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(Leiter: Univ. Prof. Dr. Thomas Rülicke)

The effect of social housing conditions on sperm production and animal welfare in male mice.

Diplomarbeit

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1. Introduction

1.1. Housing in male mice

Wild male house mice (*Mus musculus*) naturally live with several females their pups and non-dispersing juveniles in their own territory, which can reach a size of a square kilometre and more (Latham and Mason 2004). The standard laboratory housing can never completely fulfil these conditions. As a result, male mice can be afflicted by stress, high aggression levels and its consequences for health (Kappel et al. 2017). Housing conditions for male mice still need to be optimised and "any restrictions on the extent to which an animal can satisfy its physiological and ethological needs have to be kept to a minimum" (European Parliament 9/22/2010). Beyond the welfare aspect, stress levels in animals have to be minimized to generate valid results in experiments (Würbel 2001; Sherwin 2004; Castelhano-Carlos and Baumans 2009). There are many points which can be improved, like the use of gentile strains, minimizing stress and avoiding frustration, the use of an optimal cage design, enrichment and an optimal group composition, to name just a few (Van Loo et al. 2003). Here we will focus on two important parts of male housing conditions: the group size and the cage change interval (CCI), as they are both assumed to have possible effects on social stability within groups and thus on aggression levels (Van Loo et al. 2000; van Loo et al. 2001b). We further aim to assess how these housing conditions affect sperm traits, and thus potentially influence male fertility and breeding success under laboratory conditions.

1.1.1. *Group size*

General co-housing of fertile male and female mice is not applicable to prevent unplanned or not required progeny, even if this housing approach would be closest to nature. Thus, the only alternative is individual housing or housing in single sex groups, whereby co-housing of male mice is nowadays general practice in animal facilities (UK) (Kappel et al. 2017). However, both methods have their advantages and disadvantages as outlined below.

Individual lab housing provides every male its own territory (Brain 1975a) and injurious fights are prevented. On the other hand, individual housing can be problematic, as social deprivation of mice has negative effects on their physiology, like an increased heart rate, a reduced body temperature and a disruption of the normal circadian sleep pattern (Späni et al. 2003). It has

wide effects on immune functions and diseases, for example the worsening of an ongoing tumour development, a lower lymphocytes proliferation and reaction to infections (Bartolomucci 2007). Furthermore, anxious- and depressive-like behaviour, as well as alterations of neuroplasticity-related genes are induced by stress related to individual housing (Ieraci et al. 2016) and self-grooming occurs more often in individually housed mice (Garner et al. 2004a), which is associated with psychogenic distress (Reinhardt 2005).

Male laboratory mice initiate social interactions with novel male conspecifics, even if this behaviour is strain-dependent (Moy et al. 2004). Various studies showed that male mice prefer each other's company over environmental enrichment and that males will actually work for access to conspecifics (Van Loo et al. 2004; van Loo et al. 2001c; Sherwin 1996; Sherwin and Nicol 1996). Furthermore, co-housed mice show a reduction in the activity of the hypothalamic–pituitary–adrenal (HPA) axis compared to individually housed mice and improved health, e.g. a better wound healing (DeVries et al. 2007).

The main problem of grouping male mice is aggression and its consequences for animal welfare, e.g. stress, wounds, pain or even death (Weber et al. 2017; Van Loo et al. 2003; Kappel et al. 2017). Group aggression is the second leading cause of unplanned euthanasia in mice (Gaskill et al. 2017). The aggressive behaviour has a sharp onset with 4 - 5 weeks of age and it coincides with the first evidence of heightened androgen secretion (McKinney and Desjardins 1973b). It precedes the establishment of a dominance order and usually declines after a social hierarchy is established (Poole and Morgan 1973). This social stress and hierarchic encounters lead to behavioural and physiological changes not only in subordinates (Martinez et al. 1998), but also in dominant mice (Kudryavtseva et al. 2014). To name just one, urine retention, as a behaviour of avoiding possible challenges, is a problem in subordinate males and can cause nephritis in some strains (Taylor 1985).

In this study, we investigated the influence of single-, pair- and triple-housing in two commonly used lab strains on aspects of animal welfare and sperm production. The aim of our experiment was to give recommendations on whether to house males individually or to co-house them in pairs or triples in regard of individual aggression, wound and stress levels and to further investigate how the different group sizes effect sperm traits and thus potentially male fertility. The influence of group size on male welfare has already been investigated in prior studies with

the result that aggression levels are higher in bigger groups (van Loo et al. 2001b; Poole and Morgan 1973; Barnard et al. 1994). However, the group sizes in these studies were much higher and we are the first to focus on smaller group sizes, which are commonly applied in animal facilities. Also, we are the first to experimentally assess the effect of group size on male sperm production and thus its potential impact on animal breeding.

1.1.2. *Cage change*

Cage changing is an unavoidable part of mouse husbandry to maintain a healthy microenvironment and low ammonia levels (Washington and Payton 2016), both for animals and animal care personal. However, cage changing is not only time and cost intensive for animal facilities, but also stressful for mice (Rasmussen et al. 2011) and can result in peaks of aggressive behaviour (Van Loo et al. 2000). Thus, both the cage change procedure and frequency need to be optimized. For example, a passive transfer technique can reduce stress (Rasmussen et al. 2011) and transferring nesting material from the dirty cage can reduce aggression between male mice (Van Loo et al. 2000). A complete new cage can further reduce inter-male aggression compared to changing only dirty bedding (Gray and Hurst 1995). Additionally, the frequency of cage change needs to be optimized in regard of other stress-related parameters (Reed et al. 2008), animal health (Reeb-Whitaker et al. 2001) and microenvironment (Reeb et al. 1998). However, a clear recommendation does not exist, as the microenvironment is affected by the type of cage, the air change rate, the volume and type of bedding, the strain and sex of mice and their health and behaviour (Rosenbaum et al. 2009). A two week interval between cage changes is often reported as the optimum in individually ventilated cages (IVC) (Reeb-Whitaker et al. 2001; Rosenbaum et al. 2009; Reed et al. 2008), but no comparisons have been made. Beyond these animal welfare factors, an inadequate cage change regime can affect the validity of experiments, as it has long-term effects on animals like a reduced gain in body mass (Beynen and van Tintelen 1990) or a higher pub mortality (Reeb-Whitaker et al. 2001). So far, only a few possible consequences for mice physiology and behaviour have been investigated in relation to cage changing.

In this study we investigated the impact of a short (i.e. weekly) versus a long (i.e. every two weeks) cage change interval on male welfare. We investigated the wellbeing of experimental males using parameters like attack rates, prevalence and intensity of barbering and wounds,

individual corticosterone levels and spleen sizes. We further investigated the effect of cage change interval on male sperm traits, so that we can provide recommendations regarding cage change interval and male fertility.

1.2. Social status in male mice

Wild male house mice are territorial and aggressively defend their territories against intruders (Mackintosh 1970). The sole presence of an intruder induces chasing and aggressive behaviour in the resident male, however, familiar subordinate males are usually accepted (Mackintosh 1970, 1973; Hurst et al. 1993). Under a high population density, wild males become more socially tolerant. They change their behaviour in becoming more despotic, whereby a dominant mouse overrules one or more subordinate mice (Singleton and Krebs 2007). After domestication mice maintained many of their natural behaviour patterns (Latham and Mason 2004) and co-housed males usually form dominance hierarchies under laboratory conditions (Poole and Morgan 1976). The function of dominance behaviour is minimizing injurious fights within a group by the formation of a stable social hierarchy (Poole and Morgan 1973). Interestingly, the establishment of a defined social rank can have comprehensive and sometimes unexpected consequences for a male, not only for its behaviour and welfare, but also for its physiology and even its anatomy as outlined below.

Compared to subordinate males, dominant males show changes in their anatomy and physiology, like a greater size of the preputial glands (Koyama and Kamimura 1998), a faster gain of weight resulting in a higher body mass (Van Loo et al. 2000), haematological changes (Turney and Harmsen 1984), as well as altered hormone levels like a higher testosterone concentration (Koyama and Kamimura 2000). Furthermore the reproductive success is higher in dominant males (D'Amato 1988) and they have higher sperm motility compared to subordinate mice (Koyama and Kamimura 1998; Koyama and Kamimura 2000).

Subordinate males show a reduction in their activity pattern (Ely and Henry 1978; D'Amato 1988) and alterations in their hormone levels like an increased activity of the hypothalamic-pituitary-adrenal (HPA) axis and higher corticosterone concentrations (Ely and Henry 1978). As a result of chronic stress, subordinate mice further exhibit depressiveness in behavioural tests, a loss of weight and disturbance of the gastrointestinal functions (Kudryavtseva et al.

1991). Additionally, the number of fighting-induced wounds, primarily on the base of the tale and the back, is significantly higher in subordinate mice, resulting in severe welfare problems (van Loo et al. 2001b). They even show a reduced urine marking behaviour to supress competitive signalling and avoid challenges (Desjardins et al. 1973). As a result, they have the ability to contain 20 times more urine in their bladders (Desjardins et al. 1973) and particularly distended bladders are found in subordinate mice at autopsy (Nevison et al. 2000).

In this study we determined the social status of co-housed males to investigate whether and how our experimental manipulations affected the formation and stability of social hierarchies within groups. We further tested how dominant males differ in their behaviour, physiology and anatomy from subordinates to estimate how social status is related to aspects of animal welfare and reproduction.

1.3. Methods of social status determination

Dominance is usually defined as "winning in conflict situations, displaying agonistic behaviour, having first access to food, marking a territory, having a prominent order in grooming, displaying proactive courtship and showing low participation in labour" (Wang et al. 2014). Various tests have been established to assess dominance relationships and in general three criteria define the validity of such assays (Wang et al. 2011b): (i) the transitivity in group-housed mice (i.e. when mouse A is dominant over mouse B and mouse B is dominant over mouse C, than mouse A should also be dominant over mouse C), (ii) the stability of dominance relationships over time and (iii) the consistency of results between different tests. All assays have their strengths and weaknesses in considering these three criteria, their practicability and their effects on animal welfare. A "gold standard" does not exist. As a result, dominance determination was inconsistent in prior studies and protocols were adapted to individual needs, with consequences for the comparability of results. It is important that researchers justify their choice of test and that they describe it sufficiently to allow reproducibility in future experiments.

Here we applied behavioural observations to determine male social status and we aimed to investigate the advantages and disadvantages of two different behaviour observation protocols,

as well as the "Dalila effect" (barbering) in assessing male social status. We hope that our approach will facilitate the non-invasive determination of male social status in future studies.

1.3.1. *Behavioural observation*

It is assumed that dominant mice show more agonistic behaviours like starting fights, winning fights, chasing and tail rattling, while subordinates show more submissive behaviours, like getting chased and fleeing from fights. Indeed, studies in co-housed male mice have shown a correlation between social rank and agonistic behaviour (Wang et al. 2011a; Rose et al. 1971; Mondragón et al. 1987b). A higher level of aggressive behaviour in dominant animals is also known for other species, like baboons (Hausfater et al. 1982), langurs (Poirier 1970) and chickens (Rushen 1982). However, aggressive behaviour is not reserved for dominant animals, whereby animals only make use of competitive aggression if a positive outcome can be expected (Hillman 2013). This should be considered when drafting an observation protocol, as a simple quantitative analysis of agonistic behaviours is possibly not sufficient for dominance determination. However, previously used observation protocols widely differ in their duration, as well as in their interpretation: while in some studies the mouse with the most display of agonistic behaviours within a group, i.e. more than 50%, was classified as dominant (van Loo et al. 2001c; van Loo et al. 2003; van Loo et al. 2001b; Barnard et al. 1994), others are more conservative, so that mice must display at least 60% of the agonistic behaviours to be classified as dominant (Nevison et al. 2000). Furthermore, the definition of "agonistic/aggressive behaviour" is always different and most of the times unspecified. Thus, a valid test protocol should recommend when and how long observations should be performed, specify agonistic behaviours and interpret them correctly to be useful for further research.

1.3.2. *Barbering or the "Dalila effect"*

Barbering is a complex behaviour where one mouse barbers the hair and plucks the whiskers ("whisker trimming") of its cage mates. It is assumed that the dominant mouse barbers its opponents ("Dalila effect"), especially after fights, and should therefore be the only not-barbered mouse within a cage. Several studies showed that the barbering male is a highly ranked individual in other dominance assays (Wang et al. 2011c; Strozik and Festing 1981; Kalueff et al. 2006; Long 1972) and it is assumed that barbering represents an eccentric but benign

dominance behaviour (Long 1972). However, other studies reported that barbering is an abnormal behaviour with serious consequences for animal welfare and that its prevalence is affected by genetics and husbandry factors, but not by dominance status (Garner et al. 2004a). We used B6D2F1 hybrid and C57BL/6N inbred mice in our experiment, which are known to have a high barbering prevalence (Garner et al. 2004b) enabling us to study the "Dalila effect" in different group sizes and to compare the prevalence of barbering across strains.

1.3.3. *Other tests*

Several other assays exist to assess male social status, like the tube test (Wang et al. 2011c), the resident intruder test (Koyama and Kamimura 1998; Gray and Hurst 1995), the urine marking test (Desjardins et al. 1973), the food competition test (Merlot et al. 2004), the ultrasonic vocalization test (Catanzaro and Ngan 1983; Nyby et al. 1976), as well as modifications or combinations of all these assays.

To sum up, there are many possible dominance tests, each with its advantages and drawbacks regarding effort, results and impact on animal stress level. In this study, we applied two different behavioural observation protocols and investigated their pros and cons for dominance determination in two different strains of male mice. Our aim was to give recommendations on when and how long mice need to be observed to reliably determine dominance hierarchies within groups. We further compared the results with male barbering behaviour to evaluate how these different methods to assess male social status were related. We chose these protocols as they are the only ones that do not require any manipulation of mice thereby preventing additional impact on animal welfare.

1.4. Plasticity in mouse sperm

In male mice dominance plays a central role as females usually mate with the dominant, territorial male (Bronson 1979). However, female mice are known to also mate with multiple males during a single oestrus cycle (Dean et al. 2006; Thonhauser et al. 2013; Firman and Simmons 2008). The reason for this behaviour are not completely understood, even though many hypotheses have been described (Thonhauser et al. 2014). After mating with multiple partners sperm competition occurs, as the ejaculate of multiple males is blended in the reproductive tract of the female and sperm of different males has to compete for ova

fertilization. This is somehow surprising, as ejaculates in mice form a plug after copulation, which should prevent females to be fertilized by foreign males (Hartung and Dewsbury 1978). However, plugs are not effective at all times and can be removed by the following male through a repeated number of intromissions (Taylor 1985). Sperm competition should select sperm characteristics that lead to higher fertilization rates and investigations in different taxa found that sperm competition affects sperm number (Harcourt et al. 1981; Moller and Briskie 1995; Stockley et al. 1997), sperm size and form (Anderson and Dixson 2002; Gomendio and Roldan 1991). The size of testes, seminal vesicles and the anterior prostate have also been found to correlate with sperm competition level in rodents (Ramm et al. 2005). In mice, males produce more sperm when exposed to rivals in their environment (Ramm et al. 2015; Ramm and Stockley 2009) and other sperm traits, like sperm motility, improve in selection lines with a high risk of sperm competition (Firman and Simmons 2010).

