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Can live with 'em, can live without 'em: Pair housed male C57BL/6J mice show low aggression and increasing sociopositive interactions with age, but can adapt to single housing if separated

Luca Melotti[a,*,1], Niklas Kästner[a,1], Anna Katharina Eick[a], Anna Lisa Schnelle[a], Rupert Palme[b], Norbert Sachser[a], Sylvia Kaiser[a], S. Helene Richter[a]

[a] Department of Behavioural Biology, University of Münster, Badestrasse 13, 48149 Münster, Germany
[b] Department of Biomedical Sciences, University of Veterinary Medicine Vienna, Veterinärplatz 1, A 1210 Vienna, Austria

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ABSTRACT

The basic question as to whether male laboratory mice should be singly or group housed represents a major animal welfare concern within current laboratory animal legislation and husbandry. To better understand the behavioural and physiological mechanisms underlying this issue, we conducted two longitudinal experiments using C57BL/6J mice. In the first experiment (N = 32), we explored social behaviour of pair housed males from weaning to adulthood. We took weekly measures of agonistic, socio-exploratory and affiliative behaviours within two different contexts, i.e. in the undisturbed home cage and immediately after cage cleaning. In the second experiment (N = 36), we investigated whether separation of male pairs into single housing at different ages (35, 56 or 77 days of age) affected welfare-related measures such as faecal corticosterone metabolites (FCMs) and anxiety-like behaviours. In the first experiment we found that levels of agonistic behaviour were higher after cage cleaning than in the undisturbed cage as expected, but did not significantly change with age in either context. Instead, affiliative behaviour increased with age in the undisturbed home cage. In the second experiment, social separation did not affect levels of FCMs or anxiety-like behaviours at any age point. Taken together, this study shows that pair housed male mice can maintain low levels of aggression across a long period of their life and perform increasing levels of sociopositive behaviours which may serve to promote stable social relations. At the same time, our results suggest that male mice can quickly adapt to separation into single housing at different ages, from adolescence to adulthood. These findings are in line with the behavioural ecology of wild male mice, which suggests that both solitary and group living represent two alternative strategies.

1. Introduction

Among all vertebrates used in animal experimentation, mice are the most common (European Commission Report, 2013), thus the problem of providing them with housing conditions ensuring their welfare concerns a great number of research facilities worldwide. However, even the basic question as to whether male mice should be singly or group housed represents one of the main unsolved issues within the regulation of laboratory animal husbandry (Kappel et al., 2017; Weber et al., 2017). Despite a considerable male bias, especially in biomedical research (Wald and Wu, 2010; Beery and Zucker, 2011; Prendergast et al., 2014), current legislation is only providing very general guidelines on the social requirements of male mice, without offering solutions that can be easily implemented across labs. For example, the European Union (EU) Directive on the care and use of animals in research states that male mice, as members of a gregarious species, should be housed in stable groups when severe conspecific aggression does not take place. However, at the same time it allows single housing if "adverse effects or damage are likely to occur" (Directive 2010/63/EU, 2010) – *de facto* leaving it mostly up to the individual research facility to decide if and when to house male mice individually.

These rather loose recommendations stem from the fact that the social organisation of wild or free-living mice is considerably different from the one imposed by standard laboratory housing, making it difficult to simply extrapolate knowledge of the wild mice ecology to laboratory conditions. Free-living mice frequently form territories

* Corresponding author:
E-mail address: melotti@uni-muenster.de (L. Melotti).
[1] These authors contributed equally to this work.

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inhabited by small groups including a dominant male together with multiple females and their pups as well as non-dispersing juveniles (Van Zegeren, 1979). The other subordinate and sexually mature males generally disperse to form new defended territories (Wolff, 1985). For management and experimental reasons, this type of social structure is not maintained in the lab. If not kept individually, adult males are housed together in same-sex groups varying in size. Although mice are capable of showing high social flexibility in response to varying environmental conditions (Pocock et al., 2004), and non-territory-holders can share communal areas in the wild (Wolff, 1985), group housing arrangement in the lab has been found to inevitably increase the probability that agonistic encounters can escalate and cause serious injuries and distress (Weber et al., 2017). This is most likely due to a number of factors such as the inability for subordinates to disperse / avoid conflictual situations (Weber et al., 2017), failure to maintain a stable group hierarchy (e.g., Howerton et al., 2008), genetic background (strain effect: Bisazza, 1981) and/or different husbandry procedures (e.g., presence of female odour: Hurst, 2005; effect of identification method: Gaskill et al., 2017). Furthermore, previous studies on the suitability of different husbandry strategies for laboratory male mice often produced conflicting results which highlighted the presence of multiple internal and external factors that limit generalisation of findings to different laboratory conditions and husbandry protocols (Kappel et al., 2017; Weber et al., 2017). For instance, provision of different forms of environmental enrichment did not cause any differences in aggression in group housed AB/Gat male mice (Marashi et al., 2004), yet it increased aggression in male mice of a congenic strain (CS; Marashi et al., 2003), suggesting that the effect of environmental enrichment on aggression is strain-dependent.

In this jungle of often contradicting and context-specific findings, aiming at providing general recommendations that ensure the welfare of all group housed laboratory male mice may be counterproductive. One way to deal with this problem may be to address and evaluate the welfare implications of laboratory housing systems case by case (i.e., by assessing the combined effect of the above mentioned factors for each system; Kappel et al., 2017) and, using a systematic approach, to unravel the (behavioural and physiological) processes that explain how a given laboratory housing context affects relevant welfare outcomes. In this direction, one aspect that has been understudied so far is the behavioural development of group housed male mice from the juvenile phase to full adulthood. Many of the studies conducted on male mouse housing so far either relied on experimental manipulations at one or few specific time points (e.g., Arndt et al., 2009) or covered a relatively short part of the mouse life (e.g., Ferrari et al., 1998), thus potentially missing important information on how behaviour developed with age.

Another aspect that has not been addressed yet in a longitudinal and systematic way is whether preventative separation into single housing is to be preferred to group housing at any time point between adolescence and adulthood, based on welfare-related physiological and behavioural measures. On the one hand, prolonged social deprivation after weaning has been employed to induce male mouse models of neuro-psychological disorders (Valzelli, 1973), on the other hand it has been argued that the behavioural changes deriving from isolation are adaptive, indicating increased territoriality (i.e., holding a territory without intruders) rather than the onset of a pathology (Brain, 1975). Thus, it is still unclear if separation into single housing performed within a housing room allowing for visual, olfactory and auditory contact with conspecifics can be assumed to impair the welfare of male mice. Moreover, separation of male mice into single housing has been performed at disparate ages ranging from weaning (e.g., Voikar et al., 2005), adolescence (e.g., Liu et al., 2013; Kalliokoski et al., 2014) and adulthood (e.g., Arndt et al., 2009; Berry et al., 2012), thus it should be more systematically assessed whether age at social separation can have a differential effect on their welfare.

