usefulness and minimize the total number of animals used. This second cohort comprised 150 adult female mice purchased from Charles River Laboratories (Quebec) at 3 to 6 weeks of age, and housed in groups of three in mixed-strain housing. This time three strains were used, BALB/c, DBA/2 and C57BL/6, one mouse of each per cage (thus 50 cages). 50 cages were used as this was the maximum number of cages that could be observed in a single 40-60 minute session of focal observations (one per cage). They were part of an on-going longevity study, and so were housed in one of seven housing types (again randomised across racks and shelves). Six cages were standard laboratory cages, as used in Experiment 1. The other 44 cages were larger, 20H × 43L × 21W cm. Half of these were enriched with ‘comforts’ (enrichments designed to enhance comfortable rest: a sock ‘hammock’, Nestlets, a tissue to construct into nests, and a paper cup to shelter in), while half were not. Cross-factored with the presence or absence of comforts, approximately one third of the cages contained a working horizontal plastic running wheel, approximately one third contained a working metal wheel, and

approximately a third contained a locked wheel (for specifics see Table 2). The same room was used as in Experiment One, mice being provided with the same rodent chow and water *ad libitum*. Mice were kept on a 12:12 reversed dark/light cycle, with the dark cycle beginning at 1000h.

*** Table 2 about here ***

Observations

All observations were conducted in home cages when mice were seven months old. Activity budgets were again generated using scan sampling. Scans were taken every 40 minutes to an hour; scanning times were longer in Experiment Two due to the greater number of cages (120 cages were scored for activity levels as part of the larger longevity study). Sessions commenced twice daily, at 1130h and 1700, with three scans being taken per session (a total of 48 scans/cage). Scans were split between the two experimenters of Experiment One, plus a new, experienced experimenter (MW).

Social data were again collected by LH using focal sampling, from just the 50 cages used in this study. Each was watched for three minutes (reduced from the five minutes in Experiment One for practical reasons, due to the increased number of cages observed) per focal observation, with observations commencing at 1130h and 1700h per day. Data came from a total of 12 focal periods per cage, over 12 days. All bouts of social behaviours that occurred in each observation period were recorded using the ethogram in Table 1; SB was also recorded during these focal observations.

Experiment Three: Social transmission of food preferences

Animals

The subjects used were 20 pairs of females, one DBA and one C57, now aged 8 months, each pair being selected from one of 20 of the enriched cages used in Experiment Two. Twenty pairs were used because this allowed for a range of SB to be investigated, while still remaining a manageable sample size for the researchers.

Only E mice were used to ensure that all subjects came from similar housing types (only six NE cages were available), and because only in the E conditions did highly stereotypic mice attract elevated agonism from their cagemates (see Results for Experiment Two). The 20 cages used were selected on the basis of their SB (as quantified in focal observations), as follows. Of the three strains, DBA mice had the most variation in SB, and so these mice were chosen to act as demonstrators in the social learning task. Of the 44 E cages screened in Experiment Two, these 20 cages were chosen because their DBAs displayed the greatest variation in their average levels of SB, from 0.44 bouts/minute (two mice) to zero bouts/minute (four mice) C57 mice were selected to be used as observers, since a previous experiment using social transmission of food preferences had been conducted with this strain (Ryan *et al* 2008). As well, all the C57 observers in the study had very low amounts of stereotypic behaviour (only one observer mouse showed any stereotypic behaviour during focal observations), so selecting these mice reduced behavioural variation across the observers. All mice continued to be housed as they had been for Experiment Two, including in the same trios of individuals (although the BALB/cs played no active role in Experiment Three). During the social transmission test, each demonstrator was paired with a C57 observer from its home cage: thus

demonstrators and observers were familiar.