In addition to the effects of sperm competition, effects of social dominance on sperm traits have been described in mice (Koyama and Kamimura 1998; Koyama and Kamimura 2000), suggesting that male social environment is a crucial factor influencing sperm traits. As sperm and ejaculate production are costly (Dewsbury 1982), males are expected to adjust it according to their social rank and mating opportunities. Indeed, subordinate mice have been found to have lighter preputial glands (Koyama and Kamimura 1998; Bronson 1973) and lower sperm motility (Koyama and Kamimura 1998; Koyama and Kamimura 2000). The mechanism of this effect is unclear, but subordination stress is assumed to diminish sperm motility in subordinate mice (Williamson et al. 2017b). Alternatively, and not mutually exclusive, subordinates might be suppressed by odours of their dominant opponents (Koyama 2004). Even though *Koyama et Kamimura* (1998) have been the only ones to find an effect of social status on sperm traits in mice so far, this effect is known in many other taxa (Cornwallis and Birkhead 2007; Kruczek and Styrna 2009b; Rudolfsen et al. 2006; Neff 2003).

In summary it can be said, that the high variation in ejaculate composition and sperm traits in mice can depend upon many environmental and physiological conditions (Koyama and Kamimura 1998). In this study, we experimentally manipulated the social environment of male lab mice by housing them either as individuals, in pairs or in groups of three. Thus, experimental males faced a different risk of sperm competition. We aimed to investigate the effect of group

size and thus sperm competition risk and male social status on male sperm traits under standard housing conditions in two commonly used mouse strains. The first strain, C57BL/6 is the most frequently used inbred strain in research (Charles River Laboratory 2020). The second strain, B6D2F1, is a hybrid strain allowing us to investigate how genetic diversity influences mice behaviour and adaptation ability. Furthermore, both strains are not only used in biomedical research but also in behavioural research (Charles River Laboratory 2020), making our results widely applicable. We expected that males under a high risk of sperm competition, i.e. co-housed males that were kept in groups, would show improved sperm traits compared to males that were co-housed as pairs or kept individually. In addition, we expected the sperm traits of co-housed males to be related to their social status and that the variation in sperm traits of groups would be bigger than the variation of sperm traits within pairs. Overall, this study will help to optimize the keeping and breeding conditions of widely used laboratory mice and allows to bridge gaps between different research disciplines including animal behaviour, welfare and reproduction.

2. Methods

2.1. Study objectives

We experimentally manipulated male group size and cage change interval (CCI) to test their effects on male behaviour, welfare and sperm traits. We used mice with different genetic backgrounds, i.e. one inbred and one hybrid strain to assess whether potential experimental effects are dependent on male genetic diversity. We performed behavioural observations to determine male social status, their level of aggression and co-sleeping rates. Furthermore, we recorded the prevalence of barbering and wounds and compared the prevalence of barbering with our behavioural dominance classification to assess if these methods reveal similar results. We also collected animal faeces to determine testosterone and corticosterone metabolite levels in males over the course of the experiment to analyze the effects of the experimental manipulations on male hormone levels and their relationship to male behaviour and sperm traits. Furthermore, faecal corticosterone levels were used as a parameter to estimate animal wellbeing.

2.2. Experimental animals and housing

We used 168 male laboratory mice (*Mus musculus f. domesticus*) for the experiment; half of them were C57BL/6N inbred mice, half of them B6D2F1 hybrid mice. All animals were purchased from Janvier Laboratories, France. After arrival, mice were kept in triplets and were acclimatized for one week in which they were not manipulated. Animals remained in these groups until the start of the experiment at the age of 8 weeks. For the experiment, mice from the group-housed treatment remained in their respective group constellations. Mice from the pair- and single-housing treatment groups resulted from the split-up of a triplet group. Animals were randomly chosen to either group. Upon arrival, mice were earmarked to allow identification of individuals in co-housed cages.

Experimental mice were kept in individually ventilated cages (type IIL, 36.5×20.7×14 cm, Tecniplast, Buguggiate, Italy), equipped with wooden bedding (LIGNOCEL® 3–4 S, J. Rettenmaier and Söhne GmbH + Co. KG, Rosenberg, Germany), 8 cellulose pads (Pur-Zellin 4×5 cm; Paul Hartmann GmbH, Wiener Neudorf, Austria) and one cardboard tube (7.6×3.8 cm diameter, Special Diet Service, Claus GmbH, Limburgerhof, Germany) as nesting material.

Commercial mouse diet (ssniff®, V1534, Soest, Germany) and tab water were provided *ad libitum*. All animals were housed under standard laboratory conditions (temperature 21±1 °C, humidity 40–55 %) with a 12:12 h light-dark cycle and lights on at 1:00 a.m. All experimental procedures that were undertaken after 1:00 p.m. were performed under red light.

2.3. Experimental manipulations

To investigate the influence of different group sizes, 14 animals per strain were housed individually ("single-housed mice", N = 14), 28 animals per strain were kept in pairs ("pair-housed mice", N = 14) and 42 animals per strain were kept in triplets ("group-housed mice", N = 14). To assess the effect of different CCIs, half of the cages were cleaned every 7 days ("short CCI"), whereas the other half were changed every 14 days ("long CCI"). For an overview of the experimental treatment groups see Table 1. The animals were kept under their respective treatment regime for eight weeks before sacrificed by cervical dislocation to perform sperm analysis and to determine individual body and organ weights.

Table 1: Overview and distribution of experimental mice across treatment groups.

	C57BL/6N *(inbred strain)*		**B6D2F1** *(hybrid strain)*	
Cage change interval (CCI):	**7 days** *(short CCI)*	**14 days** *(long CCI)*	**7 days** *(short CCI)*	**14 days** *(long CCI)*
Group size: 1 *(single-housed mice)*	7 animals (= 7 cages)	7 animals (= 7 cages)	7 animals (= 7 cages)	7 animals (= 7 cages)
Group size: 2 *(pair-housed mice)*	14 animals (= 7 cages)	14 animals (= 7 cages)	14 animals (= 7 cages)	14 animals (= 7 cages)
Group size: 3 *(group-housed mice)*	42 animals (= 7 cages)	42 animals (= 7 cages)	42 animals (= 7 cages)	42 animals (= 7 cages)

Cages were always changed between 9:00 a.m. and 10:30 a.m. The process was standardised and according to the applied cage change routine at our institute: Cages were changed under a laminar flow to ensure hygienic standards and gloves were disinfected before touching any part inside a cage. Food from the old cage was transferred to the new one, if necessary, fresh pellets were added. Each cage was equipped with wooden bedding, 8 cellulose pads and a new

cardboard tube. Mice were transferred by picking them up by their tail. A part of the old nesting material was transferred as well to provide them some familiar odour. Water bottles were re-used for the new cage. They were exchanged once a week independent of cage change. The amount of food was usually sufficed for 14 days, but once a week cages which run short in food were filled up. The cage change was further used to visually inspect the animals and to monitor their condition and check for injuries or wounds.

2.4. Hormone measurements

Faecal testosterone metabolite (TM) and corticosterone metabolite (CM) levels were measured over the course of the experiment to analyze the effects of the experimental treatments on hormone levels and the correlation of hormone levels with animal behaviour and sperm traits. For this purpose, faecal samples were collected at the end of week 2, 4, 6 and 8 of the experiment. The collection was always timed with a cage change to avoid any interference with the cage change interval and to prevent additional mouse handling and stress. For faeces collection, mice were individually transferred to a clean and empty mouse cage type II (26.7 × 20.7 × 14 cm) covered with paper towel to absorb any urine thereby avoiding any contamination of faecal samples. Faeces collection always started at 9:30 a.m. and lasted for 30 min. Afterwards, all mice were returned to a new home cage (keeping their respective group constellations) and faecal samples were immediately collected with forceps and stored in Eppendorf tubes at -20 °C until further processing. A minor adaptation of the commonly applied cage change protocol was undertaken here, as mice were transferred from their individual faecal sample collection boxes to their new home cage. They received new food and a freshwater bottle, and no old nesting material was transferred.

CMs and TMs were extracted from faeces using a method described by *Palme and Möstl* (1997) for ruminants and adapted and validated for mice by *Touma et al.* (2003) for CM and a method described by *Auer et al.* (2020) for TM. Faecal samples were homogenized with a mortar and pestle. An aliquot of 0.05 mg was shaken with 1 ml of 80 % methanol for 30 min on a multi-vortex. The suspension was centrifugated for 10 min at 2500 × g. An aliquot of the supernatant was diluted (1:10) with assay buffer (Tris/HCl 20 mM, pH 7.5) and frozen at -20 °C until analysis. To determine the amount of faecal CM, a 5α-pregnane-3β,11β,21-triol-20-one enzyme immunoassay (EIA) was used, which is well suited to assess CM in faecal samples of mice

(Touma et al. 2003). To determine the amount of faecal TM, an EIA with an antibody produced in rabbits against testerone-3-CMO:BSA (working dilution 1:20,000) and 5α-androstane-3β,17β-diol-3-HS-DADOO-biotin as label (dilution 1:160,000) was used (Auer et al. 2020).

2.5. Behavioural observations and social status determination

We applied two different observation protocols, namely evening observations and post cage change observations to assess the level of aggression within groups and to determine male social status. In addition, we evaluated male barbering behaviour to determine whether and how barbering was related to male social status. Finally, we also performed observations to quantify the co-sleeping rates of mice within groups.

2.5.1. *Evening observations*

During evening observations male agonistic behaviour was observed every day from Monday to Friday for 120 min between 1 p.m. and 5 p.m. during the dark phase under red light. Therefore, all cages containing co-housed mice were simultaneously observed and all behaviours described in Table 2 were recorded.

Table 2: Ethogram of recorded agonistic behaviour.

Behaviour	*Characterisation of the behaviour*
Chasing	One mouse is following another mouse, while the other mouse is fleeing.
Fighting	Mice are in physical combat with each other. Fighting can include biting. If possible, it is recorded who started the fight.
Fleeing	One mouse runs off from another mouse. This can occur after a fight.
Tail rattling	Mouse is in a tense posture and its tail is rattled on the ground.
Submissive upright	Mouse stands on its hind-paws and stretches its fore-paws off the body during an interaction.
Submissive downright	Mouse lies on its back and stretches its fore-paws off the body during an interaction.
Selfgrooming	Mouse grooms its fur after a fight.
Anogenital sniffing	Mouse sniffs at the anogenital region of another mouse.

Each mouse received positive scores for showing dominant behaviours and negative scores for showing submissive behaviours during the observation period. Which behaviours were considered dominant and which submissive are listed in Table 3 including the respective scores. Behavioural scores were summed up on a weekly basis to assess dominance relationships over time and to determine whether social hierarchies were stable.

Table 3: Behavioural scores of specific behaviours.

Dominant behaviour	***Behavioural score***
Chasing	1
Attacking (starting a fight)	1
Fighting (no attacker can be determined)	0.5
Tail rattling	0.5
Anogenital sniffing	0.1

Submissive behaviour	***Behavioural score***
Getting chased	-0.5
Getting attacked	-0.5
Fleeing from a fight	-1
Submissive upright/downward	-0.5
Selfgrooming	-0.1

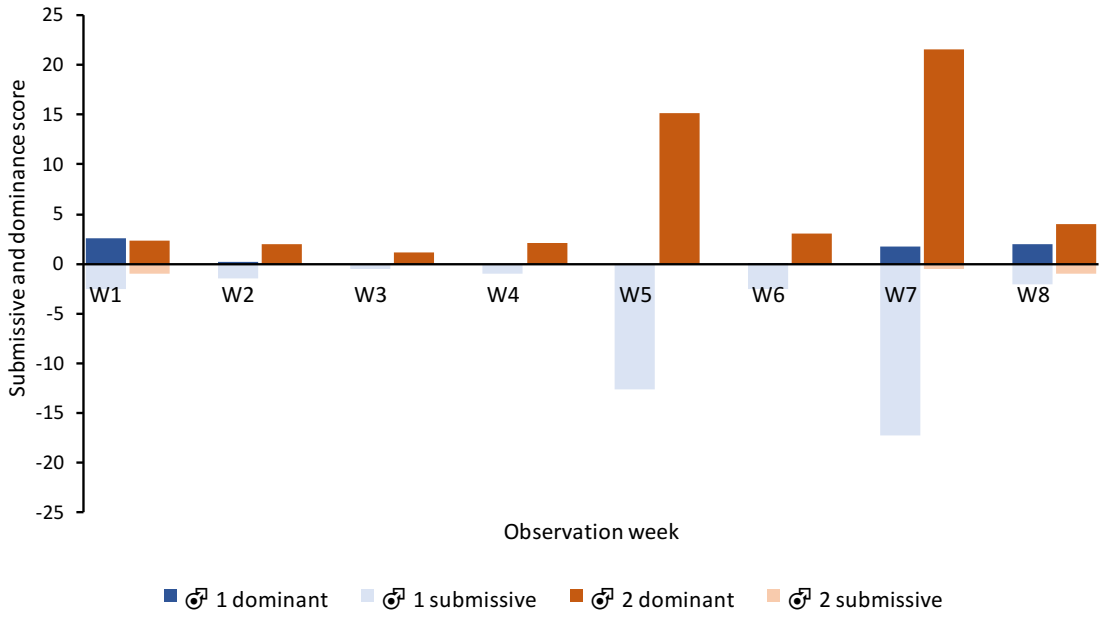
An observation week started with the day of cage change for the short CCI group, or in case of the long CCI group, on the same day with or without the cage change and lasted for 7 days. The scores for dominant and submissive behaviours were added up and the weekly social status was determined using the following criteria (examples in Figure 1 for pair-housed mice and Figure 2 for group-housed mice):

- A mouse was classified dominant for the observation week, if:
    - Its dominance behaviour score was at least 2. Thus, each mouse had to show a minimum of dominance related behaviours in order to be classified as such.
    - The score for dominant behaviours between mice had to differ in at least 50 % in order to classify one as dominant. Thus, each group had to show a distinct difference in their score to be classified.

- A mouse was classified submissive for the observation week, if:
    - Its submissive behaviour score was at least -2. Thus, each mouse had to show a minimum of submissive behaviours to be classified as such.
    - The score for submissive behaviours between mice had to differ in at least 50 % in order to classify one as submissive. Thus, each group had to show a distinct difference in their score to be classified.
- No dominance status was assigned when one of these criteria was not fulfilled.