Against this background, we conducted two longitudinal and complementary experiments. For these, we chose mice of the C57BL/6J strain (thereafter C57) as this is one of the most commonly used laboratory strains (The Jackson Laboratory, 2018). In the first experiment, we aimed to explore social behaviour of male mice housed together in pairs from weaning to adulthood and to identify relevant factors that can explain or even predict the success or failure of group housing. To this end, we measured agonistic, socio-exploratory and affiliative behaviours over a period of nine weeks within two different contexts, namely in the undisturbed home cage and immediately after cage cleaning. In addition to the temporal development of the behavioural measures, we analysed the consistency of agonistic behaviours over time and across context. As cage cleaning is considered to be a critical factor for outbreaks of aggression in male mice (Gray and Hurst, 1995; Van Loo et al., 2000) we predicted that overall levels of agonistic behaviour would be higher in this situation. Lastly, we expected that pair mates with more similar weights would show increased levels of agonistic behaviour (Andersen et al., 2000; Van Loo et al., 2000). In the second experiment, we aimed to investigate whether separation of male pairs into single housing at different ages affects welfare-related measures such as adrenocortical activity, anxiety-like behaviours, activity levels and body weight. We expected separation to affect these parameters differently depending on the time point of separation.

2. Methods

2.1. Animals and housing

Subjects were 32 (Experiment 1) and 36 (Experiment 2) C57BL/6J (hereafter C57) male mice purchased from Charles River Laboratories, Sulzfeld, Germany. At postnatal day (PND) 21, they were delivered to the Department of Behavioural Biology, University of Münster, Germany, where they were randomly assigned to pairs and housed in transparent Makrolon type III cages (l × b × h: 37 cm × 21 cm × 15 cm). Cages were kept in a room housing only male mice. Animals had ad libitum access to standard rodent food (Altromin 1324, Altromin GmbH, Germany) and tap water (for the additional items provided in the cage, see Section 2.3.2). The housing room temperature and humidity were maintained at ca. 22 °C and 50%, respectively. A 12:12 light:dark cycle (lights off at 9:10 am) was maintained and experimental procedures were conducted during the dark phase under red light. During cage cleaning, which was performed weekly, each mouse was examined for the presence of any skin wounds. However, in both experiments no skin wounds were found on any of the mice. To allow individual identification of the two cage mates, the left or right ear were marked via ear cuts. For husbandry and experimental procedures, mice were handled using “loose tail handling”, which consisted of collecting the mouse by the proximal part of the tail, immediately placing it on the experimenter´s arm, and letting it freely move on the arm while holding the tail.

All procedures complied with the regulations on animal experimentation within the EU (European Communities Council DIRECTIVE 2010/63/EU). They were conducted in accordance with the institution’s animal care and use guidelines and approved by the national and local authorities.

2.2. Experimental design

This study was comprised of two experiments. Experiment 1 aimed at assessing how aggressive, social-exploratory and affiliative behaviours develop in stable male pairs from weaning to adulthood. Experiment 2 aimed at investigating whether separation into single housing at different ages has implications for welfare-related measures such as faecal corticosterone metabolites (hereafter FCMs), anxiety-like behaviours, activity levels and body weight.

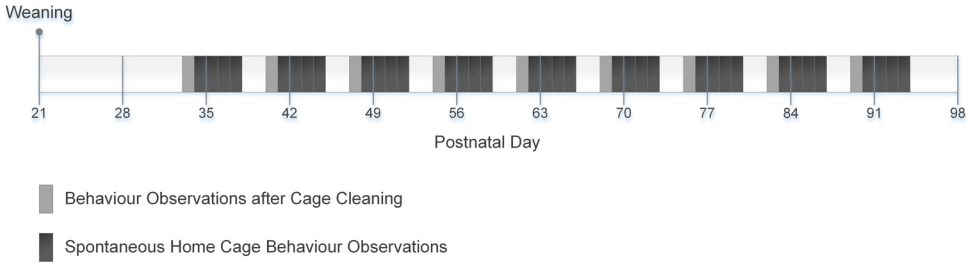

Fig. 1. Timeline of Experiment 1. Agonistic, social-exploratory and allo-grooming behaviours were recorded every week between PND 33 and 94, both after cage cleaning and as spontaneous (i.e., undisturbed) behaviours in the home cage. Behaviour after cage cleaning was observed on the same week-day for all animals, while spontaneous home cage behaviour was recorded once per week (four cages per day across four consecutive days).

2.3. Experiment 1

Experiment 1 was performed between PND 33 and 94 and consisted of weekly observations of agonistic, social-exploratory and affiliative behaviours in two different contexts: as spontaneous (i.e., undisturbed) behaviours in the home cage and directly after cage cleaning (Fig. 1).

2.3.1. Spontaneous home cage behaviour

Spontaneous behaviour in the home cage was video-recorded with infrared cameras (EH1000H-4 Nano cameras, AVer Information Inc., Taiwan) once a week between PND 34 and 94 during the first hour of the dark phase (nine hours recorded per pair). Behaviours were observed continuously at the cage level (i.e., without distinguishing between the two cage mates) and consisted of dominant agonistic behaviours (*tail rattling*, *mounting*, *chasing*, *attack*, *sustained attack*, *escalated fighting*), submissive agonistic behaviours (*defensive upright posture* and *fleeing*), social-exploratory behaviours (*approaching*, *body sniffing*, *facial sniffing*, *ano-genital sniffing*, *following*) and affiliative behaviour (*allo-grooming*). Ethogram and behavioural pattern definitions were derived from previous literature (Jansen et al., 2010; Kloke et al., 2011; Heiming et al., 2013) and are shown in Table 1. The behaviour frequencies within the same behavioural category were summed for each week of observation, and rates (per minute) across the whole experiment were calculated for agonistic (dominant plus submissive), social-exploratory and affiliative behaviours. The pronouncedly aggressive behaviour "sustained attack" was never observed throughout the experiment.