Flavour selection for the social learning test

Non-subject mice in pilot trials were tested to determine suitable flavours for the experiment. Two flavours picked were those that no researchers or technicians had consumed in the past six months (shatavari powder and ashwagandha root powder), while two others had been very little ingested (marjoram and anise seed): important because rodents can pick up flavour preferences from humans (Galef 2001). Flavours were ground up (if needed) and mixed into powdered rodent chow at appropriate concentrations. Mice were each given a choice of two flavours in a specialised test apparatus designed for this purpose: a polyethylene cage (37 x 21 x 19 cm) with two food magazines affixed to one side (Tecniplast SPA, Buguggiate, VA, Italy). The food magazines had removable food trays that hold the flavoured foods; the food trays each had an apron to catch spilled food, so allowing for precise measurements of food intake (Valsecchi *et al* 1989). Each flavour was available for consumption in one of the two compartments, counterbalanced, for a period of four hours. Dishes were weighed and the amount of food eaten was measured at one, two and four hours. Both powdered diets were measured at wet weight. We confirmed whether all mice consumed each flavoured food (defined as eating more than 0.1g), and calculated the coefficient of variation across individuals to assess how consistent consumption levels were across mice. The two flavours eaten by all mice with the lowest coefficients of variation were chosen for Experiment Three. These were 2% shatavari powder (Rootalive Inc., Canada) and 2% marjoram (McCormick®, Canada). In these pilot tests, mice consumed more marjoram (mean 0.738 $\pm$ 0.586g) than shatavari (mean 0.589 $\pm$ 0.508g); however, because these two flavours were never offered in a

pairwise combination, their relative appeal to naïve mice was unknown.

Social transmission of food preferences

The protocol described here is adapted from Valsecchi & Galef 1989. Ten cages were tested daily (thus 10 DBA demonstrators and 10 C57 observers), for two days. The demonstrators' variation in SB was balanced across days (*ie* mice spanning similar ranges were tested each day), and the tester was blind to these during the test.

All cages of subjects were food deprived for 16 hours before trials began, largely over the 12 hour light phase (when food consumption is very low: *eg* Clipperton *et al* 2008), thus starting at 1730h, in order to ensure food consumption the morning of the following day. At 0930h each demonstrator mouse was moved to a clean empty cage and fed a powdered diet composed of 2% shatavari powder and powdered rodent chow. Food was presented for two hours to these demonstrators, in jars approximately 7 cm in diameter and 5 cm in depth (with a perforated stainless steel disc placed on the top of the food to prevent digging and spillage). Weights of food given were measured before and after consumption, to ensure that each demonstrator consumed the diet (greater than 0.2g).

Food was removed from the cage, and each demonstrator was then immediately paired with its corresponding observer (the C57 from its homecage) by placing the observer mouse in the test cage with the demonstrator and the two familiar mice were allowed to interact for one hour. Demonstrators were then moved back to their home cages. Observers were instead each moved to a specialised Tecniplast test cage (as used to screen potential flavours at the start of this study), with each flavour of food available for

consumption in one of the two compartments, counterbalanced across cages. The observer mice in these test apparatuses were also provided with a small amount of bedding and water *ad libitum*. Weights of each food were taken before testing and after two hours (after which each observer mouse was then returned to its homecage). The amounts of each diet consumed by each observer mouse were then analyzed, to determine effects of demonstrator levels of SB on observer preferences.

Statistical analyses

Statistical analysis was performed using JMP® 11 software, and general linear models (GLMs). Appropriate transformations were performed in order to satisfy the assumptions of parametric models as best as possible; in practice this typically meant that homogeneity of variance was achieved but strict normality of residuals was not; realistically, this is of small concern as these tests are robust to deviations from normality (Rasch & Guiard 2004). All results were considered significant at $P = 0.05$ or lower (and presented as trends if between 0.05 and 0.10). Two-tailed tests were used throughout, to be conservative, even though we made directional predictions. Tukey's tests were used to investigate the drivers of any significant interactions between categorical variables.

In Experiments One and Two, cage was treated as a random effect, and nested within housing type (EE / NE). Strain, housing and their interactions were included as fixed effects in every model. Behavioural variables analysed were stereotypic behaviour, and 'still but awake' (both calculated as a percentage of all observations); along with the number of aggressive + displacement acts given or received per minute of observation (pooled under the term 'agonism'), the number of aggressive acts recorded per minute of

observation, and time spent nesting together recorded per minute of observation. To avoid problems of non-orthogonality, sequential tests were used when continuous independent variables were included, with the term of interest placed last in the model (Doncaster & Davey 2007, Grafen & Hails 2002).