Mice that were most often classified as dominant over the course of the 8 weeks were considered as overall dominant (D). In group-housed mice only one mouse could become dominant (D), both others were defined as subordinates (S). If two mice were dominant over the same number of weeks, the mouse which was submissive more often was defined as subordinate (S). If mice showed no agonistic behaviour or too little to fulfil the criteria, no social status was assigned (NA). We recorded whether and how often changes in social hierarchies within groups occurred.

a)



b)

	week 1		week 2		week 3		week 4	
mouse ID	dom. score	sub. score	dom. score	sub. score	dom. score	sub. score	dom. score	sub. score
♂1	2,6	-2,5	0,2	-1,5	0	-0,5	0	-1
♂2	2,3	-1	2	0	1,1	0	2,1	0

	week 5		week 6		week 7		week 8	
mouse ID	dom. score	sub. score	dom. score	sub. score	dom. score	sub. score	dom. score	sub. score
♂1	0	-12,6	0,1	-2,5	1,8	-17,2	2	-2
♂2	15,1	0	3,1	0	21,6	-0,5	4	-1

c)

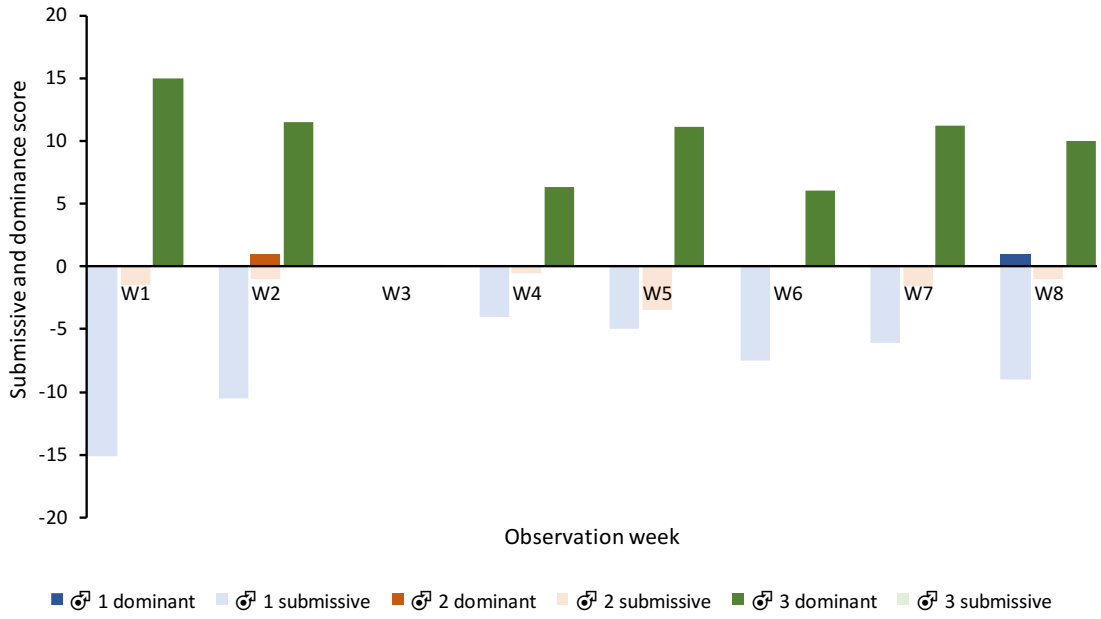
mouse ID	week 1	week 2	week 3	week 4	week 5	week 6	week 7	week 8
♂1	S	---	---	---	S	S	S	S
♂2	---	D	---	D	D	D	D	D

d)

	total number		
mouse ID	**D**	**S**	status
♂1	0	5	**S**
♂2	6	0	**D**

Figure 1: Example of the social status determination of co-housed mice. Mice in this example were hybrids, pair-housed and experienced a long CCI. ***(a)*** *Bar plot showing the individual scores for dominance and submissive behaviours during evening observations over the course of the experiment.* ***(b)*** *Table, depicting the respective behavioural scores for dominant and submissive behaviours.* ***(c)*** *Calculation of social status on a weekly basis.* ***(d)*** *Sum of all dominant and submissive weeks over the course of the experiment and the assigned overall social status.*

a)



b)

	week 1		week 2		week 3		week 4	
mouse ID	dom. score	sub. score	dom. score	sub. score	dom. score	sub. score	dom. score	sub. score
♂1	0	-15,1	0	-10,5	0	0	0	-4
♂2	0	-1,5	1	-1	0	0	0	-0,5
♂3	15	0	11,5	0	0,1	0	6,3	0

	week 5		week 6		week 7		week 8	
mouse ID	dom. score	sub. score	dom. score	sub. score	dom. score	sub. score	dom. score	sub. score
♂1	0	-5	0	-7,5	0	-6,1	1	-9
♂2	0	-3,5	0	0	0	-1,6	0	-1
♂3	11,1	0	6	-0,1	11,2	-0,1	10	0

c)

mouse ID	week 1	week 2	week 3	week 4	week 5	week 6	week 7	week 8
♂1	S	S	---	S	S	S	S	S
♂2	---	---	---	---	S	---	---	--
♂3	D	D	---	D	D	D	D	D

d)

	total number		
mouse ID	**D**	**S**	status
♂1	0	7	**S**
♂2	0	1	**S**
♂3	7	0	**D**

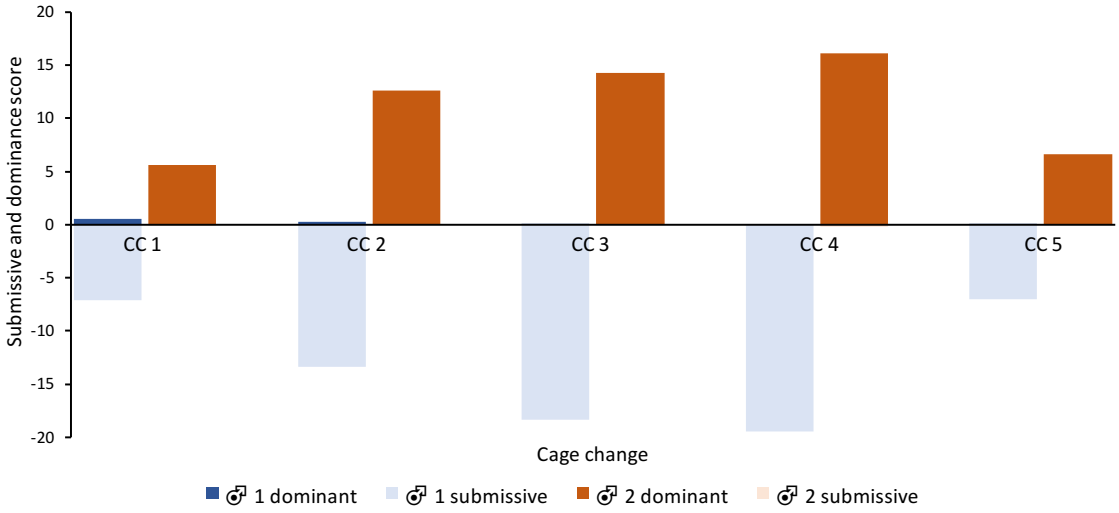
Figure 2: Example of the social status determination of co-housed mice. Mice in this example were inbred, group-housed and experienced a short CCI. ***(a)*** *Bar plot showing the individual scores for dominance and submissive behaviours during evening observations over the course of the experiment.* ***(b)*** *Table, depicting the respective behavioural scores for dominant and submissive behaviours.* ***(c)*** *Calculation of social status on a weekly basis.* ***(d)*** *Sum of all dominant and submissive weeks over the course of the experiment and the assigned overall social status.*

2.5.2. *Post cage change observations*

During post cage change observations, the agonistic behaviour of males was observed for 30 min directly after each cage change, between 9 a.m. and 11 a.m. during the light phase. Thus, depending on the CCI 4-8 cages were observed simultaneously. Cages with short CCI were observed 9 times, whereas cages with long CCI were observed 5 times over the course of the experiment. All behaviours described in Table 2 were recorded.

Each mouse received positive scores for showing dominant behaviours and negative scores for showing submissive behaviours during the observation period (for score calculation see Table 3). We assessed the social status of mice using the same evaluation criteria as described above for evening observation. The dominant status was calculated after every cage change to assess dominance relationships over time and to determine whether hierarchies were stable (example in Figure 3).

a)



b)

	cage change 1		cage change 2		cage change 3		cage change 4		cage change 5	
mouse ID	dom. score	sub. score	dom. score	sub. score	dom. score	sub. score	dom. score	sub. score	dom. score	sub. score
♂1	0,5	-7,1	0,3	-13,4	0,1	-18,4	0	-19,5	0,1	-7
♂2	5,6	-0,1	12,6	0	14,3	0	16,1	-0,2	6,6	0

c)

mouse ID	CC1	CC2	CC3	CC4	CC5
♂1	S	S	S	S	S
♂2	D	D	D	D	D

d)

	total number		
mouse ID	**D**	**S**	status
♂1	0	5	**S**
♂2	5	0	**D**

Figure 3: Example of the social status determination of co-housed mice. Mice in this example were hybrids, pair-housed and experienced a long CCI. ***(a)*** *Bar plot showing the individual scores for dominance and submissive behaviours during evening observations over the course of the experiment.* ***(b)*** *Table, depicting the respective behavioural scores for dominant and submissive behaviours.* ***(c)*** *Calculation of social status after every cage change.* ***(d)*** *Sum of all dominant and submissive weeks over the course of the experiment and the assigned overall social status.*

We compared both observation protocols regarding their weekly and overall consistency in male social status classification. We also determined how fast the respective protocols allowed the classification of male social status.

2.5.3. *Barbering and wound score*

To alternatively determine the social status of co-housed mice and to test whether barbered mice differ in some behavioural or physiological parameters (i.e. attack rate, sperm traits, organ mass or CM and TM levels) form non-barbered mice, we assessed individual barbering scores.

In addition, we also determined individual wound scores to investigate the effect of different housing conditions and male genetic background on the number of wounds and fur changes.

After sacrifice the fur of each male was scanned for wounds and for barbering. The results (separated for barbering and skin abnormalities) were graded into four categories: (-) no abnormalities, (+) low changes, (++) obvious changes and (+++) high changes (for stage classifications see table 4 and 5). The scoring of all animals was performed by the same person who was blind for the treatment groups of the animal. The mouse with no or the least barbering in a group was classified as dominant (D), the barbered mice as submissive (S). If mice of a group showed no barbering or an equal barbering score, no social status was assigned (NA).

Table 4: Classification of fighting induced wounds.

	Wound score		**Stage characterisation**
Non-wounded mice	-	no wound	No visible wounds.
Wounded mice	+	low wound	Wounds are only visible after manual examination of fur and skin.
	++	intermediate wound	At least one wound, which is so big that no manual examination is needed to see it.
	+++	high wound	More than one wound, which is so big that no manual examination is needed to see it. Injuries are so severe, that the individual needs to be separated or euthanized.

Table 5: Classification of barbering behaviour.

	Barbering score		**Stage characterisation**
Non-barbered mice	-	no barbering	No hair loss.
Barbered mice	+	low barbering	Loss of whiskers.
	++	intermediate barbering	Loss of whiskers and hair loss close to snout.
	+++	high barbering	Loss of whiskers and hair loss close to snout and eye area/forehead.

2.5.4. *Co-sleeping*

We determined the co-sleeping rate to investigate if different housing conditions and genetic background influenced the co-sleeping rate and to test whether co-sleeping was correlated with attack rates and stress levels.

On 5 days per week (Monday-Friday) we recorded mouse sleeping behaviour and classified mice as either co-sleeping, sleeping separately or not sleeping (see Table 6). Observations were performed at 9 a.m. (±15 min) during the resting phase of the mice and before experimental manipulations started.

Table 6: Ethogram of recorded sleeping behaviours.

Behaviour	*Characterisation of the behaviour*
Sleeping/resting	The mouse lies at one place with minimal movement, the eyes can be closed or opened. "Very short movements during a long resting period (e.g. turning) are not considered an interruption." (Van Loo et al. 2004, p. 181)
Not sleeping/resting	The mouse shows locomotive behaviour or lies at on place with obvious movement, e.g. autogrooming.
Co-sleeping	The mice are sleeping/resting, and their bodies are in contact with one another.
Separate sleeping	The mice are sleeping/resting, and their bodies are not in contact with one another.

2.5.5. *Attack rate*

To investigate whether and how our experimental manipulations influenced male aggression and wellbeing we assessed individual attack rates in co-housed mice. The attack rates were determined based on evening observations and the behaviours considered in attacks are summarized in Table 7. We calculated an active (actively attacking others) and passive (being attacked by others) attack rate for each male by adding up the scores associated with the display of attacks. We calculated a weekly attack rate and used the mean values of all eight weeks of observation to determine the overall active and passive attack rate per male.

Table 7: List of agonistic behaviours that were considered in attacks and their associated scores.

Active attacking behaviour	***Attack score***
Chasing	1
Starting a fight	1

Passive attacking behaviour	***Attack score***
Getting chased	1
Getting attacked and involved in a fight	1

We further assessed attack rates from post cage change observations to specifically assess the influence of cage change on male level of aggression. As the number of cage change was dependent on the CCI, mean attack rates in the short CCI treatment were based on 5 observations, whereas mean attack rates in the long CCI were based on 9 observations.

2.6. Sperm traits and reproductive organs

To investigate whether and how our experimental manipulations affected male reproductive physiology, we investigated male sperm traits and organ weights. To assess individual sperm traits and to determine organ weights, animals were dissected at the end of the experiment. As we could not process all mice simultaneously, we scarified a subset of mice (N = 12) per day, that were balanced for treatment groups. Furthermore, the daily dissection order of these animals was randomized.

2.6.1. *Reproductive organs*

Mice were euthanized by cervical dislocation before dissections were performed. Body mass was measured with a pharmacy scale (which was also used for all other weight measurements) *post mortem*. Then mice were placed on their back and the abdomen was sprayed with 70 % EtOH before the abdominal cavity was opened with scissors and the following organs were extracted:

1. The *caudae epididymides,* which were used for subsequent sperm analyses (see below).
2. The testicles, which were separated from the rest of the epididymis, meso, vessels and fat, before being weighed.
3. The *glandulae vesiculosae,* which were removed together with the prostate, the *glandulae ampullaris*, the urinary bladder and spermatic duct by cutting through the cranial end of the *pars membranacea urethrae* and the *musculus urethralis* with scissors and extracting the whole convolute with forceps. Once removed, the urinary bladder was cut off at its cervix, as well as both spermatic ducts, before the *glandula vesiculosa*, the *glandula ampullaris*, the *pars anterior prostatae* (*glandula coagulationis*) and the *prostata glandularis* were weighed together.
4. The spleen, which was separated from the *omentum majus* and the vessels at its hilum, before being weighed.

2.6.2. *Sperm analysis*

We analysed various sperm traits to investigate the effects of our experimental treatment and male social status on sperm production. All sperm parameters were analysed with a computer assisted sperm analyser (CASA, [SCA, Microptic, Spain]). The CASA system provided information regarding sperm number (M/sample), sperm motility (%) and sperm swimming velocity (µm/sec), i.e. curvilinear velocity (VCL). We further assessed the decline in sperm motility over time as a proxy for sperm longevity. Therefore, sperm motility was re-measured 2 h after the initial measurement.

For the sperm measurement, both *caudae epididymides* were extracted and put into a petri dish containing a 200 µl drop of TYH (Toyoda, Yokoyama and Hosi, in 1971) medium covered with paraffine oil. The medium was pre-heated to 37 °C and always located on a heating plate when

the sample was processed. The tissues were generously perforated with iris micro-scissors, before the petri dish was left in an incubator for 10 min at 37 °C and 5 % CO_2, to enable the sperm to disperse into the medium. The sperm suspension was then shaken carefully for 1 min to homogenize it before the tissues were removed and 10 µl of the sperm suspension were transferred into a petri dish containing 190 µl THY covered with paraffine oil. This 1:20 dilution was made to reach an optimal sperm concentration for CASA. 5 µl of the diluted and homogenized sperm suspension were then extracted and transferred into a Leja Slide and sperm movement was recorded under a microscope (Nikon Ci-L; Basler Ace colour camera suitable for SCA). We recorded 10 videos per sample and used the average values for statistical analyses. The petri dish was then returned to the incubator and the procedure was repeated after 2 h. After automated sperm recording, a manual correction of all videos was performed. Manual corrections were performed by one person who was blinded for the experimental treatments.

2.7. *Ethical statement*

This study has been discussed and approved by the institutional ethics and animal welfare committee of the University of Veterinary Medicine Vienna (ETK-21/02/2019) in accordance with good scientific practice guidelines and national legislation.