2.3.2. Behaviour after cage cleaning and body weight

Cages were cleaned and mice were weighed once a week between PND 33 and 90, and behavioural observations were conducted immediately following this procedure. Each new cage was provided with fresh wood shavings as bedding (Allspan, Höveler GmbH & Co. KG, Germany), a clean transparent red plastic house (Mouse™, Tecniplast, Germany) as well as a wooden stick (pinewood) as enrichment, and a paper tissue (Katrin Basic System towel M, Metsä Tissue GmbH, Germany) as nesting material. The same cage lid was kept for each cage throughout the experiment, and a small quantity of soiled bedding was transferred from the old to the new cage to provide familiar odours (but see also discussions on the implications of these procedures for aggression; Gray and Hurst, 1995; Van Loo et al., 2000). While the cage order was kept the same (i.e., each mouse pair always experienced the cage cleaning procedure at roughly the same time of day), the picking order of the two cage mates was randomised across cage cleaning days. For each cage, as soon as the second cage mate was weighed and then transferred to the new cage, the experimenter continuously recorded behaviour frequencies at the cage level (i.e., without distinguishing between the two cage mates) for 15 min. Observed behaviours were agonistic, social-exploratory and affiliative behaviours as described in Section 2.3.1 and Table 1. Behaviour frequencies within the same behavioural category were summed for each week of observation, and rates (per minute) across the whole experiment were calculated for agonistic (dominant plus submissive), social-exploratory and affiliative behaviours. To assess whether similarity in body weight was related to higher levels of aggression (Andersen et al., 2000), the relationship between body weight ratio (average weight of the lighter cage mate divided by average weight of the heavier one) and agonistic behaviour at the cage level was explored.

2.4. Experiment 2

In Experiment 2 (see Fig. 2 for experimental timeline), cages were randomly divided into three experimental groups of six cages (12 mice per group). The mice of the first experimental group (PND 35 Group) were separated from their cage mate at 35 days of age and then singly housed in same size type III cages for the rest of the experiment. The

Table 1
Ethogram with definitions of behavioural patterns recorded.

Behavioural Category	Behaviour	Definition
Dominant Agonistic Behaviours	*Tail rattling*	Mouse performs fast waving movements with its tail
	Mounting	Mouse puts both forepaws on the back of the other mouse, approaching it either from the rear or from the side
	Chasing	Mouse runs after the other mouse while the other mouse displays "Fleeing" behaviour
	Attack	Mouse launches at the other mouse so that its nose/mouth gets in contact with it; this may include grasping the skin of the other mouse with the teeth and pulling it
	Sustained Attack	Series of attacks occurring within 1 second from one another, performed by the focal mouse towards the other mouse
	Escalated Fighting	Mice attack each other with continuous physical contact including wrestling and rolling in the bedding
Submissive Agonistic Behaviours	*Defensive Upright Posture*	Mouse stands on its hind paws in an upright posture and keeps its forepaws stretched out in front of its body; the behaviour ends when at least one forepaw touches the ground again
	Fleeing	Mouse rapidly locomotes away from the other mouse, which is performing threat or aggressive behaviour
Social-Exploratory Behaviours	*Approaching*	Mouse locomotes directly towards the other mouse until the distance between the two mice falls at least below one body length
	Body Sniffing	Mouse touches the body of the other mouse (except the face and the ano-genital areas) with its nose
	Face Sniffing	Mouse touches the face of the other mouse with its nose
	Ano-genital Sniffing	Mouse touches the ano-genital region of the other mouse with its nose
	Following	Mouse follows the other mouse keeping at a maximum distance of one body length
Affiliative Behaviour	*Allo-Grooming*	Mouse grooms the other mouse by licking its fur and/or stroking its fur with the forepaws; the behaviour ends when allo-grooming is not performed for at least two seconds

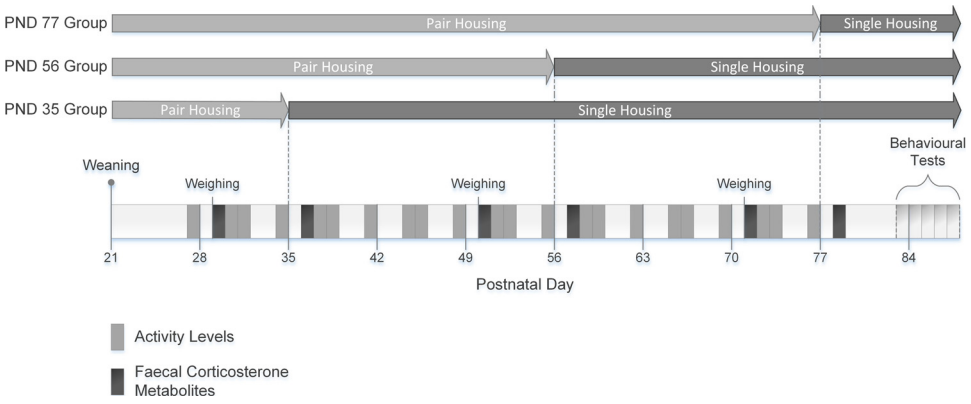

Fig. 2. Timeline of Experiment 2. After weaning, animals were pair-housed and allocated to three experimental groups in which the cage mates were separated into single housing at different time points for each group (PND 35, 56 and 77 Groups, respectively). Activity levels in the home cage were recorded throughout the experiment (PND 27–76). Faeces were collected and corticosterone metabolites were analysed six days before and one day after each separation event, to allow for comparisons between pre- and post-separation. Additionally, mice were weighed six days before every separation event. Between 83 and 87 days of age, mice were tested for anxiety-like behaviour, exploratory locomotion and social interest.

second and third experimental groups (PND 56 Group and PND 77 Group) underwent the same procedure but at later time points: the separation into single housing occurred at 56 and 77 days of age, respectively. FCMs sampling was performed six days before (29, 50 and 71 days of age) and one day after (36, 57 and 78 days of age) each separation event, to allow for comparisons between pre- and post-separation levels of stress hormones. To assess an effect of the housing condition on the weight development, all animals were weighed six days before every separation event (Day 29: basal weights; Day 50: effect of first separation; Day 71: effect of second separation). Additionally, activity levels of each mouse were recorded during repeated scan sampling sessions from 27 to 76 days of age (see Section 2.4.2). After all mice were singly housed, each animal was tested for anxiety-like behaviour and exploratory locomotion in three established paradigms: the Elevated Plus Maze test (EPM; Day 83), the Novel Cage test (NC; Day 84) and the Open Field test (OF; Day 85). Additionally, a Social Interest test (SI) was performed (Day 86 or 87).

2.4.1. Analysis of faecal corticosterone metabolites

Adrenocortical activity was assessed non-invasively by measuring corticosterone metabolites in the faeces (FCMs; Touma et al., 2003). Mice were transferred to a Makrolon type III cage that contained some bedding material, a red plastic house and a paper tissue, either individually or as a pair depending on the experimental group. They were kept in this cage for three hours and then brought back to their home cage. Faeces produced during this period were collected into 1.5 ml reaction tubes and frozen at −20 °C. Faecal samples were then dried (80 °C for two hours) and homogenised, and aliquots of 0.05 g were extracted with 1 ml of 80% methanol (Palme et al., 2013). Then, FCMs were analysed by means of a 5α-pregnane-3β,11β,21-triol-20-one enzyme immunoassay, previously established and successfully validated to evaluate adrenocortical activity in mice (for details see Touma et al., 2003, 2004). Intra- and inter-assay coefficients of variation were below 10 and 12%, respectively.