Pooled analyses were also conducted, combining data from Experiments One and Two to assess the consistency or otherwise of effects across the two studies, and to run some analyses with greater power. The BALB/c mice were excluded from this pooled dataset, since not present in both studies. In these analyses, cage (again a random effect) was nested within both housing type and experiment, and for categorical variables, all possible two- and three-way interaction terms were included. These analyses aimed to investigate: 1) whether mice in enriched cages were more often out of sight than mice in non-enriched; 2) if so, whether housing type effects on behaviour could still be detected when this problem was controlled for statistically, by incorporating all ‘out of sight’ observations (active and inactive pooled) into all relevant models; 3) whether housing type effects on aggression or agonism could still be detected if stereotypic behaviour was statistically controlled for, and *vice versa*; and 4) how consistent relationships between SB and social interactions were across the two studies.

In Experiment Three, the weight of shatavari-flavoured food (the diet eaten by the demonstrators) eaten by each observer was expressed as a proportion of all food eaten, and regressed against how stereotypic each demonstrator was (as a % time budget). Observers were also divided into two groups according to whether the shatavari-flavoured food was qualitatively preferred (*ie* making up more than 50% total weight of

food consumed) over the control novel food. The stereotypic behaviour levels of the two groups' demonstrators were then compared in a GLM, with 'test day' and its interaction as blocking factors.

Results

Experiment One

Compared to mice in enriched (E) cages, mice in non-enriched (NE) cages performed more SB ($F_{1,34} = 63.35$, $P = <0.0001$), and more 'still but awake' behaviour ($F_{1,32} = 6.85$, $P = 0.01$). They also performed more acts of agonism (aggression + displacement) ($F_{1,32} = 5.59$, $P = 0.024$; see Figure 1), although not aggression when considered on its own. For receiving agonism, there was an interaction between strain and housing type (strain*housing: $F_{1,58} = 14.53$, $P = 0.003$), caused by C57 mice in NE cages receiving more aggression than those in enriched cages (Tukey's test, $P = 0.006$). There were no effects on the receipt of aggression when considered on its own. There was no effect of housing type on nesting together.

*** Figure 1 about here ***

Performance of agonistic behaviour in enriched and non-enriched cages.

Experiment Two

No behavioural differences were found between the various large enriched housing types, and so all were pooled as 'enriched cages' for ease of subsequent analysis. Compared to mice in enriched cages, mice in NE cages were more stereotypic ($F_{1,48} = 9.70$, $P =$

0.0031). For 'still but awake' behaviour there was a strain*housing interaction ($F_{2,96} = 3.21, P = 0.04$), because C57s in NE cages performed more than C57s in enriched cages (Tukey's test, $P = 0.010$). There were no effects of housing type on all acts of agonism ($F_{1,48} = 1.04, P = 0.31$), but for being aggressive, there was a strain*housing interaction ($F_{1,96} = 3.30, P = 0.041$), because C57 mice in NE cages were more aggressive than those in large enriched cages (Tukey's test, $P = 0.035$; see Figure 2). There were also no housing type effects on the receipt of agonism ($F_{1,48} = 1.28, P > 0.10$), but for receiving aggression *per se* there was a trend for NE mice to receive more ($F_{1,48} = 3.21, P = 0.08$). Mice in NE cages also spent significantly more time nesting together than those in enriched cages ($F_{1,48} = 22.34, P < 0.0001$).

**** Figure 2 about here ****

There were no relationships between SB and giving or receiving agonism. However, a significant interaction with strain was found when correlating SB with the performance of aggression (Strain*SB: $F_{1,125.8} = 12.16, P < 0.0001$). Upon further analysis, it was found that C57 mice that performed more aggression had lower levels of SB ($F_{1,46} = 16.00, P = 0.0002$). A significant interaction with housing type was also found regressing SB against receiving aggression (housing*SB: $F_{1,137.8} = 17.77, P < 0.0001$), because in enriched cages, mice that performed more SB also received more aggression ($F_{1,115.3} = 9.77, P = 0.002$). Finally, mice that performed higher levels of SB also spent more time nesting with cage mates ($F_{1,109} = 15.523, P < 0.0001$).