All animals were checked daily for their general condition, especially for fighting induced wounds. During the two hours of daily behavioural observations and cage change any critical increase of aggression in the co-housed animals could be noticed, even though a certain amount of aggression can be regarded as normal (Bisazza 1981a; Brain and Parmigiani 1990). Thus, groups with specifically high levels of aggression could be spotted and separated as soon as possible, since it can be a serious welfare problem (Bisazza 1981a; Brain and Parmigiani 1990). Over the course of the study two groups were separated due to their high levels of aggression.

2.8. Statistics

All statistical tests were performed with IBM SPSS statistics 25 for MS Windows.

To test the consistency between the two *observation protocols*, we calculated Spearman's correlation between the agonistic behaviours recorded in the evening observation and the post cage change observation.

To test which factors affected the *passive attack rate* (i.e. mean number of weekly received attacks per mouse), we performed a linear mixed model (LMM) with passive attack rate as dependent variable and strain, CCI and group size as fixed factors. Cage ID was included as random factor to control for the non-independence of mice within a cage. To assess the relationship between male aggression, stress and testosterone levels Spearman's rank correlations were calculated between passive attack rates and mean corticosterone levels and active attack rates and testosterone levels. To test whether attack rates change over time, we performed a generalized linear mixed model (GLMM with Poisson distribution) with passive attack rates per week as dependent variable and observation week, strain, CCI, and group size as fixed factors. Mouse ID and cage ID were included as a random factor to control for the non-independence of mice within a cage.

To test which factors affected the *co-sleeping rate*, we performed a linear model (LM) in which we included the co-sleeping rate as dependent variable and strain, CCI and group size as fixed factors. Furthermore, to test if high attack rates, TM and CM levels are related to different sleeping patterns, we run Spearman's correlations between the co-sleeping rates and mean TM and CM levels per group and mean attack rate per group.

To test which factors affected male *sperm traits*, we performed LMMs with sperm number, sperm motility, sperm longevity, and sperm velocity as dependent variables and strain, group size, CCI and male social status as fixed factors. Cage ID was always included as random factor to control for the non-independence of mice within a cage. Furthermore, for the LMM on sperm number male relative testes mass was added as a covariate and for the LMM on sperm motility and sperm velocity, sperm number was added as a covariate. To assess how sperm traits are related to male body mass and hormone levels, we calculated Spearman's correlations between sperm traits (sperm number, sperm motility, sperm velocity parameters, sperm longevity), body mass, mean CM and mean TM levels. To investigate the intra-group variance in animals sperm traits we calculated the absolute difference in sperm number, sperm motility, sperm velocity parameters, sperm longevity between two mice (i.e. for cages containing pair-housed mice) or between the mouse with the highest and the lowest value (i.e. for cages containing group-housed mice). We performed LMs and included intra-group variation of sperm number, sperm motility,

sperm velocity and sperm longevity as dependent variables and strain, group size and CCI as fixed factors.

To test which factors affected *body and organ mass*, we performed LMMs with the respective body and organ masses (relative testes, seminal vesicles and spleen weight) as dependent variables and strain, group size, CCI and social status as fixed factors. Cage ID was included as a random factor to control for the non-independence of mice within a cage. Furthermore, Spearman's correlation was calculated between body and organ masses (i.e. relative testes, seminal vesicle and spleen) and mean TM and CM levels to investigate their relationships. To investigate the intra-group variance in the animals body and organ mass traits we calculated the difference in body weight and relative weight of testes, seminal vesicle and spleen between two mice (i.e. for cages containing pair-housed mice) or between the mouse with the highest and the lowest value (i.e. for cages containing group-housed mice). We performed LMs with the intra-group variation of body weight and relative testes, seminal vesicle and spleen weight as dependent variables and strain, group size and CCI as fixed factors.

To test which factors affected *hormone levels*, we performed LMMs with mean faecal TM and CM levels as dependent variables and strain, group size, CCI and social status as fixed factors. Cage ID was included as a random factor to control for the non-independence of mice within a cage. To test whether hormone levels change over time, we performed LMMs with mean faecal TM and CM levels as dependent variables and the time point of faeces collection, strain, CCI, group size and male social status as fixed factors. Mouse and cage ID were included as a random factor to control for the non-independence of mice within a cage. To investigate the intra-group variance in the animals hormone levels we calculated the difference in mean faecal TM and CM levels between two mice (i.e. for cages containing pair-housed mice) or between the mouse with the highest and the lowest value (i.e. for cages containing group-housed mice). We performed LMs with the intra-group variation of mean faecal TM and CM levels as dependent variables and strain, group size and CCI as fixed factors.

We tested for all models if model assumptions were fulfilled and transformed data if necessary. In case data transformation did not help to fulfil model assumptions, we applied non-parametric statistics to confirm the results.

3. Results

3.1. Determination of male social status

We used two different observation protocols to determine the social status of co-housed males: the post cage change observation (30 min of observation after every cage change) and the evening observation (2 h of observation during the dark phase). Both protocols revealed similar results as we could show that their scores for dominance (Spearman correlation: $r_{133} = .808$, $p = .000$; Figure 4) and submissive behaviours (Spearman correlation: $r_{133} = .783$, $p = .000$) were significantly correlated. Scores for dominance and submissive behaviours are directly related in pair-housed mice, but not in group-housed mice.

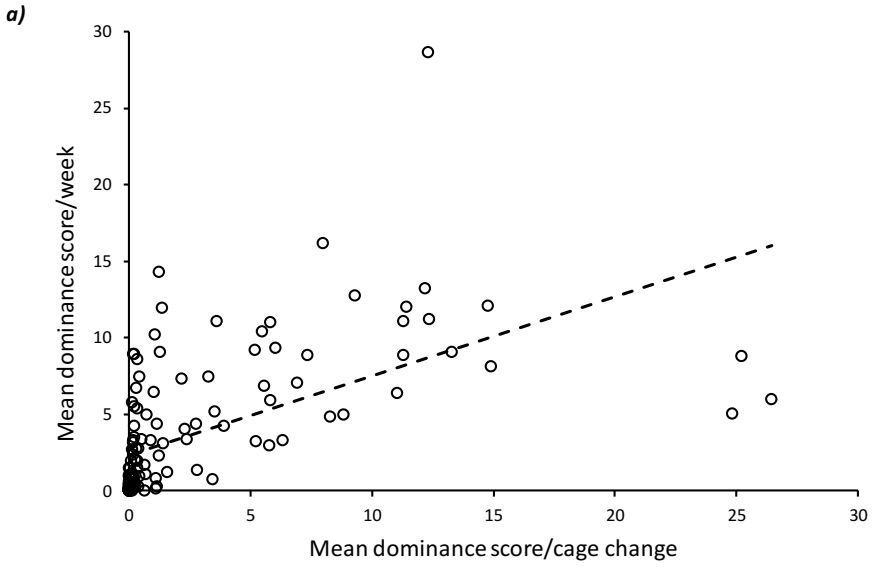


Figure 4: Relation of individual dominance scores measured by applying post cage change observation (mean score/cage change) and evening observation (mean score/week).

A clear dominance hierarchy could be assigned in 66.7 % of cages (36/54) when applying post cage change observations and in 64.8 % of cages (35/54) when using evening observations (more detailed information in Table 8). Dominance classification was identical in 83.1 % between observation protocols. During post cage change observations, mice from the long CCI group were observed for 2.5 h and mice from the short CCI group for 4.5 h. During evening observations mice from both CCI groups were observed for 80 h. Interestingly, mice showed significantly more agonistic behaviours per hour during post cage change observations (average

dominance score per mouse: 10,5) compared to the evening observations (average dominance score per mouse: 0,64) (paired t-Test: t = 9.142, p = .000).

*Table 8: Number of cages showing a clear dominance hierarchy after evening observations and post cage change observations depending on genetic background, group size and CCI. *2 cages were excluded from the analyses since mice showed severe fighting.*

	Evening observation		**Post cage change observation**	
Group:	**Established social status**	**No established social status**	**Established social status**	**No established social status**
Inbred mice	10	16*	9	17*
Hybrid mice	26	2	26	2
Pair-housed mice	18	10	20	8
Group-housed mice	18	8*	15	11*
Short CCI	18	10	17	9
Long CCI	18	8*	18	8*

For those groups where we could determine male social status within the experimental period, we compared the progress in dominance classification between observation protocols by looking at the determination of male social status on a weekly basis (Figure 5). The post cage change observation protocol enabled a correct determination of male social status in almost 70 % of cases already after the first observation period. This rate then showed a steady but flat rise over time. In comparison, evening observations during the first week were ineffective in inferring male social status, however, this changed swiftly as male social status could be correctly assigned in 81 % after two weeks and more than 94 % after 4 weeks of observation.

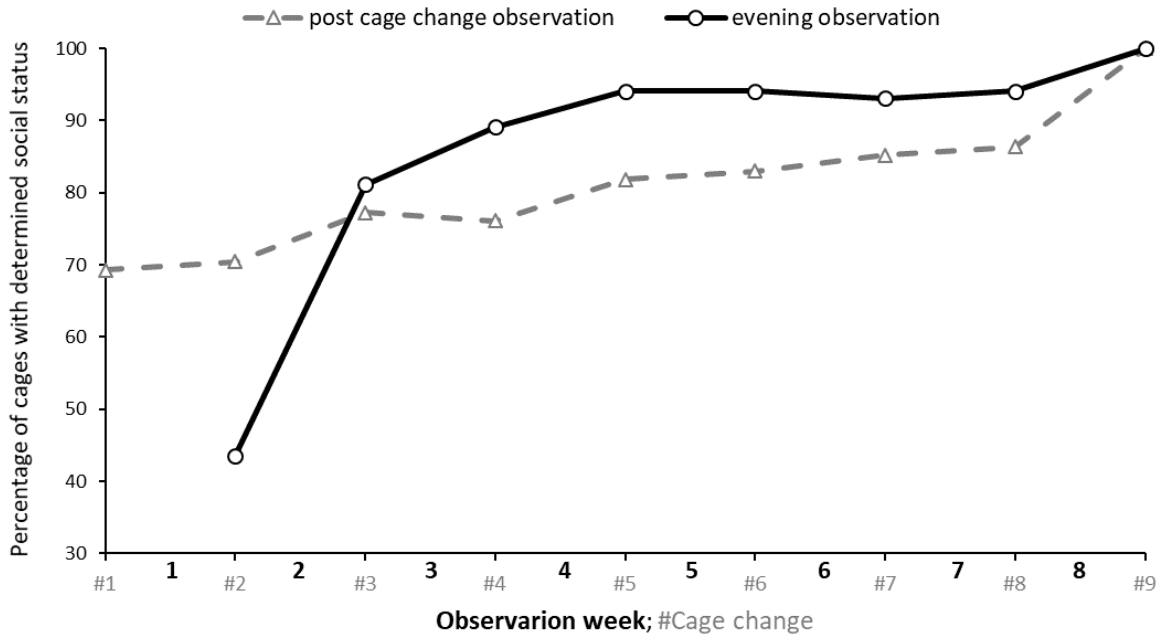


Figure 5: Determination of male social status over time based on post cage change observations and evening observations.

Experimental animals formed stable social hierarchies, as changes in social status within groups occurred rarely and were observed in only 4 % of cages (2/54) when applying evening observations and in 2 % of cages (1/54) when applying post cage change observations.

3.2. Male level of aggression

We found a significantly positive correlation between the attack rate in post cage change observations and evening observations (*active attack score*: Spearman correlation: $r_{133} = .704$, $p = .000$; Figure 6; *passive attack score:* Spearman correlation: $r_{133} = .725$, $p = .000$). Scores for active and passive attack rates were directly related in pair-housed mice, but not in group-housed mice.

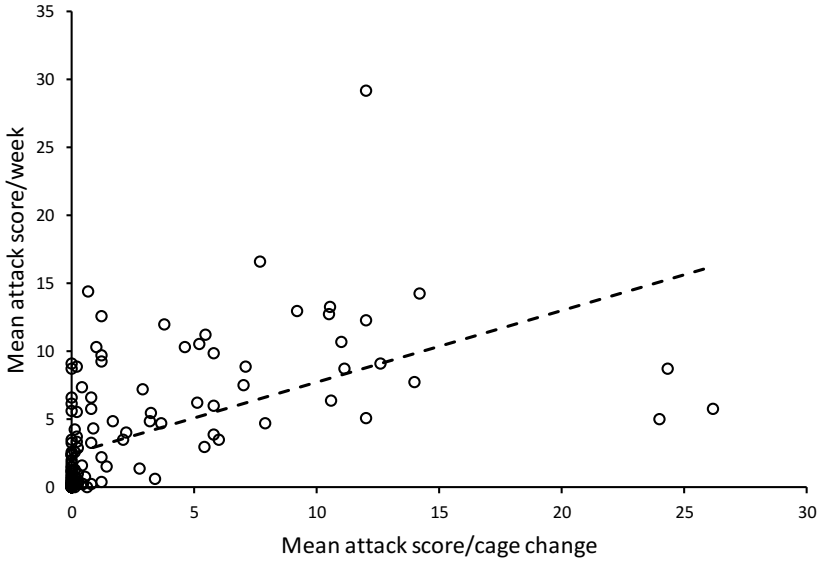


Figure 6: Relationship between mean passive attack scores per cage determined by post cage change observation and evening observation.

We used the evening observations to determine the mean passive attack rate per mouse over the course of the experiment. The passive attack rate per week was variable over the course of the experiment (GLMM: $F_{7,990} = 6.384$, $p = .000$; Figure 7) with peak values at week 5 and 7. Passive attack rates per mouse differed significantly between the two strains (LMM: $F_{1,30} = 29.272$, $p = .000$; Figure 8), showing that the B6D2F1 hybrid males face on average more attacks from their cage mates than C57BL/6N inbred males. The group size did not affect the passive attack rates per mouse (LMM: $F_{1,30} = .236$, $p = .631$). Furthermore, mice in cages with a short CCI did not receive more attacks than mice in cages with long CCI (LMM: $F_{1,30} = .579$, $p = .453$), even though attacks occurred significantly more frequent on days with cage change than on days without cage change (paired t-Test: $t = 5.127$, $p = .000$; see peaks in Figure 7 and Figure 8).