2.4.2. Activity levels

Activity levels were recorded three days per week between 9:15–17:15, from 27 to 76 days of age. On each of these days, six 30-min scan sampling sessions were performed at an hour interval from each other. During a scan sampling session, the experimenter moved between cages following always the same order and recorded the frequencies of inactive and active behaviour. Inactive behaviour was defined as the animal lying still on its side or sitting, without performing self-grooming, for at least five seconds. Active behaviour was scored if the animal showed any locomotor behaviour, including self-grooming. To allow comparability between experimental groups, frequencies of the two cage mates (whether they were still housed together or not) were averaged, and overall frequencies were calculated for the period from 27 to 34 days of age (all mice still housed in pairs; Phase 1), the period from 37 to 55 days of age (first experimental group singly housed; Phase 2) and the period from 58 to 76 days of age (first and second experimental groups singly housed; Phase 3). For the data analysis, the proportion of active behaviour on total (active and inactive) behaviour was calculated for each phase.

2.4.3. Behavioural tests

Behavioural tests were performed on separate days during the dark phase and in the same order (EPM – NC – OF – SI). Testing order of individuals was randomised for every behavioural test, and each test apparatus was cleaned with 70% ethanol between animals (full evaporation of ethanol was ensured before each animal was tested). The experimenter was blinded to the experimental groups during testing.

2.4.3.1. Elevated Plus Maze test. Mice were tested in the EPM (Pellow et al., 1985; Lister, 1987, 1990) at the age of 83 days. The apparatus consisted of a plus-shaped maze elevated 50 cm above the floor. The maze comprised four arms (30 × 5 cm each) and a central square (5 × 5 cm). Two opposite arms were surrounded by 20 cm high wooden walls (closed arms), the two remaining arms only had a 0.4 cm high border to prevent the mice from falling from the maze (open arms). The apparatus was made of wood painted light grey and the surface of the maze was covered by a grey PVC inlay. The illumination level in the centre square was set to 25 lx. A webcam (Logitech Webcam Pro 9000) was placed directly above the centre of the field and the behavioural measures were collected using the Anymaze software (v. 4.75, Stoelting Co., Wood Dale, USA). Mice were individually placed in a transport container and taken to a testing room adjacent to the housing room. After one minute in the transport container (which ensured that all animals were awake / similarly active), the mouse was placed in the centre square of the EPM with its head always facing the same closed arm. Then the experimenter left the room and the mouse was left to freely explore the apparatus for five minutes, after which it was returned to its home cage. Behavioural measures taken were relative time on open arms (time on open arms/(time on open arms + time on closed arms)), relative number of open arm entries (open arm entries/(open arm entries + closed arm entries)), latency to enter an open arm and distance travelled on the open arms as measures of anxiety-like behaviour, and the total distance travelled as measure of exploratory locomotion.

2.4.3.2. Novel Cage test. Mice underwent the NC (Richter et al., 2016a,b) at 84 days of age. Each mouse was individually transferred into a test arena consisting of a standard Makrolon type III cage located in the housing room and filled with 11 of bedding material (approximate depth of bedding: 1.5 cm; Allspan, Höveler GmbH & Co.KG, Langenfeld, Germany). Each mouse was placed with the head always facing the same corner of the test arena. During the following five minutes, the frequency of rearing behaviours was recorded directly

by the experimenter as a proxy measure of exploration. Rearing behaviour was defined as the mouse standing on its hind paws (Fuss et al., 2013). The behaviour ended when both front paws touched the ground again. Once the five minutes had passed the mouse was returned to its home cage, and the test arena was cleaned with 70% ethanol and re-filled with new bedding material for testing the next mouse.

2.4.3.3. Open Field test. Mice were tested in the OF (Archer, 1973; Bodden et al., 2015) at 85 days of age. The apparatus was made of white plywood and consisted of a square arena (80 × 80 cm) surrounded by walls (height: 37.5 cm) and with an illumination level set to 35 lx at the centre of the arena. Mice were individually taken to an adjacent test room using a transport container and after one minute they were placed in the test arena with their head always pointing towards the same corner. In the following five minutes the experimenter left the room and behavioural measures were automatically recorded by Anymaze. Measures taken were time spent in the centre (defined as the area at least 20 cm from the walls), number of entries to the centre and distance travelled in the centre as measures of anxiety-like behaviour, and total distance travelled as proxy measure of exploratory locomotion. After the five minute period the mouse was taken back to its home cage.

2.4.3.4. Social Interest test. Mice were tested in a modified version of the SI (Lukas et al., 2011; Kästner et al., 2017) to measure their interest in investigating an unfamiliar conspecific. Testing occurred on two consecutive days (Days 86 and 87), with one randomly selected half of the animals being tested on the first day and the other half on the second day. Mice were individually placed in a test arena located on one side of the housing room, so to minimise any direct interference (e.g., visual contact) from other conspecifics housed in the same room. The test arena consisted of a standard Makrolon type III cage filled with 1 l of bedding material (same bedding as in the NC) and covered with a transparent and perforated plastic lid. Each mouse was placed in the arena so that its head always pointed towards the same corner, and after an habituation period of one minute, a cylindric wire mesh cage (diameter: 10 cm; height: 8 cm) was placed in the middle of the arena and the mouse was left to explore the arena and mesh cage for an additional three minute period. The mouse was then transferred to a "waiting cage" (standard Makrolon type II cage), and a stimulus unfamiliar male mouse of the same strain (derived from the mouse stock of the Dept. of Behavioural Biology, Münster) was introduced into the wire mesh cage. The subject mouse was placed again in the arena with its head facing the same corner, and was left to explore it for three minutes. During this period, the experimenter directly recorded the time the subject mouse spent investigating the stimulus mouse in the wire mesh cage. Such behaviour was defined as the nose of the subject mouse being less than one cm away from the wire mesh cage / stimulus mouse. The subject mouse was then returned to its home cage, the test arena and wire mesh cage were cleaned with 70% ethanol, and the arena was re-filled with new bedding material for testing of the next mouse.

2.5. Data analysis

All statistical analyses were carried out using SPSS (IBM SPSS v. 25) and graphs were drawn with SigmaPlot (v. 12.5). Statistical analyses were considered to be significant at $P < 0.05$. P values between 0.05 and 0.1 were set as statistical trends.

2.5.1. Experiment 1

As most of the outcome measures did not meet parametric assumptions, data were analysed using non parametric tests. Consequently, data are presented as median (M) and interquartile range (IQR).