Pooled analyses

Across the two studies pooled, mice in enriched cages were out of sight significantly

more often than those in NE cages: ($F_{1, 74.3} = 24.03, P < 0.0001$). All potential housing type effects on behaviour therefore were reinvestigated to check they were not mere side-effects of enriched mice being harder to observe. For SB, there remained a strong overall effect of housing type, enriched mice being less stereotypic ($F_{1,75} = 36.28, P < 0.0001$). However, experiment*strain*housing was also significant ($F_{1, 101} = 6.65, P = 0.013$), with Tukey's tests revealing that while enrichment reduced SB in both strains in Experiment One ($P < 0.005$), in Experiment Two it only did so for DBAs ($P < 0.0001$). Being 'still but awake' was consistently reduced by enrichment (with no interactions with strain or experiment) ($F_{1, 78} = 6.89, P = 0.010$).

Enriched mice also still performed fewer agonistic acts ($F_{1, 87} = 5.85, P = 0.018$), and fewer acts of aggression *per se* ($F_{1,78} = 4.27, P = 0.042$): housing effects that did not interact with experiment (or strain), *ie* were consistent across populations. The pooled analyses also revealed a three-way effect of strain*housing*experiment effect on agonism received ($F_{1,112} = 8.83, p = 0.004$). A Tukey's test showed that this was driven by enrichment only reducing the receipt of agonism in C57s in Experiment One ($P = 0.006$). When looking at the receipt of aggression only, the pattern was the same as described for agonism (strain*housing: $F_{1,104} = 6.83, P = 0.01$). Finally, for nesting together, the tests revealed another three-way effect of strain*housing*experiment ($F_{1,104} = 6.124, P = 0.015$), driven by both strains in Experiment Two doing more co-nesting in NE housing (for C57s: Tukey's $P < 0.0001$; for DBAs, $P = 0.0005$), but no such effects in Experiment One.

If SB was added as a covariate, then the effects of housing type on the giving and receipt

of agonistic behaviour were all reduced or even eliminated. Enrichment effects on the performance of aggression thus became non-significant ($F_{1,85} = 0.42$, $P = 0.51$); enrichment effects on the production of agonistic behaviours were reduced to a trend ($F_{1,96} = 3.54$, $P = 0.06$). This suggests that at least in part, the effects of housing type on agonism reflected its effects on SB. In contrast, if performing aggression or all agonism were added as covariates, or if receiving aggression or all agonism were, the effects of housing type on SB remained very similar: enriched mice remained significantly less stereotypic ($P < 0.0001$ in all models), with experiment*strain*housing also remaining significant ($P < 0.05$ in all models), and Tukey's tests again revealing that while enrichment reduced SB in both strains in Experiment One ($P < 0.01$ in all models), in Experiment Two it only did so for DBAs ($P < 0.01$ in all models). This in turn thus suggests that the effects of housing type on SB were *not* dependent on its effects on agonism.

The last pooled analyses re-investigated the potential relationships between SB and social interactions. No relationships were found between performing SB and being dominant or aggressive (nor were there any significant interactive effects). For receiving all acts of agonism, however, across both studies together there was a near positive trend with SB ($F_{1,176} = 2.69$, $P = 0.102$). Furthermore, for receiving aggression only, there was a significant interaction with housing type ($F_{1,153} = 16.57$, $P < 0.0001$). Splitting the data by housing type revealed a strong positive relationship between performing SB and receiving aggression within enriched mice, regardless of strain or experiment ($F_{1,121} = 8.79$, $P = 0.004$), but not within NE mice ($F_{1,45} = 0.001$, $P = 0.98$). Finally, for co-

nesting, there were no consistent patterns, but instead two significant three-way interactions, and a trend effect for a third (SB*housing*strain: $F_{1,122} = 7.58$, $P = 0.007$; SB*strain*experiment: $F_{1,130} = 4.43$, $P = 0.037$; SB*housing*experiment: $F_{1,155} = 2.72$, $P = 0.101$). Splitting data into subsets to try and investigate why, revealed no significant main effects of SB.

*** Table 3 about here ***

Experiment Three

A regression revealed no significant linear relationship between how stereotypic the demonstrators were and how much shatavari-flavoured diet was selected by their observers ($F_{1,16} = 0.16$, $P = 0.692$). However, the 20 observers divided into two equal sized groups as to whether or not they chose to eat more shatavari-flavoured diet than marjoram-flavoured control. When these groups' demonstrators were compared, the observers who chose to eat more shatavari tended to have had less stereotypic demonstrators than observers who ingested equal amounts of the two flavours or ate more marjoram ($F_{1,16} = 3.58$, $P = 0.077$; see Figure 3). The amount of aggression received by these demonstrators in their home cages, in contrast, appeared unrelated to whether or not their observers favoured the shatavari-flavoured diet ($F_{1,16} = 0.96$, $P = 0.34$).