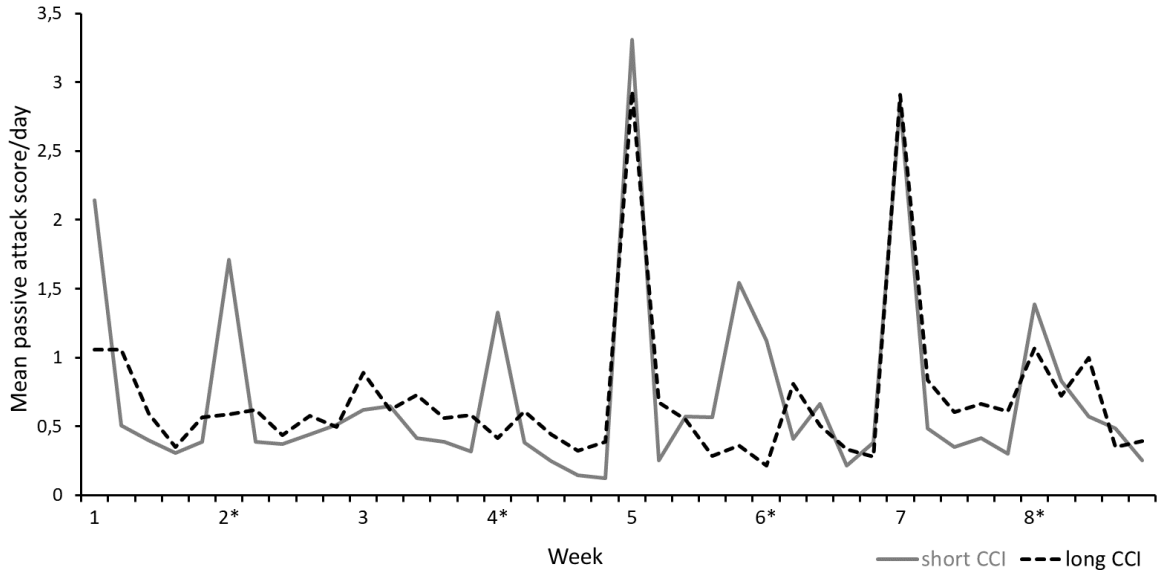


*Figure 7: Mean passive attack rates in mice (counted during evening observation) with short CCI (continuous grey line) and long CCI (dashed black line). *Days of no cage change for cages with a long CCI.*

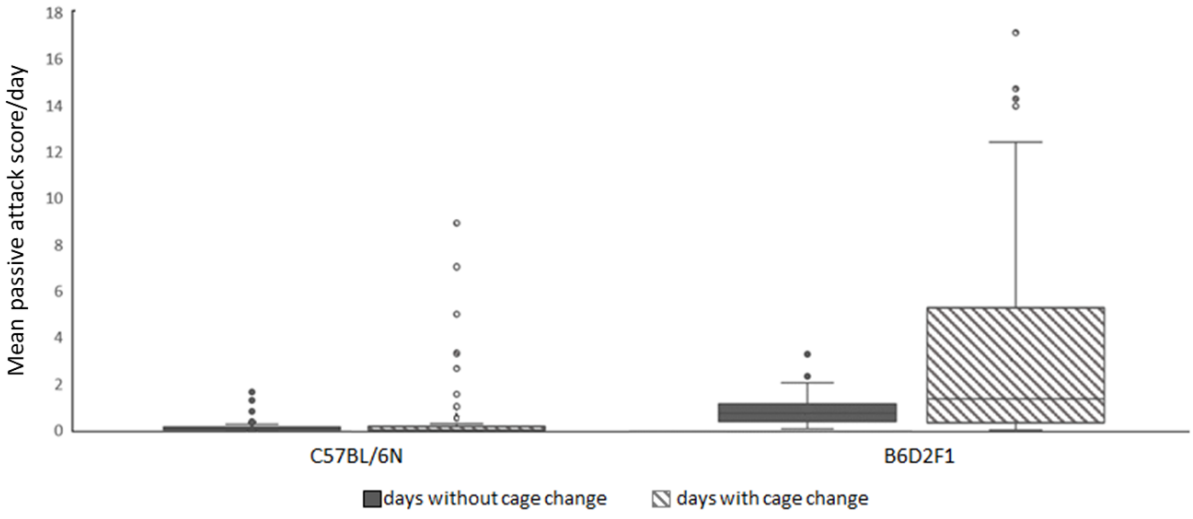


Figure 8: Difference in mean passive attack rate between C57BL/6N inbred mice and B6D2F1 hybride mice depicted for days with and without cage change.

Interestingly, we found a significantly positive correlation between attack rates and individual stress hormone levels: Those mice that attacked more (Spearman correlation: $r_{133} = .260$, $p = .002$) and received more attacks (Spearman correlation: $r_{133} = .269$, $p = .002$) showed higher CM levels. Furthermore, passive attack rates were also significantly positively correlated with TM levels (Spearman correlation: $r_{133} = .221$, $p = .010$;), whereas we found no correlation between active attack rates and TM levels (Spearman correlation: $r_{133} = .136$, $p = .129$).

3.3. Male fighting and injuries

During the experiment two cages had to be excluded from the experiment and mice had to be separated because of severe fighting. All of them were C57BL/6N inbred mice, that were group-housed, with a long CCI. The other 136 co-housed experimental mice had no visible wounds.

3.4. Male barbering behaviour

At the end of the experiment 80 % (128/162) of mice did not show any signs of barbering, 6 % (10/162) showed a low level of barbering, 12 % (20/162) an intermediate level and 2 % (4/162) a high level of barbering.

Comparing the social status of mice determined by barbering (non-barbered mice are usually classified as dominant, barbered mice as subordinate) versus behavioural observations was difficult as mice showed no barbering in 63 % (34/54) of cages (Figure 9). Among the 37 % (20/54) of cages where mice showed signs of barbering, only 40 % (8/20) performed agonistic behaviours during observations. In summary, both barbering and agonistic behaviour was simultaneously observed only in 22% (12/54) of cages. One of these 12 cages had to be excluded as mice showed an equal level of barbering so that no social status could be identified based on barbering. Among the remaining 11 cages the prevalence of barbering was in accordance with the observed social status of mice in only 60 % (16/27).

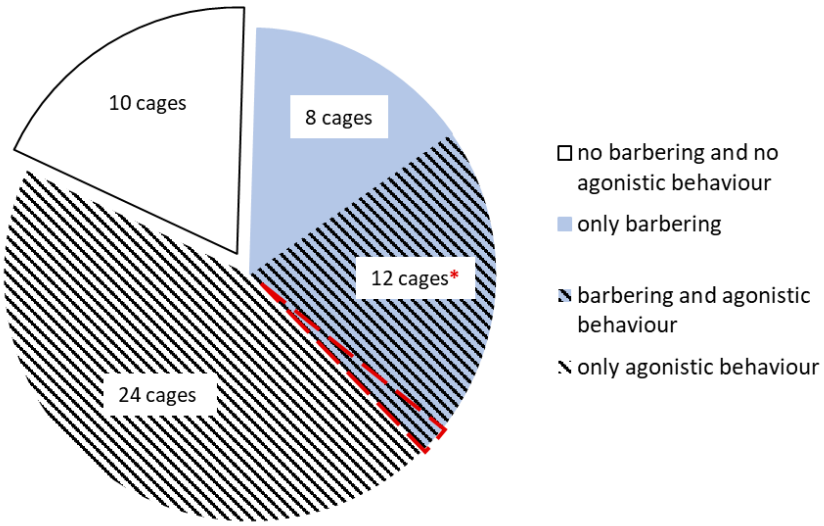


*Figure 9: Prevalence of barbering and agonistic behaviour in 54 observed cages. *One cage was excluded because all mice showed an equal level of barbering.*

We found a significant effect of group size on the prevalence of barbering (LM: Wald-$\chi^2 = 13.801$, $p = .001$; see Table 9). Post-hoc tests revealed that barbering prevalence was significantly higher in group-housed than in single (Bonferroni $p = 0.004$) and pair (Bonferroni $p = 0.004$) housed mice, whereas the difference between single-housed and pair-housed mice was not significant (Bonferroni $p = 0.792$).

Table 9: Number of barbered and not barbered mice depending on male genetic background, group size and CCI. Mice with a low (+), intermediate (++) and high (+++) barbering score were counted together.

		Barbering		**No barbering**	
		number	percentage	number	percentage
Genetic background	*C57BL/6N mice*	14	18 %	64	82 %
	B6D2F1 mice	20	24 %	64	76 %
Group size	*Single-housed*	1	4 %	27	96 %
	Pair-housed	6	11 %	50	89 %
	Group-housed	27	35 %	51	65 %
CCI	*Short*	19	23 %	65	77 %
	Long	15	19 %	63	81 %

No difference in the level of aggression was detected between barbered and non-barbered mice as active (Mann–Whitney U test: U = 1622, N = 134, $p = .818$) and passive attack rates (Mann–Whitney U test: U = 1809, N = 134, $p = .461$) were comparable between groups. Furthermore, barbered and non-barbered mice did not differ in their mean CM (Mann–Whitney U test: U = 1670, N = 134, $p = .986$) or TM (Mann–Whitney U test: U = 1752, N = 134, $p = .659$) levels.

Interestingly, we found that the prevalence of barbering was related to sperm traits in co-housed mice. Barbered mice had significantly fewer (Mann–Whitney U test: U = 1280, N = 134, $p = .046$; Figure 10a) but faster swimming sperm in regard of VCL (i.e. curvilinear velocity) (Mann–Whitney U test: U = 2068, N = 134, $p = .038$; Figure 10b) compared to non-barbered mice. Sperm VSL (i.e. straight-line velocity) tended to be higher in barbered compared to non-barbered mice, though this result was marginally non-significant (Mann–Whitney U test:

U = 2017, N = 134, p = .070). Sperm motility (Mann–Whitney U test: U = 1841, N = 134, p = .368) and longevity (Mann–Whitney U test: U = 1615, N = 134, p = .790) did not differ between barbered and non-barbered mice.

Barbered and non-barbered mice did not differ in their body mass (Mann–Whitney U test: U = 1390, N = 134, p = .153), however, we found that barbered mice had a significantly lower relative seminal vesicle mass (Mann–Whitney U test: U = 1173, N = 128, p = .046; Figure 10c) compared to non-barbered mice. There was no difference in their relative testes mass (Mann–Whitney U test: U = 1758, N = 134, p = .637) or their relative spleen mass (Mann–Whitney U test: U = 1751, N = 134, p = .663).

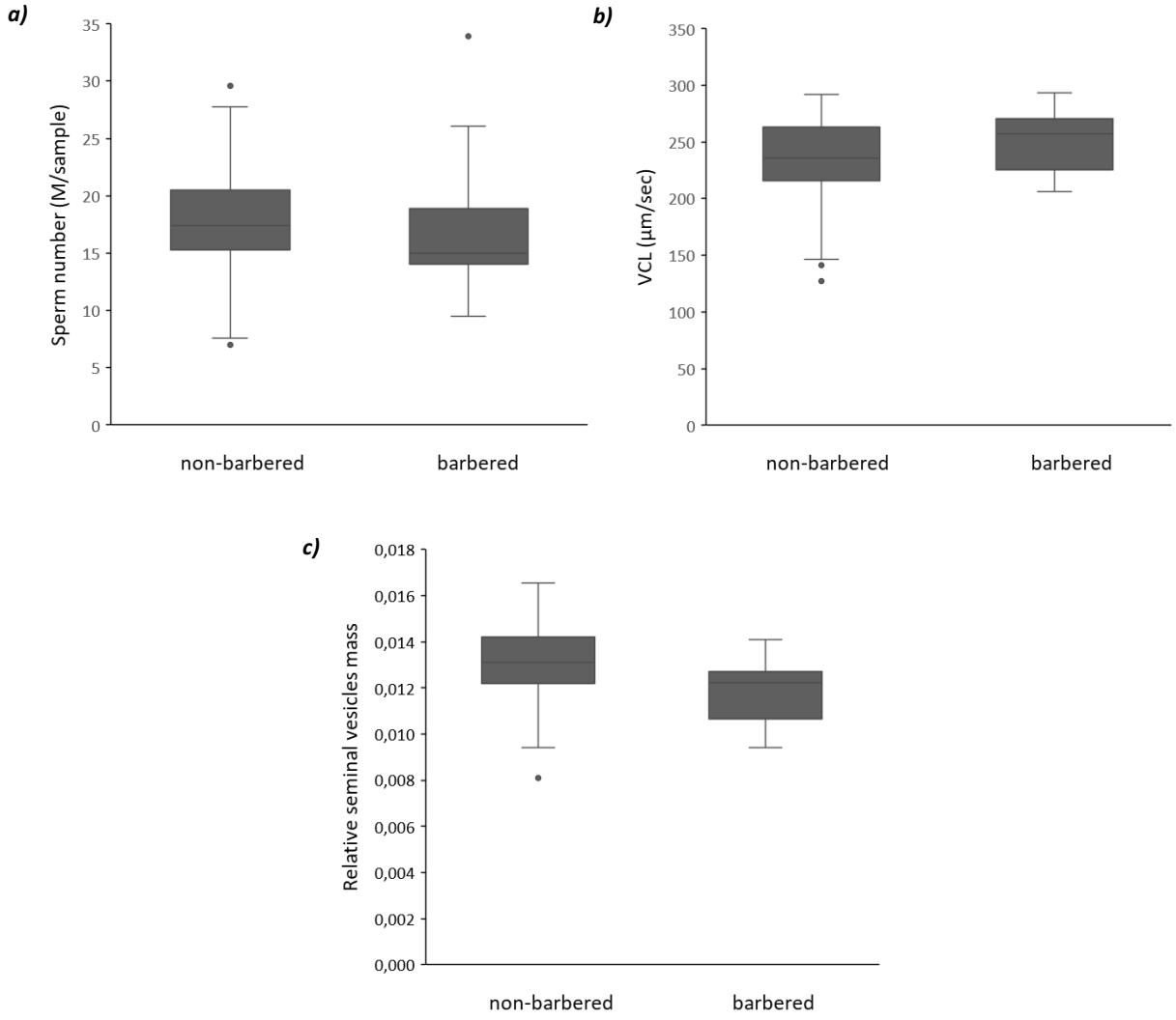


Figure 10: Comparison of barbered and non-barbered mice in regard of their ***(a)*** *sperm number,* ***(b)*** *sperm swimming velocity (VCL) and* ***(c)*** *relative seminal vesicles mass.*

3.5. Male co-sleeping rates

Overall, co-sleeping was common and was recorded on average in 73 % of observations, whereas separate sleeping was recorded in only 1 %. In 26 % of the observations mice were not sleeping. The likelihood for co-sleeping was not related to group size (LM: $F_{1,53} = .000$, $p = .998$) as we did not find a difference in incidence of co-sleeping between pair-housed or group-housed males. However, co-sleeping was significantly affected by male genetic background, and C57BL/6N inbred males showed more co-sleeping than B6D2F1 hybrid males (LM: $F_{1,53} = 5.731$, $p = .020$; Figure 11a). Also, CCI affected co-sleeping, which was higher in groups with a short CCI compared to a long CCI (LM: $F_{1,53} = 4.658$, $p = .036$; Figure 11b).

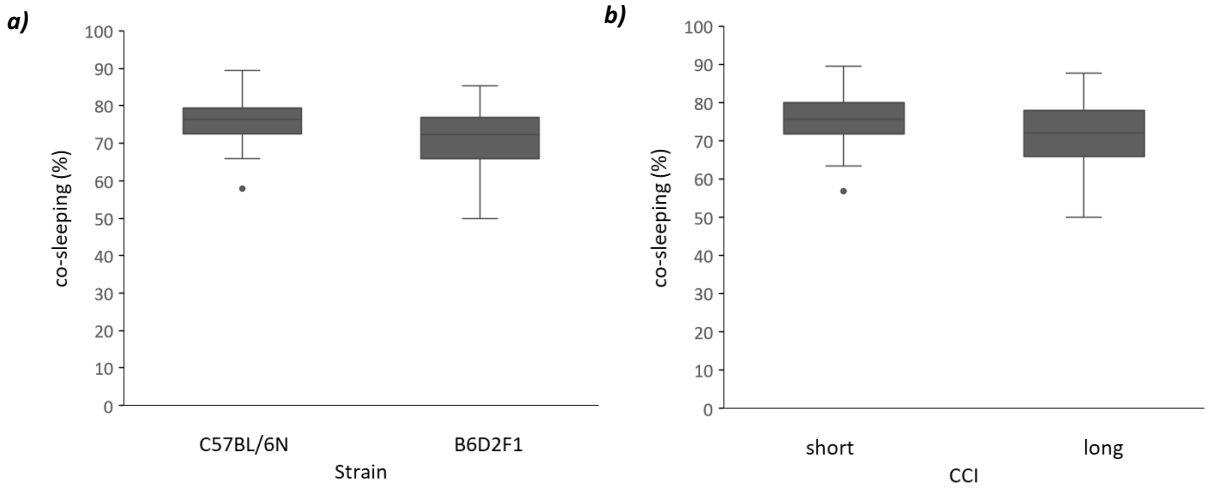


*Figure 11: Difference in co-sleeping incidence (percentage of days mice showed co-sleeping) between **(a)** C57BL/6N inbred mice and B6D2F1 hybrid mice and **(b)** mice from groups with a short CCI and mice from groups with a long CCI.*

Mice of the B6D2F1 hybrid strain slept in 1,3 % of (14/1104) observations separately, whereas mice of the C57BL/6N strain were never observed sleeping separately (0/1023). Co-sleeping was significantly negatively correlated with attack rates (Spearman correlation: $r_{53} = -.355$, $p = .008$; Figure 12). Furthermore, the more attacks per week, the higher the likelihood that co-housed mice were sleeping separately (Spearman correlation: $r_{53} = .380$, $p = .005$).