Behavioural data derived from both contexts (spontaneous home cage behaviour & behaviour after cage cleaning) were analysed for an effect of age (in days) using related-samples Friedman´s tests. In cases of a significant effect indicated by the Friedman's test, *post hoc* comparisons between individual weeks of age were carried out via Dunn's tests, using Bonferroni correction for multiple comparisons.

To investigate an effect of the cage cleaning on social behaviours, comparisons between spontaneous home cage behaviours and behaviours after cage cleaning were made at the cage level (N = 16) using related-samples Wilcoxon signed rank tests. For this purpose, data of all weeks were averaged.

The consistency of agonistic behaviour across contexts was investigated by assessing the relationships between agonistic behaviour during undisturbed home cage observation and after cage cleaning within each week, using Spearman's rank correlations. Furthermore, to elucidate the predictive value of assessing the level of aggression between paired-housed mice after cage cleaning, the temporal consistency of agonistic behaviour in this context was explored. For this purpose, the intraclass correlation coefficient (ICC) was calculated using a two-way mixed design assessing the consistency of the mean of the experimental weeks (Shrout and Fleiss, 1979; Landers, 2015; Koo and Li, 2016). Consistency was evaluated based on the ICC estimate and on the lower bound of the 95% confidence interval (Koo and Li, 2016).

The relationship between body weight ratio and overall agonistic behaviour in both contexts was analysed using Spearman's rank correlations.

2.5.2. Experiment 2

All data were analysed using general linear models. Normality of the data was assessed through visual inspection of the studentized residuals´ distribution. Where data did not meet parametric assumptions, they were transformed (square root or logarithm transformation). Homogeneity of variance (fixed factors) was examined through visual inspection of the residuals´ plot and using Levene's test of equality of error variances.

Concentrations of FCMs (cage level, n = 18) were analysed for an effect of experimental group and age using a mixed design ANOVA, with "experimental group" set as fixed factor and "age" as repeated measure. The same procedure was followed for body weight (individual level, n = 36) and activity data (cage level, n = 18), with the difference of replacing "age" with "experimental phase" as repeated measure. When sphericity was violated as indicated by Mauchly's test of sphericity, the model´s degrees of freedom were corrected using the Greenhouse-Geisser correction (FCMs and body weight). Data from the behavioural paradigms (EPM, NC, OF and SI; individual level, n = 36) were analysed for an effect of the fixed factor "experimental group" using one-way ANOVAs.

When main effects or interactions were significant, *post hoc* comparisons were performed using Sidak correction for multiple comparisons.

3. Results

3.1. Experiment 1

3.1.1. Development of behaviour with age

There was no significant effect of age on the level of agonistic behaviour, both for the spontaneous home cage behaviour ($\chi^2(8) = 12.76$, $p = 0.12$) and after cage cleaning ($\chi^2(8) = 13.20$, $p = 0.10$). In contrast to this, there was a significant context-dependent effect of age on social-exploratory behaviour: while rates increased over time for the spontaneous home cage behaviour ($\chi^2(8) = 35.35$, $p < 0.001$; *post hoc* comparisons with Bonferroni corrected $p < 0.05$ for days: 35 < 91; 49 < 77, 84, 91; 56 < 91), they decreased over time after cage cleaning ($\chi^2(8) = 37.94$, $p < 0.001$; significant Bonferroni-corrected comparisons for days: 35 > 77; 42 > 70, 77;

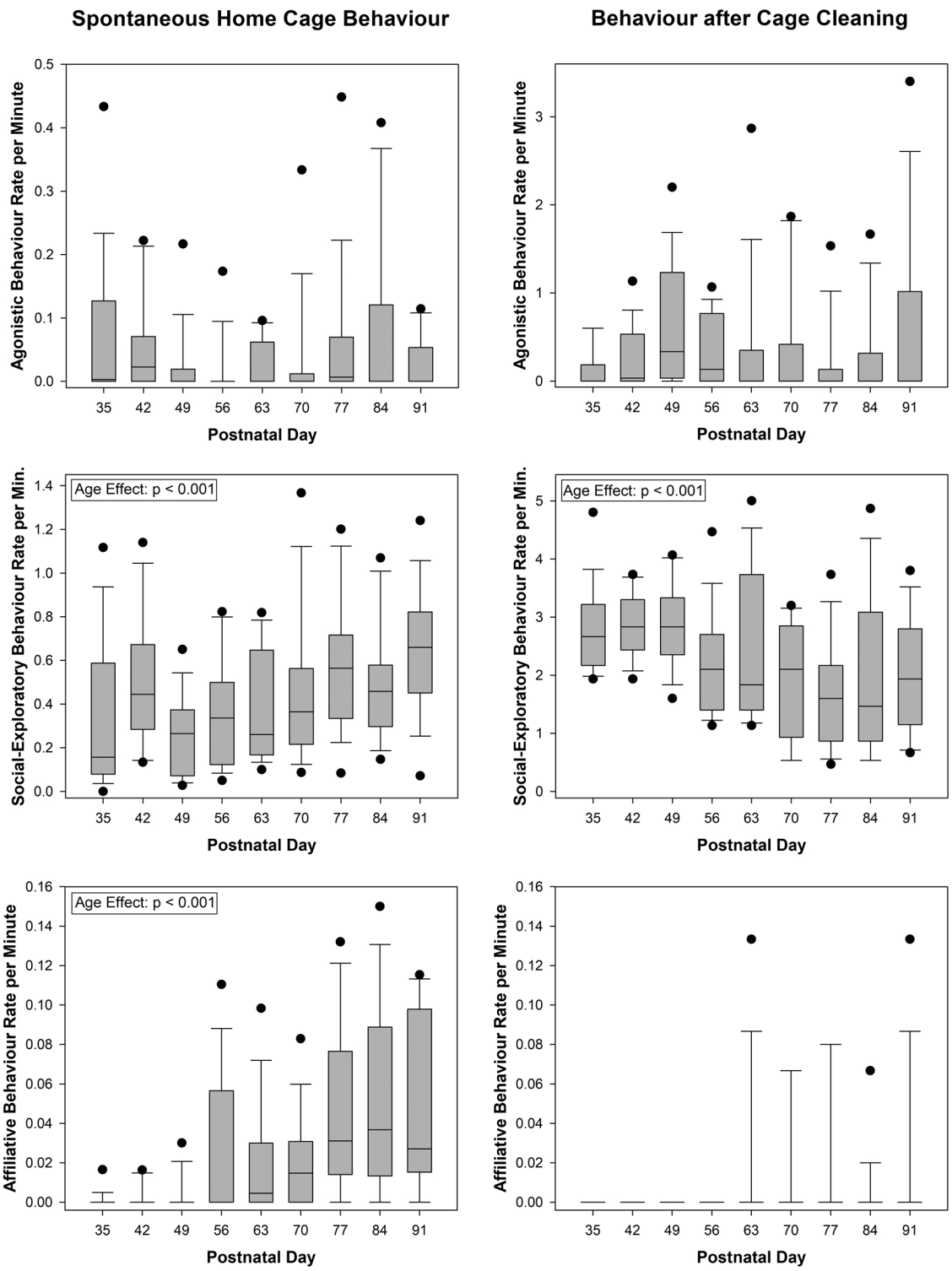

Fig. 3. Temporal development of behaviour. Social behaviours of pair-housed male mice observed as spontaneous, i.e. undisturbed, home cage behaviour or directly after cage cleaning (N = 16 pairs). The box represents the middle 50% of the data, while the upper and lower whiskers include the middle 80% of the data; the upper and lower round dots indicate the 95th and 5th percentiles, respectively. The horizontal line within the box represents the median. Y axes are scaled differently to improve readability of the results.