*** Figure 3 about here ***

Discussion

In Experiments One and Two, mice raised and housed in standard non-enriched (NE) cages broadly performed more stereotypic behaviour than those in enriched cages, just as

456 expected. NE mice also spent significantly more time being 'still but awake'. Being
457 inactive despite being awake has previously been found to increase in barren enclosures
458 in mice (Tilly *et al* 2010), and similar effects have been seen in other species too
459 (reviewed Meagher & Mason 2012). In mink, this behaviour appears to indicate
460 boredom-like states (Meagher & Mason 2012). In mice, its welfare significance is
461 unknown, and it was not the focus on our research; however, we do flag this behaviour as
462 a potentially interesting topic for future study.

463
464 In terms of social interactions within the home cage, in Experiment Two, mice in NE
465 cages spent more time nesting together than those in enriched cages, but this effect was
466 not consistent across both studies. However, across both Experiments One and Two
467 together, as predicted, agonistic social interactions were consistently more frequent in NE
468 housing. As reviewed in the Introduction, this finding joins several previous studies in
469 showing that barren housing can have adverse social effects on laboratory rodents. Such
470 effects occur beyond this taxonomic group too: NE housing can promote agonistic
471 interactions between conspecifics in non-rodent species (*eg* as reviewed Diez-Leon *et al*
472 2013, Diez-Leon & Mason subm.). For example, NE conditions can exacerbate
473 aggressive behaviour in primates (Honess & Marin 2006, Márquez-Arias *et al* 2010) and
474 newly weaned pigs (Schaefer *et al* 1990), while in farmed mink, NE males are less
475 successful with females in a mate choice experiment (Diez-Leon *et al* 2013). It would be
476 interesting to explore such effects further, perhaps using less enriched NE cages to create
477 more contrast, or studying males, as the more aggressive sex. Possible mechanisms for
478 elevated agonism could also be investigated; these include greater levels of frustration in

NE mice (since frustration can exacerbate aggression; reviewed Papini 2003); reduced behavioural competition, with NE mice having fewer opportunities than enriched mice to perform behaviours other than aggression (Turner *et al* under review); reduced abilities of NE mice to physically remove themselves from each other, and/or use enrichment objects to hide in order to diffuse social tension; and more abnormal brain development, perhaps increasing agonistic interactions by making mice poorer at learning and/or more prone to repeat activities that are counter-productive.

This last idea led us to investigate how SB and agonistic behaviours inter-relate. Experiment One yielded no information on this, perhaps because of its relatively small sample size, but Experiment Two yielded several interesting results, as did analyses of both datasets pooled. One of our predictions, based on studies of dopamine transporter knockout mice (Rodríguez *et al* 2004) was that stereotypic mice would show increased levels of aggression. However, our results did not support this. Our second prediction was that highly stereotypic mice would receive high levels of aggression, and this received more support. Receiving aggression positively correlated with SB, although only in enriched cages: stereotypic enriched mice consistently received the most aggression from their cagemates. Why this was only manifest in the enriched cages is unclear, but could perhaps reflect the masking effects of other potentially abnormal behavioural changes in non-enriched mice (*eg* more time spent still but awake).

At least in the enriched populations, this pattern is thus consistent with the most stereotypic mice being perceived as abnormal by cagemates, or acting socially oddly in competitive situations, thence becoming targets for aggression. Another potential

explanation, however, is that mice receiving high levels of aggression then develop more SB in response to increased stress (*cf eg* Akre *et al* 2011). To tease apart these two possibilities, analyses were run to investigate whether housing type effects on agonism were still detectable if variation in SB was statistically controlled for, and conversely, whether housing type effects on SB were still detectable if variation in agonism was statistically controlled for. These analyses revealed that effects of housing type on SB were still evident even when variation in agonism was factored out, but the converse was not true: housing effects on agonism were reduced or even eliminated when variation in SB was factored out. These patterns are consistent with SB being a key driver of the housing effect on agonistic social interactions, just as predicted. Future research should test this hypothesis experimentally, for instance in longitudinal studies to assess which behavioural differences appear first, and/or by moving mice between cages to investigate whether transferring high or low SB individuals to new social groups differentially influences the expression of agonistic behaviour by their cagemates. We also recommend that any such future studies use video rather than direct live observation, to assist the quantification of more nuanced aspects of social interaction (*eg* affiliative versus aggressive allogrooming: Warne 1947, Grant & Macintosh 1963).