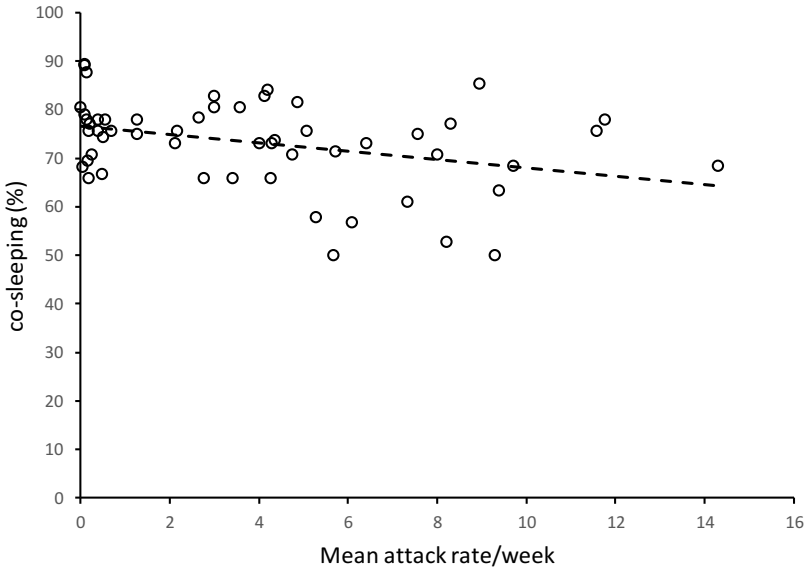


Figure 12: Relationship between mean attack rates and co-sleeping incidence.

3.6. Male sperm traits

Sperm quantity (i.e. the absolute sperm number per sample) tended to differ between the two strains, as B6D2F1 hybrid males had marginally less sperm than C57BL/6N males (LMM: $F_{1,78} = 3.48$, $p = .066$). We found no significant effects of CCI (LMM: $F_{1,72} = .114$, $p = .736$) or group size (LMM: $F_{2,96} = .498$, $p = .609$) on male sperm number. There was also no difference between dominant or submissive mice or mice without dominance status in regard of their sperm number (LMM: $F_{2,141} = .287$, $p = .751$). However, we found a significantly positive correlation between male relative testes mass and sperm number (LMM: $F_{1,149} = 17.892$, $p = .000$; Figure 13). Surprisingly, sperm number did neither correlate with body mass (Spearman correlation: $r_{161} = .125$, $p = .113$) nor with male corticosterone or testosterone levels (*CM:* Spearman correlation: $r_{161} = .049$, $p = .540$; *TM:* Spearman correlation $r_{161} = .041$, $p = .607$).

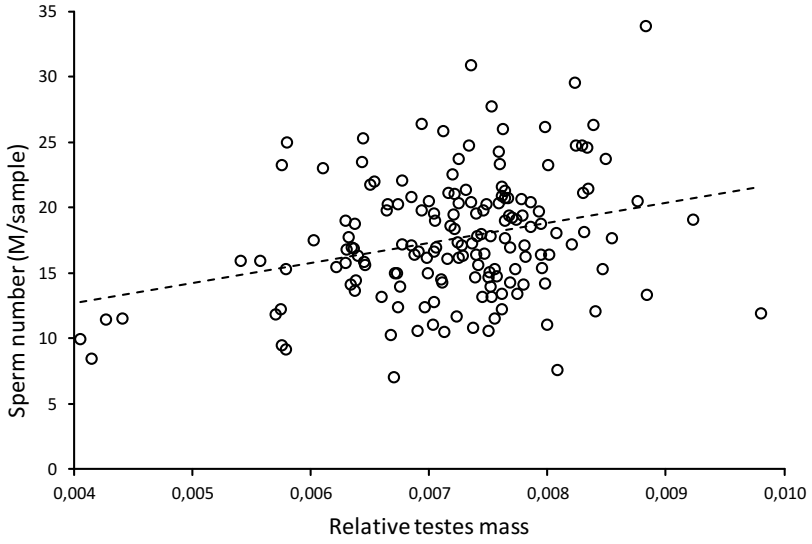


Figure 13: Relationship between sperm number and relative testes mass.

The intra-group variation in sperm number was bigger in group-housed than in pair-housed mice (LM: Wald-$\chi^2_{1,53}$ = 7.825, p = .005; Figure 14). The different genetic backgrounds (LM: Wald-$\chi^2_{1,53}$ = .781, p = .377) and CCI (LM: Wald-$\chi^2_{1,53}$ = 1.059, p = .303) had no effect on intra-group variation.

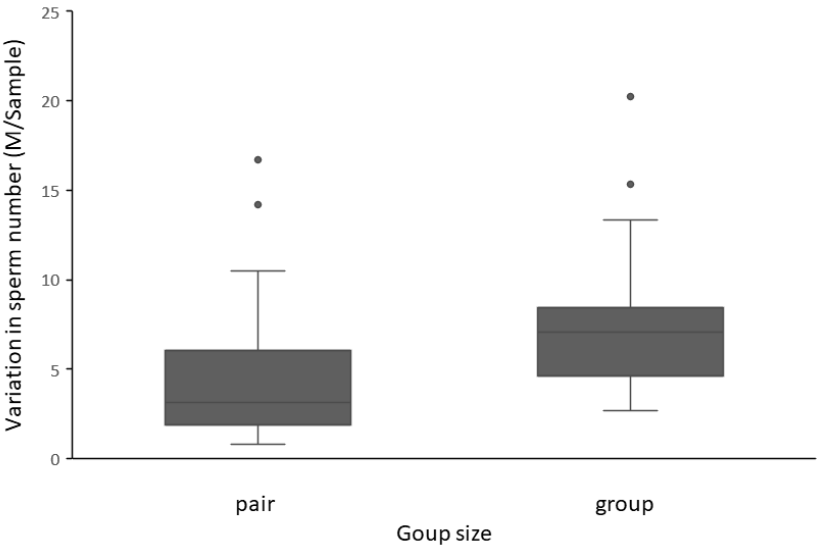


Figure 14: Comparison of the intra-group variation in sperm quantity between pair-housed and group-housed males.

Sperm motility (i.e. the percentage of motile sperm per sample) was significantly affected by group size (LMM: $F_{2,90}$ = 3.306, p = .041; Figure 15a) and post-hoc tests revealed that single-

housed mice had a lower sperm motility than group-housed mice (Bonferroni $p = .046$), whereas the difference between single and pair-housed mice (Bonferroni $p = .392$) and pair-housed and group-housed mice (Bonferroni $p = 1.000$) was not significant. CCI (LMM: $F_{1,69} = .000$, $p = .986$) and male genetic background (LMM: $F_{1,66} = .994$, $p = .322$) did not affect sperm motility. Also, we found no difference in sperm motility between dominant and subordinate males (LMM: $F_{2,139} = .250$, $p = .779$).

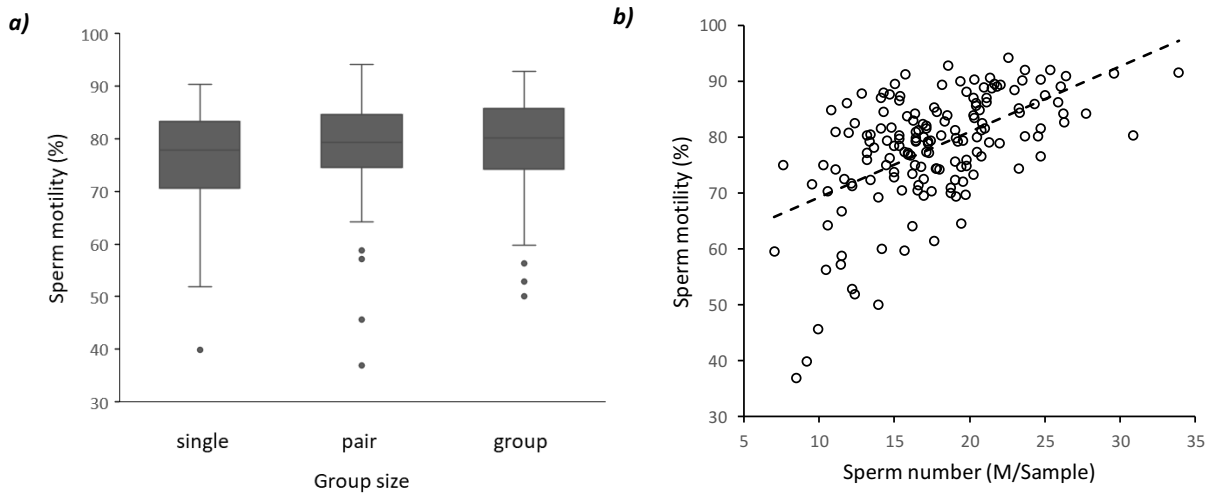


Figure 15: ***(a)*** *Difference in sperm motility between single-, pair- and group-housed males.* ***(b)*** *Relationship between individual sperm quantity and sperm motility.*

No association was found between body mass and sperm motility (Spearman correlation: $r_{161} = .102$, $p = .195$), though we found that sperm number was positively correlated with sperm motility (LMM: $F_{1,158} = 76.657$, $p = .000$; Figure 15b). Male CM and TM levels were not related to sperm motility (*CM:* Spearman correlation: $r_{161} = .150$, $p = .056$; *TM:* Spearman correlation: $r_{161} = -.083$, $p = .296$).

The intra-group variation in sperm motility was bigger in group-housed than in pair-housed mice (LM: Wald-$\chi^2_{1,53} = 6.128$, $p = .013$; Figure 16). Neither male genetic background (LM: Wald-$\chi^2_{1,53} = .009$, $p = .924$) nor CCI (LM: Wald-$\chi^2_{1,53} = .155$, $p = .693$) affected intra-group variation in sperm motility.

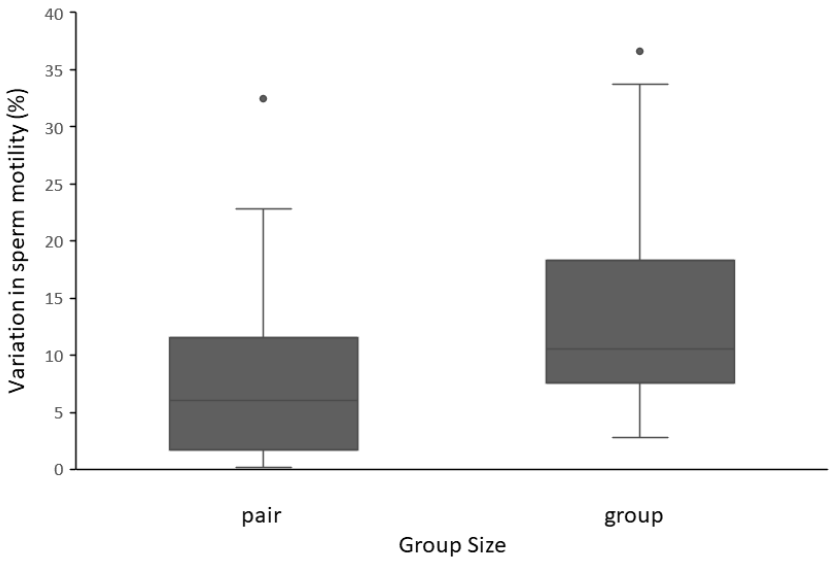


Figure 16: Comparison of intra-group variation in sperm motility in pair-housed and group-housed males.

Sperm motility after 2 h, which was assessed as an indicator of *sperm longevity,* was neither affected by male genetic background, (LMM: $F_{1,80} = 2.325$, $p = .131$) group size (LMM $F_{2,86} = 1.262$, $p = .288$) or CCI (LMM: $F_{1,80} = .857$, $p = .357$). Furthermore, we could not find any difference in sperm longevity between dominant or submissive males (LMM: $F_{2,136} = .746$, $p = .476$). Sperm longevity was significantly positively correlated with sperm number (LMM: $F_{1,174} = 53.647$, $p = .000$) and male body mass (Spearman correlation: $r_{161} = .189$, $p = .016$). Male CM and TM levels were not related to sperm longevity (*CM:* Spearman correlation: $r_{161} = .111$, $p = .160$; *TM:* Spearman correlation: $r_{161} = .105$, $p = .184$). The intra-group variation in sperm longevity was not affected by group size (LM: Wald-$\chi^2_{1,53} = 2.105$, $p = .147$), male genetic background (LM: Wald-$\chi^2_{1,53} = .672$, $p = .412$) nor CCI (LM: Wald-$\chi^2_{1,53} = .310$, $p = .577$).

Sperm *VCL* was significantly affected by male genetic background as B6D2F1 hybrids had higher VCL than C57BL/6N inbred males (LMM: $F_{1,73} = 189.926$, $p = .000$; Figure 17). We found no effect of group size (LMM: $F_{2,78} = .467$, $p = .629$) or CCI (LMM: $F_{1,73} = 2.806$, $p = .098$) on sperm swimming velocity. Also, there was no difference in sperm velocity between dominant or submissive males (LMM $F_{2,127} = .187$, $p = .830$).

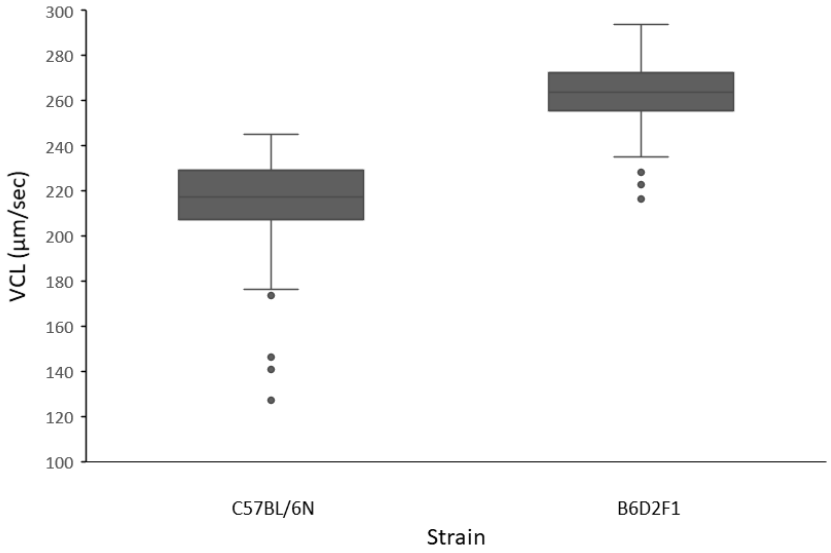


Figure 17: Difference in VCL between C57BL/6N inbred males and B6D2F1 hybrid males.