49 > 70, 77, 84). Rates of affiliative behaviour increased with age for the spontaneous home cage behaviour ($\chi^2(8) = 54.17$, $p < 0.001$; significant Bonferroni-corrected comparisons for days: 35 < 77, 84, 91; 42 < 77, 84, 91; 49 < 77, 84, 91), while they did not significantly change after cage cleaning ($\chi^2(8) = 10.05$, $p = 0.26$). The effect of age on behaviour is summarised in Fig. 3.

3.1.2. Comparison between spontaneous home cage behaviour and behaviour after cage cleaning

Overall, significantly more agonistic behaviour was displayed after cage cleaning (M = 0.292; IQR = 0.463) than in the spontaneous home cage behaviour observations (M = 0.030; IQR = 0.058; z = 3.11, p = 0.002). Similarly, significantly more social-exploratory behaviours were shown after cage cleaning (M = 2.280; IQR = 1.180) compared to

the spontaneous home cage behaviour observations (M = 0.400; IQR = 0.226; z = 3.52, p < 0.001). In contrast, the overall level of affiliative behaviour was significantly lower after cage cleaning (M = 0.004; IQR = 0.007) than in the spontaneous home cage behaviour observations (M = 0.024; IQR = 0.015; z = -2.95, p = 0.003).

3.1.3. Agonistic behaviour: consistency and relationship with body weight ratio

To investigate if agonistic behaviour was consistent across spontaneous home cage behaviour and *post* cage cleaning contexts, which would indicate that both contexts reflected the same agonistic behavioural "dimension", the relationship between overall agonistic behaviour rates (i.e., all weeks averaged) of these two contexts was assessed. There was a statistical trend for a positive correlation (r_s = 0.46, p = 0.07).

In order to assess whether the levels of agonistic behaviour of each cage were consistent across time, an intraclass correlation coefficient was calculated for agonistic behaviour rates after cage cleaning, which indicated moderate to good consistency (Koo and Li, 2016) across weeks of observation ($ICC_{average}$ = 0.81; CI lower bound = 0.62).

There was a strong trend for a positive correlation between body weight ratio (M = 0.972; IQR = 0.032) and agonistic behaviour after cage cleaning (i.e., the more similar the weights of the cage mates were, the more agonistic behaviour was performed; r_s = 0.49 p = 0.05). However, body weight ratio did not correlate with agonistic behaviour in the spontaneous home cage behaviour context (r_s = 0.06 p = 0.81).

3.2. Experiment 2

3.2.1. Effect of experimental group and time on faecal corticosterone metabolites, body weight and activity

Concentrations of FCMs were significantly affected by sampling day (F(1.83,27.42) = 30.05, p < 0.001): they decreased over time as indicated by *post hoc* comparisons (Sidak; p < 0.05 for days: 29 > 57, 71, 78; 36 > 57, 71, 78; 50 > 71, 78; 57 > 71, 78) and visual inspection of the data (Fig. 4). There was also a trend for a main effect of experimental group (F(2,15) = 2.88, p = 0.09) where PND 35 Group tended to have higher levels of FCMs than PND 56 Group (p = 0.09). However, the change in concentration of FCMs over time was not differentially affected by experimental group (no significant interaction: F (3.66,27.42) = 0.86, p = 0.49).

Body weight was affected by a two-way interaction between experimental group and experimental phase (F(2.65,43.78) = 4.71, p = 0.008; Fig. 5) with the main effects of experimental phase (F (1.33,43.78) = 340.77, p < 0.001) and experimental group also being significant (F(2,33) = 7.14, p = 0.003). While groups did not differ concerning body weight at the beginning of the experiments, animals of the PND 35 Group weighed significantly less after being singly housed (Phase 2) compared to the (pair housed) PND 56 and PND 77 Groups (p = 0.002, p < 0.001, respectively). Similarly, animals of the PND 56 Group weighed significantly less after being singly housed (Phase 3) compared to the pair housed animals from PND 77 Group (p = 0.03).

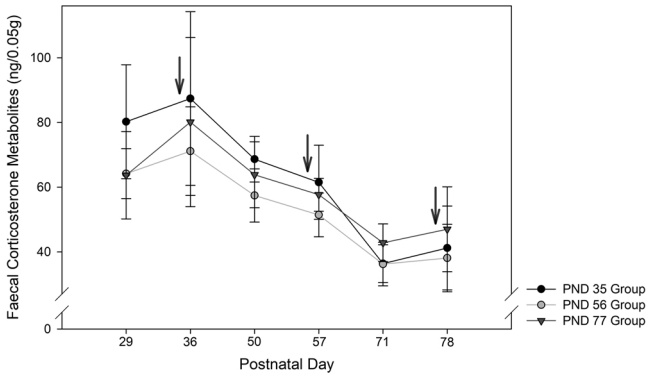

Fig. 4. Concentrations of faecal corticosterone metabolites by experimental group and across sampling days (N = 18). Arrows indicate the separation into single housing events (PND 35, 56 and 77). Data are shown as mean ± SD. Line breaks on the y axis were added for better readability of the results.

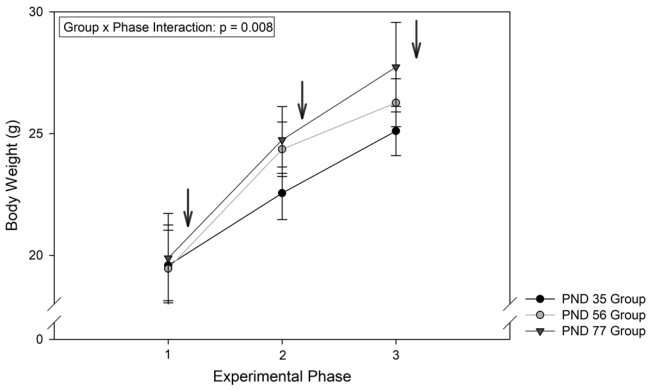

Fig. 5. Body weights by experimental group and across experimental phases (N = 36). Weighing occurred six days before each separation into single housing. Separation events are indicated by arrows (PND 35, 56 and 77). Data are shown as mean ± SD. Line breaks on the y axis were added for better readability of the results.