Experiment Three then investigated the social abilities of stereotypic mice in a different way. We tested the hypothesis that stereotypic mice would be less effective demonstrators in a social learning task. This was inspired by previous findings of demonstrator effects in rodent studies (see Introduction). Mixed-strains were used, as this is how mice were housed in the experiment; as far as we know this is the first study to investigate the social transmission of food preferences between different strains of mice.

As predicted, low SB demonstrators appeared to possibly be more effective than high SB demonstrators at inducing a qualitative relative preference in observer mice for a novel flavour the demonstrators had recently eaten. However, since this is the first ever indication of such an effect, and it was also merely a trend, we recommend that repeat studies are now conducted to assess whether this result can be replicated. Such studies should use flavours *a priori* shown to be equally preferred by naïve observers (we did not have such data), to allow a clearer, more quantitative assessment of the social transmission of preference. They should also involve videoing the interactions between the demonstrator and observers -- particularly oronasal investigations, since olfactory cues are essential for the social transmission of food preferences (Valsecchi & Galef 1989) -- to both identify how these vary in quality or quantity (*cf eg* Choleris *et al* 2011), and assess the impact of any SB performance during this interaction phase. Replicate studies might also benefit from using feeding regimes for the demonstrators that have been shown to induce weaker, more variable flavour preferences in observers, since social effects might be more easily detected using such paradigms (*cf* Galef *et al* 1998).

Overall, the findings from these three experiments together suggest that non-enriched mice who develop SB do not just have a motor symptom consistent with autism, but possibly also the social and communicative deficits that characterise this condition (*eg* Silverman *et al* 2010, Patterson 2011). Future research should therefore test this hypothesis further, both in the ways already suggested, and also by investigating whether low SB mice are preferred as social or sexual partners to high SB mice (*cf* the mink studies of Diez-Leon *et al* 2013).

Animal welfare implications and conclusions

This study shows that in addition to reducing levels of stereotypic behaviour, housing female mice in larger, enriched laboratory cages decreases the undesirable social behaviours of aggression and displacement, with obvious implications for their welfare. Furthermore, the two effects seemed related, with high SB mice appearing more prone to being the targets of aggression. Since being aggressed and subordinate is stressful (Lumley *et al* 2000, Bartolomucci *et al* 2005), this suggests that in mice, the welfare of highly stereotypic individuals is of particular concern. It also suggests that these mice may be abnormal in ways that render them socially impaired – an idea tentatively supported by high SB mice tending to be relatively ineffective demonstrators in a social learning task. Whether SBs and their underlying causes truly render mice socially compromised, and if so, how, needs future research, as it could have welfare implications, not only for high SB individuals but also for their cagemates, and not only in mice, but all captive species prone to SB.

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Tables and figures

Table 1 Behaviours recorded during scan and focal sampling in Experiments One and Two (adapted from Grant & Mackintosh 1963; Tilly *et al* 2010; Clipperton-Allen *et al* 2011)

Table 2. Types of cages and number of cages per housing type for Experiment Two

Table 3: Overview of how the home cage behaviours of non-enriched (NE) and enriched mice compare across both studies

Figure 1: The effects of housing type on the performance of agonistic behaviours; there was a significant main effect of housing type and no interaction with strain (see text for details). Data presented are means and SEs of raw data.

Figure 2: Performance of aggression (bouts/min) in enriched and non-enriched cages in Experiment Two (data shown are means and SEs of raw data); housing type interacted with strain, an effect driven by the elevated aggression of NE C57s (see text for details)

762 Figure 3: Level of stereotypic behaviour in the demonstrators of shatavari flavour,
763 compared for observers who ate either more marjoram ($n = 10$) or more shatavari ($n = 10$)
764 in a two choice test. Shown are means and SEs of raw data (see text for details)