VCL was significantly correlated with male body mass and heavier males had faster swimming sperm (Spearman correlation: $r_{161} = .471$, $p = .000$; Figure 18a). Furthermore, VCL was also positively correlated with sperm number (LMM: $F_{1,146} = 11.253$, $p = .001$) and male CM (Spearman correlation: $r_{161} = .158$, $p = .044$) and TM levels (Spearman correlation: $r_{161} = .311$, $p = .000$; Figure 18b).

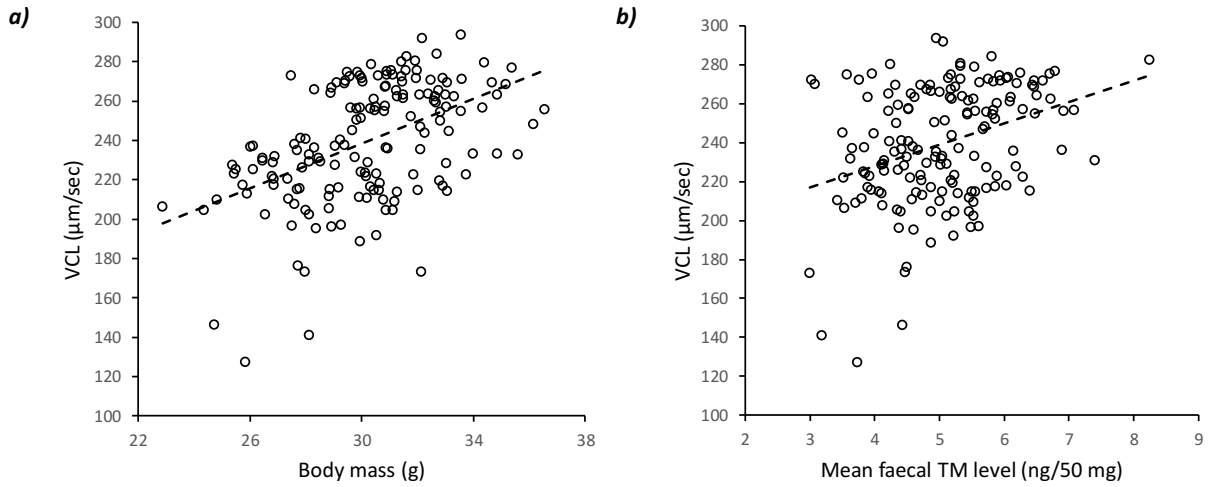


*Figure 18: Relationship between VCL and **(a)** body mass and **(b)** mean faecal TM levels over the course of the experiment.*

The intra-group variation in VCL was not affected by group size (LM: Wald-$\chi^2_{1,53} = .237$, $p = .627$), male genetic background (LM: Wald-$\chi^2_{1,53} = .019$, $p = .890$) nor CCI (LM: Wald-$\chi^2_{1,53} = .670$, $p = .413$).

3.7. Male body and organ mass

The two strains differed in their *body mass*, whereas the B6D2F1 hybrid males were significantly heavier than the C57BL/6N inbred males (LMM: $F_{1,71} = 30.675$, $p = .000$; Figure 19a). Furthermore, we found that body mass differed depending on male social status (LMM: $F_{2,132} = 5.341$, $p = .006$; Figure 19b) and post-hoc tests revealed that dominant and subordinate males did not differ in their body mass (Bonferroni $p = .626$), whereas mice without dominance status were significantly lighter than dominant (Bonferroni $p = .000$) and subordinate (Bonferroni $p = .000$) mice. The housing conditions did not influence male body mass as we found no effects of group size (LMM: $F_{2,83} = .018$, $p = .982$), or CCI (LMM: $F_{1,61} = .097$, $p = .757$). Interestingly though, we found a significant positive correlation between body mass and both mean CM levels (Spearman correlation: $r_{161} = .355$, $p = .000$) and mean TM levels (Spearman correlation: $r_{161} = .260$, $p = .001$).

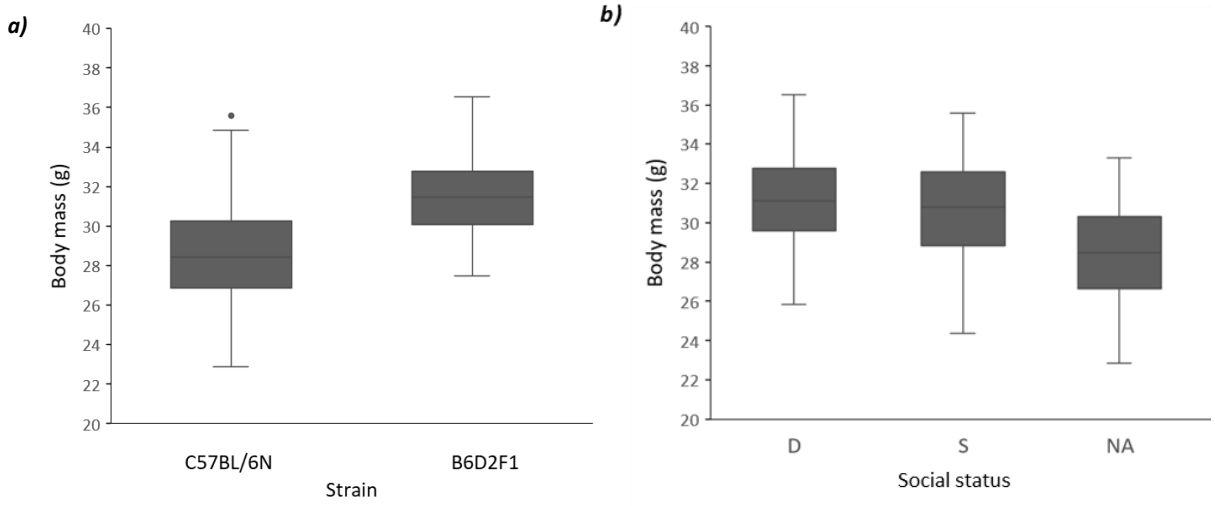


Figure 19: Difference in body mass between ***(a)*** *C57BL/6N inbred mice and B6D2F1 hybrid mice and between* ***(b)*** *dominant males (D), subordinate males (S) and males with no assigned social status (NA).*

The *relative testes mass* (i.e. the testes mass corrected for body mass) was significantly higher in B6D2F1 hybrid than in C57BL/6N inbred males (LMM: $F_{1,604} = 21.591$, $p = .000$; Figure 20a). Furthermore, we found an effect of group size on relative testes mass (LMM: $F_{2,527} = 4.219$, $p = .015$; Figure 20b). Post-hoc tests revealed that testes of pair-housed mice were significantly lighter than testes of group-housed mice (Bonferroni $p = .011$) and tended to be lighter than testes of singe-housed mice, though not significantly (Bonferroni $p = .071$). Single-housed and group-housed mice did not differ in their relative testes mass (Bonferroni

$p = 1.000$). CCI (LMM: $F_{1,601} = .993$, $p = .319$) had no effect on relative testes mass and we could not find any difference in relative testes mass between dominant or submissive males (LMM: $F_{2,188} = .029$, $p = .972$). As expected, we found that relative testes mass was related to mean TM levels (Spearman correlation: $r_{161} = .301$, $p = .000$), and males with relatively heavy testes showed increased levels of TM. There was no relationship between relative testis mass and mean CM levels in males (Spearman correlation: $r_{161} = -.100$, $p = .207$).

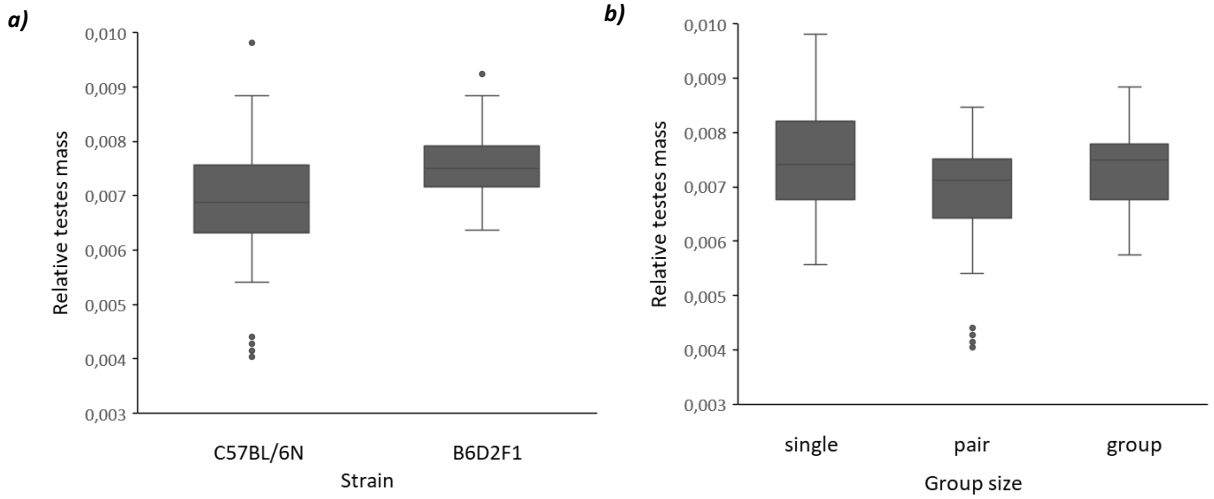


Figure 20: Difference in relative testes mass between ***(a)*** *C57BL/6N inbred mice and B6D2F1 hybrid mice and* ***(b)*** *single-, pair- and group-housed males.*

The *relative seminal vesicle mass* (i.e. the seminal vesicles mass corrected for body mass) was significantly higher in the C57BL/6N inbred than in the B6D2F1 hybrid males (LMM: $F_{1,71} = 15.596$, $p = .000$; Figure 21a). Furthermore, we found an effect of group size on relative seminal vesicles mass (LMM: $F_{2,85} = 6.690$, $p = .002$; Figure 21b). Single-housed mice had significantly heavier seminal vesicles than pair-housed (Bonferroni $p = .006$) and group-housed (Bonferroni $p = .002$) mice, whereas pair-housed and group-housed mice did not differ in their relative seminal vesicles mass (Bonferroni $p = 1.000$). However, CCI did not affect relative seminal vesicle mass (LMM: $F_{1,310} = .404$, $p = .526$) and we could not find any difference in relative seminal vesicles mass between dominant or submissive males (LMM: $F_{2,127} = 1.202$, $p = .304$). Relative seminal vesicle mass was significantly negatively correlated with mean CM levels (Spearman correlation: $r_{155} = -.259$, $p = .001$), whereas it did not correlate with mean TM levels (Spearman correlation: $r_{155} = .129$, $p = .109$).

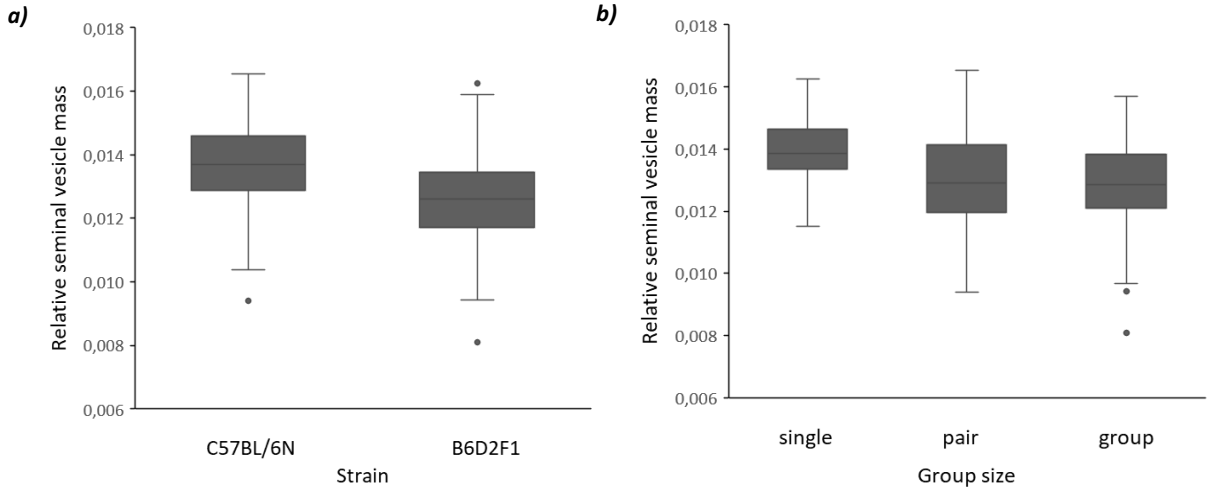


Figure 21: Difference in relative seminal vesicles mass between ***(a)*** *C57BL/6N inbred mice and B6D2F1 hybrid mice and* ***(b)*** *single-, pair- and group-housed males.*

The *relative spleen mass* (i.e. the spleen mass corrected for body mass) was significantly affected by group size (LMM: $F_{2,155} = 3.684$, $p = .027$; Figure 22) and single-housed mice had a significantly lighter spleen than pair-housed (Bonferroni $p = .013$) and group-housed (Bonferroni $p = .016$) mice, whereas pair-housed and group-housed mice did not differ in their relative spleen mass (Bonferroni $p = 1.000$). CCI had no effect on relative spleen mass (LMM: $F_{1,155} = .135$, $p = .714$) and neither had male genetic background (LMM: $F_{1,155} = .881$, $p = .349$). Also, relative spleen mass did not differ between mice depending on their social status (LMM: $F_{2,155} = 1.048$, $p = .353$). Finally, relative spleen mass did not correlate with mean TM levels (Spearman correlation: $r_{161} = -.050$, $p = .525$), or CM levels, though the later was marginally non-significant (Spearman correlation: $r_{161} = .133$, $p = .090$).

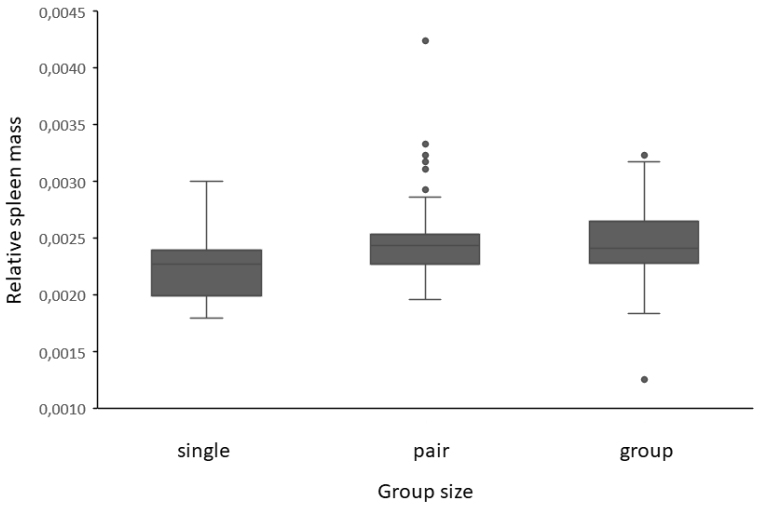


Figure 22: Difference in relative spleen mass between single-, pair- and group-housed males.

3.8. Hormone levels

Overall, we found that mice with a high CM levels also tended to have slightly higher TM levels, though this result was marginally non-significant (Spearman correlation: $r_{161} = .149$, $p = .059$).