Relative activity levels were affected by a two-way interaction between experimental group and experimental phase (F(4,30) = 7.36, p < 0.001; Fig. 6) with the main effects of experimental phase (F (2,30) = 468.71, p < 0.001) and experimental group (F(2,15) = 4.03, p = 0.04) also being significant. *Post hoc* comparisons revealed that during Phase 1, when all animals were still pair housed, PND 56 Group was more active than PND 77 Group (p = 0.01). While from Phase 1 to Phase 2 (only PND 35 Group singly housed) activity increased in animals of all groups (p < 0.001), the increase was strongest in the PND 35 animals, which were significantly more active than PND 77 animals (p = 0.004) and, as a trend, PND 56 animals (p = 0.099). From Phase 2 to Phase 3 (both PND 35 and 56 Groups singly housed) activity levels did not change significantly in PND 35 and PND 56 animals (p > 0.95) while they increased in the still pair housed PND 77 animals (p = 0.002). Consequently, activity levels of PND 35 and 77 animals did not differ anymore during Phase 3 (p = 0.99), while PND 56 animals were significantly less active than PND 77 Group (p = 0.04) and, as a trend, PND 35 Group (p = 0.06).

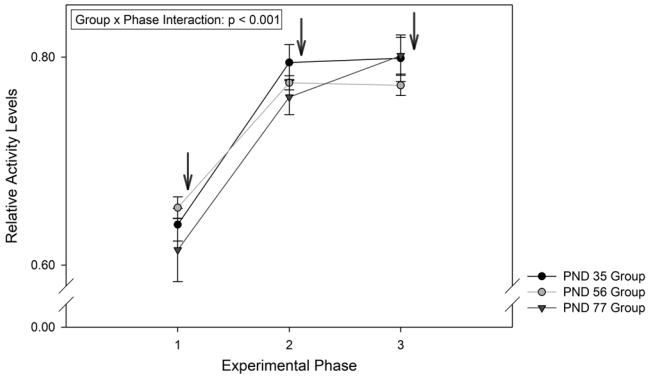

Fig. 6. Relative activity levels (proportion on total observations) by experimental group and across experimental phases (N = 18 pairs). Arrows indicate the separation into single housing events (PND 35, 56 and 77). Data are shown as mean ± SD. Line breaks on the y axis were added for better readability of the results.

Table 2
Effects of experimental group on the measures from the behavioural tests (N = 36; one-way ANOVAs).

Behavioural test	*Measure*	*Mean ± SD*	*Experimental Group Effect*	
			Test Statistics	*P value*
Elevated Plus Maze	Relative time spent on open arms	0.293 ± 0.133	F(2,33) = 1.18	0.32
	Relative number of open arm entries	0.367 ± 0.118	F(2,33) = 0.38	0.69
	Latency to enter an open arm (s)	19.01 ± 19.82	F(2,33) = 0.25	0.78
	Distance travelled on the open arms (m)	2.041 ± 1.173	F(2,33) = 1.49	0.24
	Total distance travelled (m)	10.28 ± 1.978	F(2,33) = 0.68	0.52
Novel Cage	Frequency of rearing behaviours	64.89 ± 13.12	F(2,33) = 1.46	0.25
Open Field	Time spent in the centre (s)	13.61 ± 4.643	F(2,33) = 0.46	0.64
	Number of entries to the centre	8.861 ± 3.506	F(2,33) = 0.21	0.81
	Distance travelled in the centre (m)	2.358 ± 0.869	F(2,33) = 0.02	0.99
	Total distance travelled (m)	34.62 ± 5.662	F(2,33) = 0.46	0.63
Social Interest	Time investigating stimulus mouse (s)	71.31 ± 14.53	F(2,33) = 1.63	0.21

3.2.2. Effect of experimental group on anxiety-like behaviour, exploratory locomotion and social interest

There was no significant effect of experimental group on any of the measures of anxiety-like behaviour (EPM: relative time spent on open arms, relative number of open arm entries, latency to enter an open arm, distance travelled on the open arms; OF: time spent in the centre, number of entries to the centre, distance travelled in the centre), exploratory locomotion (EPM: total distance travelled; NC: frequency of rearing behaviours; OF: total distance travelled) or social interest towards an unfamiliar conspecific (SI: time spent investigating stimulus mouse), as summarised in Table 2.

4. Discussion

By using a systematic and longitudinal approach, the present study investigated how aggressive, social-exploratory and affiliative behaviours develop in stable pairs of male mice from the juvenile phase to adulthood (Experiment 1), and whether separation into single housing at different ages has implications for welfare (Experiment 2). We assessed this in the C57 strain, being among the most commonly used mouse laboratory strains.

4.1. Experiment 1 – temporal development of behaviour in pair housed male mice

Although male mice in this experiment were housed in pairs to the age of 95 days, i.e. about 45 days after sexual maturity, levels of agonistic behaviour remained relatively low and did not significantly vary with age in both contexts investigated. This is in contrast to previous literature showing that aggressive behaviour in male CD1 and Rockland-Swiss albino mice increases when reaching sexual maturity (Barkley and Goldman, 1977; Terranova et al., 1993; Kawai et al., 2003). However, it corresponds to a previous finding where, contrary to other strains, group housed C57 mice did not fight and did not seem to form a well-defined hierarchy (Bisazza, 1981). Thus, this result may be specific to the C57 strain and is particularly relevant since aggression outbreaks represent the main concern for the welfare of group housed male mice (Van Loo et al., 2003; Weber et al., 2017). Another possible explanation for the low levels of aggression observed in our study may be represented by the specific housing conditions adopted in the facility. The low animal density in the housing room and in the home cage, the absence of females in the housing room and the use of an open cage system are all factors that may have contributed to reduce inter-male aggression.

Interestingly, other than aggression, non-agonistic behaviours did change over time depending on the context. In the undisturbed home cage, both social exploratory and affiliative behaviours increased with age whereas, after cage cleaning, social-exploratory behaviour decreased over time and affiliative behaviour barely occurred. The difference between contexts is further highlighted by the results of the direct comparison between them: levels of agonistic and social exploratory behaviours were overall higher after cage cleaning than during undisturbed home cage observations, while levels of affiliative behaviour were lower.

Therefore, the longitudinal assessment of spontaneous home cage behaviour in our study suggests that male mice are capable to share the same socio-positive behaviours that are shown to be expressed in female mice (Terranova et al., 1993). Individually housed mice exposed to repeated social encounters with same-sex conspecifics showed decreasing levels of affiliative behaviour (i.e., allo-grooming) across adolescence (Terranova et al., 1993). In contrast, we demonstrate here that male mice housed in stable pairs not only can show sustained performance of this behaviour over time, but even increase it during adulthood. Affiliative behaviour in general is associated with the consolidation of social relations and with reduced aggression in several animal species (Lindberg, 2001). Therefore, the increasing performance of such behaviours with age may explain why aggression remained at relatively low levels in the current study. Allo-grooming, in particular, is an affiliative behaviour involved in the moderation of social tensions (Spruijt et al., 1992) and has been identified as a promising indicator of positive affective state and improved welfare (Boissy et al., 2007). Social affiliation in male mice has been investigated mainly as the time spent in proximity of another male (e.g., a familiar male placed at the centre of an open field test: Pieper et al., 1997; or an unfamiliar male within a social interaction test: An et al., 2011). However, the continuous and long-term assessment of allo-grooming as in our study may represent a better measure of improved welfare in group housed male mice.

The higher level of agonistic behaviours immediately after cage change is in line with previous studies that identified the cage cleaning procedure as a risk factor for escalations of aggression (Gray and Hurst, 1995; Van Loo et al., 2000). In this context, *inter*-pair differences in agonistic behaviour after cage cleaning were consistent over time, suggesting that in situations where aggression levels are relatively low, early observations of this behaviour may predict aggression levels later in life. The predictive value of this measure is corroborated by the fact that the two contexts (agonistic behaviour after cage cleaning and in the undisturbed home cage) tended to correlate.

Another factor showing to be potentially relevant for the prediction of agonistic behaviour was body weight ratio between cage mates. Similarity in weight tended to predict the levels of agonistic behaviour, with more aggression occurring in pairs with similar weight. On the one hand, similarity in weight might lead to a less stable hierarchy and thus to higher levels of aggression (Andersen et al., 2000). On the other hand, a more stable social organisation formed early on may have induced differences in weight gain between cage mates (Van Loo et al., 2000).

4.2. Experiment 2 – effects of separation of male mice into single housing at different ages

Levels of FCMs were not differentially affected by separation events at any age point considered between adolescence and adulthood. While there is general agreement that basal corticosterone levels tend not to differ between single and group housing conditions in male mice (Arndt et al., 2009; Berry et al., 2012), there are conflicting results with respect to adrenocortical reactivity in response to social separation (greater reactivity in singly housed males: Brain, 1975; Berry et al., 2012; no difference in reactivity: Arndt et al., 2009). In our study, we did neither find a short-term increase in levels of FCMs following social separation nor any long-term effects of separation time, indicating no major impact of pair versus single housing on the animals' degree of stress.

In contrast to FCMs, body weight was affected by social separation. While it increased in all animals over the course of the experiment, separation induced a lower weight gain both at PND 35 and 56. At first glance, it may appear that a reduced weight gain may indicate impaired welfare (i.e., a stress response). However, as we did not find effects on the animals´ stress response, this might rather be due to the relatively higher energy consumption needed for thermoregulation by singly housed males, which could not benefit from direct social contact (Gordon et al., 1998). Furthermore, mild agonistic encounters have been found to increase body weight in mice (Bodden et al., 2015; Kästner et al., 2018), hence the cessation of these might as well have contributed to the reduction in weight gain.

Our results concerning home cage activity suggest different effects of separation depending on age. Relative to the other experimental groups, younger males that were separated into single housing displayed an increase in their activity levels, while older males decreased their activity after separation. Even if the present finding alone doesn´t explain whether age dependent changes in activity levels may reflect different responses to social separation, it opens to the possibility that more subtle behavioural modifications may underlie short-term changes in welfare (e.g. the onset of stereotypies; Fureix et al., 2016).

Notably, there was no long-term effect of the different separation times on anxiety-like behaviour, exploratory locomotion or social interest for any of the measured parameters. Our finding is in line with some studies showing that, compared to group housing, separation into single housing does not affect anxiety-like behaviour (Rodgers and Cole, 1993; Arndt et al., 2009) but contrasts with other findings indicating that social separation is anxiogenic (Ferrari et al., 1998; Chourbaji et al., 2005; Berry et al., 2012; Liu et al., 2013).

4.3. Pair vs. single housing – what have we learned?

The general recommendation across international regulation bodies is that male mice should be group-housed in the absence of injurious aggression (Directive 2010/63/EU, 2010; US National Research Council, 2011). However, the assessment of the main welfare-related measures from this study (levels of FCMs and anxiety-like behaviours) supports the conclusion that, under standard laboratory conditions and in the absence of serious outbreaks of aggression, the welfare of male mice is not differentially affected by pair or single housing. In particular, separation into single housing did not impair or improve welfare at different time points, ranging from adolescence to early adulthood.

Looking at the behavioural ecology of wild mice from which laboratory strains were derived, it is not uncommon that sexually mature male mice may choose to live alone and protect their own territory. In fact, the most common outcome when reaching sexual maturity within a deme of adult females and juveniles controlled by a dominant adult male would be to disperse (Van Zegeren, 1979). Thus, individual housing within a laboratory room that allows for visual, olfactory and auditory contact with conspecifics could mimic male dispersal under reproductive competition in the wild, where the cage environment becomes an own territory without intruders (see Brain, 1975, for a similar reasoning). Nonetheless, mice can be extremely flexible in adapting to the most disparate environmental conditions varying in space, food availability and population density (Pocock et al., 2004; Bronson, 1979). In particular, e.g. under ecological constraints such as high population density, it is possible for male mice under semi-natural conditions to adopt a social organisation in which males live together in the same area (Wolff, 1985). Even when highly territorial males are present, males not owning any territory can coexist between owned territories (Noyes et al., 1982; Wolff, 1985). Therefore, the behavioural ecology of the mouse supports the co-existence of group and solitary living as alternative strategies, which may explain our finding. In order to generalise our results to the laboratory mouse however, further research comparing different strains that show varying levels of intermale aggression is needed.

4.4. Conclusions

We demonstrated that under standard laboratory conditions, the welfare of pair housed C57 male mice is not affected by separation into single housing at different ages from adolescence to adulthood. At the same time, we showed how pair housed male mice can maintain low levels of aggression across a long period of their life and perform increasing levels of sociopositive behaviours which may serve to promote stable social relations. In fact, the behavioural ecology of wild male mice supports the coexistence of both solitary and group living as two alternative strategies. Thus, our results highlight the extreme flexibility towards different housing and social conditions which male mice have inherited from their wild ancestors.

Declarations of interest

None.

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