We found an effect of group size on mean faecal CM levels (LMM: $F_{2,112} = 10.519$, $p = .000$; Figure 23a). Post-hoc tests revealed that single-housed mice had significantly lower CM levels than pair-housed (Bonferroni $p = .009$) and group-housed (Bonferroni $p = .000$) mice, whereas pair-housed and group-housed mice did not differ in their mean faecal CM levels (Bonferroni $p = 1.000$). Male genetic background (LMM: $F_{1,77} = 1.487$, $p = .226$) and CCI (LMM: $F_{1,71} = .070$, $p = .792$) had no significant effect on CM levels. Interestingly, mean CM levels differed depending on male social status (LMM: $F_{2,111} = 4.607$, $p = .012$; Figure 23b): Subordinate mice had significantly higher mean CM levels than mice that did not establish a social status (Bonferroni $p = .023$). No difference was found between subordinate and dominant mice (Bonferroni $p = .104$) or dominant mice and those that did not establish a social status (Bonferroni $p = 1.000$).

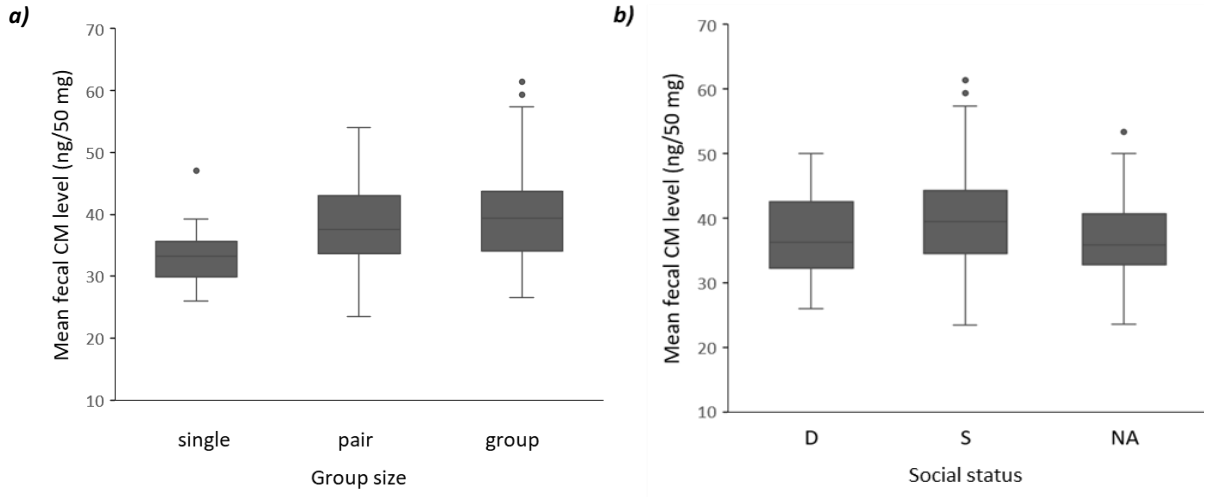


Figure 23: Difference in mean faecal CM levels between (a) single-, pair- and group housed males and (b) dominant males (D), subordinate males (S) and males with no assigned social status (NA).

CM levels changed significantly over the course of the experiment (LMM: $F_{3,569} = 6.356$, $p = .000$; Figure 24): CM levels were comparatively high at the beginning, then decreased before starting to rise again.

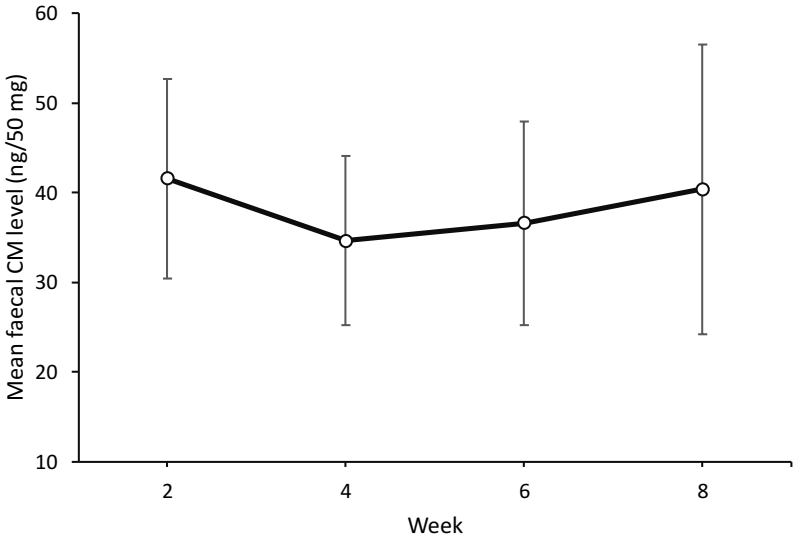


Figure 24: Male mean faecal CM levels over the course of the experiment.

Mean TM levels were significantly higher in B6D2F1 hybrid males than in C57BL/6N inbred males (LMM: $F_{1,72} = 19.015$, $p = .000$; Figure 25). However, we found no effect of group size (LMM $F_{2,89} = 1.680$, $p = .192$) or CCI (LMM: $F_{1,71} = .124$, $p = .726$) on male TM levels and there was no difference in mice depending on their social status (LMM: $F_{2,140} = .325$, $p = .723$). TM levels showed significant variation over the course of the experiment (LMM:

$F_{3,572} = 24.447$, $p = .000$; Figure 25): TM was high at the beginning, then decreased and finally started to rise again at the end.

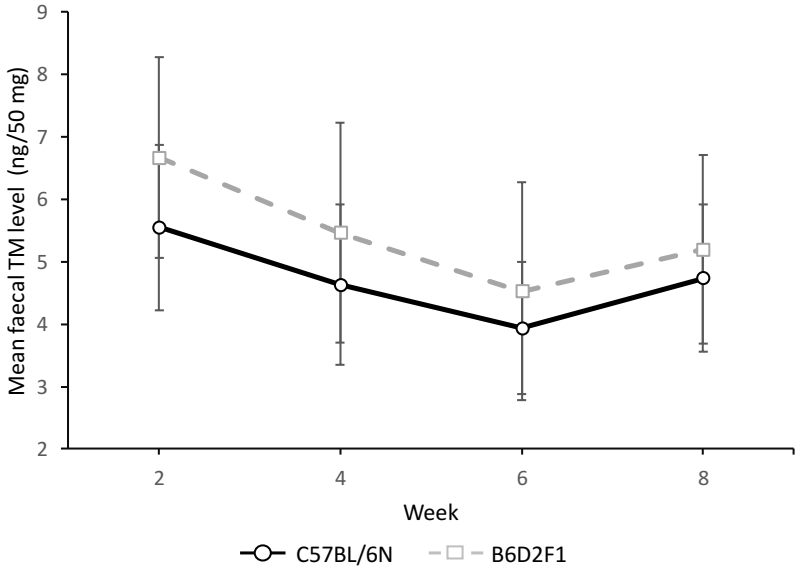


Figure 25: Male mean faecal TM levels over the course of the experiment in C57BL/6N inbred males (continuous black line) and B6D2F1 hybrid males (dashed grey line).

4. Discussion

4.1. Determination of male social status

We applied both, evening and post cage change observations to assess male social status. Our comparison provided several interesting results and we start our discussion in highlighting the benefits of the post cage change observation protocol. First, evening observation took up a lot of observation time over the course of the experiment, i.e. 80 h, compared to 2,5-4,5 h for post cage change observation. However, it provided only about 50 % more information about agonistic behaviour. Hence, the cost-effectiveness ratio for evening observation was disproportionately low. The additional information gained by the time intensive evening observations was not required to reliably assess male social status, as both observation protocols revealed highly similar results: dominance and submissive behaviours were highly correlated and dominance classification was identical in 83,1 % of cases. Even though dominance and submissive behaviours were highly correlated, we found some differences in the behaviour profile of mice between the observation protocols. These differences might be explained by the timing of our observations. For example, after each cage change dominant mice have to re-establish their social status in the "new" territory, as their scent marks, which were used for identification and hierarchical perception were removed (Gosling et al. 1996; Nevison et al. 2000). In comparison, mice at the evening observations might show more behaviour related to maintaining rather than establishing their social hierarchies. Thus, the behaviour pattern of mice after cage change can vary from the behaviour pattern of mice during the evening observation, because they are observed in a different context.

With both protocols we could classify male social status in 65 % of the groups. In previous experiments this number varied from 25 % (Koyama and Kamimura 2000), 36 % (Koyama and Kamimura 1998) to 88 % (van Loo et al. 2003), probably because different strains and modified observation assays were used. With our evening observation protocol 4 weeks of observations were sufficient to determine the social hierarchy within a group and further observations affected the social status classification in less than 5 %. Dominance behaviour has been shown to be dynamic (Williamson et al. 2017b) and an alteration in social hierarchies within groups can always occur. In our experiment social hierarchies within groups were very stable, as alterations occurred in only 2-4 %, probably because small groups of male mice evolve highly

despotic hierarchies (Poole and Morgan 1973). If social hierarchies have to be determined in a short period of time, we recommend performing post cage change observations, as already little observation effort is sufficient to classify the majority of social hierarchies within groups: In our case, already one observation period (i.e. 30 min) was sufficient to determine the social rank in 70 % (61/88) of mice that showed agonistic behaviour. In comparison, we needed two weeks of evening observations with several hours of observation time to achieve this level of dominance classification. Post cage change observation produced reliable results faster, probably because males show more agonistic behaviour directly after cage change, while agonistic behaviours during evening observation occur only rarely. This is aggravated by the fact that during evening observations cages were observed simultaneously for a longer period of time under red light. This required a high level of concentration and we cannot assure that some interactions might have been missed. To sum up, we can say that the post cage change observation protocol reliably assessed male social status, while being more time efficient. However, the evening observation was still worth investigating, as it revealed interesting information about attack rates, which we will discuss later.

An alternative method to determine male social status is the evaluation of barbering behaviour. However, this method is controversially discussed in the literature (Garner et al. 2004a) and in our study comparing the social status determined by barbering and by behavioural observations was problematic, as in only 22% of the cages mice showed both barbering and agonistic behaviour that would allow a comparison. In these cases, dominance classification was only identical in 60% of cases. The assumption that barbering is a characteristic of dominance behaviour (the so called “Dalila effect”) is discussed controversially: while studies show a clear correlation between dominance in tube tests and barbering (Wang et al. 2011c; Kalueff et al. 2006; Strozik and Festing 1981), others do not (Garner et al. 2004a). Furthermore, one study reports that self-barbering is as common in individually housed mice as in group-housed mice (Garner et al. 2004a). Even if self-barbering was not common in our strains (barbering prevalence in individually housed males was 3,5% compared to 32,5% in co-housed males), it could have confounded our results, as we could not distinguish between barbering and self-barbering in co-housed males. The question is, whether barbering *per se* is an adequate proxi to assess social hierarchies within groups. Our results did not support this approach, as barbering occurred in both dominant and subordinate males. However, more tests are required

to compare how other methods to determine social status (i.e. tube test, resident intruder test, territory urine marking, etc.) relate to barbering. Even if barbering was not a reliable indicator of male social status in our study, it was still worth investigating, as barbering represents an abnormal behaviour (Garner et al. 2004b) and hence a welfare problem and barbered and non-barbered males differed in some physiological traits, which we will highlight later.

4.2. Housing effects on male aggression and refinement considerations

4.2.1. *Male attack rate and stress hormone level*

As expected, we found that the level of intra-group aggression was related to individual stress levels and the higher the attack rates within a group, the higher the individual CM levels were. This is in line with previous studies showing that a high level of fighting results in an increase of urine and serum corticosterone levels (Goldsmith et al. 1978; van Loo et al. 2001a). Our finding is the first to show this effect using faecal CM analysis, indicating that our assay provides a non-invasive alternative to reliably assess animal stress levels. Given that high attack rates were related to elevated CM levels in all group members, independent of whether an individual initiated or received an attack, our finding highlights that conflicts are not only stressful for the loser, but also for the winner of an agonistic interaction (Beckett and Purkaystha 1975; Kappel et al. 2017).

Intra-group aggression was preliminarily determined by mouse strain, whereas CCI and group size had no significant effects. We found B6D2F1 hybrid males to be more aggressive than C57BL/6N inbred males. Genetically defined differences in aggression levels of male mice are widely known and have previously been reported (van Loo et al. 2003; Bisazza 1981b; Mondragón et al. 1987a; Guillot and Chapouthier 1996). For example, C57BL/6N inbred mice are known to be comparatively meek, showing the least inter-male aggression compared to DBA/2 inbred mice, Swiss albino outbred CD-1 mice and wild mice (Parmigiani et al. 1999). The reason for these strain specific differences can be linked to breeding processes, as artificial selection of different genotypes can influence the neuroendocrine physiology of mice, which in turn can then lead to modifications in social behaviour and thus altered aggression and anxiety levels (Parmigiani et al. 1999). Aggressive behaviour is an important category in mouse phenotyping and currently 31 allelic compositions are found to increase aggression toward male

conspecifics (Mouse Genome Informatics 2020). The increased inter-male aggression in B6D2F1 hybrid males found in our study might thus be explained by their allelic compositions and genetic background, as one of their parental strains, the DBA/2 strain, is known to be aggressive (Parmigiani et al. 1999). Interestingly, we could show that the mean faecal CM levels of males did not differ between the C57BL/6N and B6D2F1 strain, despite the clear differences in their attack rates. An explanation could be that B6D2F1 hybrid mice have lower basal corticosterone levels compared to C57BL/6N inbred mice. Differences in basal corticosterone levels between different strains (e.g. male BALB/cAnNCrlBR mice and male Swiss derived CRL:CD-1(ICR)BR mice) can occur (van Loo et al. 2003), even if a difference between other parental strains (BALB/cByJ and C57BL/6ByJ) and their F1 strains have not been found (Roy et al. 2007).

Interestingly, in co-housed mice group size did neither effect inter-group aggression, nor individual stress hormone levels. Similarly, a previous study in outbred MF1 mice also reported no effect of group size on the level of agonistic behaviours (Hunt and Hambly 2006). The only studies that found a positive relationship between group size and male agonistic behaviour tested males in comparatively larger groups (Barnard et al. 1994; van Loo et al. 2001a). Not surprisingly, effects only became apparent when comparing males in groups of 3 to males that were kept in groups bigger than 6. Small groups (up to 5 males/cage) usually evolve a despotic hierarchy with one dominant mouse, resulting in a decline in the level of intra-group aggression (Poole and Morgan 1973) and thus potentially also in the stress level. In contrast, bigger groups (9 or more males/cage) have permanently high levels of aggression, resulting from changes in their dominance hierarchy and additional aggression between subordinate males (Poole and Morgan 1973). In our experiment both pair-housed and group-housed males showed stabile hierarchies as only males from 1 of the 54 co-housing groups changed their social status during the eight weeks of observation. Another indicator that groups sizes of 2 or 3 facilitates the formation of stable hierarchies and constrains intra-group aggression in mice is that only 4 % of co-housed mice in our experiment showed fighting induced wounds. Additionally, we found that group size had no overall effect on faecal CM levels, indicating that the stress level of males did not differ between pair-housed or group-housed males. This is in line with similar studies that found no effect of group size on urine (van Loo et al. 2001b) and faecal corticosterone levels (Hunt and Hambly 2006). In summary, our data suggest that group sizes

of 2 and 3 do not differentially impact on male welfare in the investigated strains and we can recommend both co-housing regimes.

Interestingly, we found that single-housed males had significantly lower mean CM levels than co-housed males. This suggests that both being suppressed by a dominant cage mate, as well as defending a dominance status is stressful, while this challenge is missing in single-housed mice (Kappel et al. 2017). Similarly, previous studies have shown that individual housing lowers faecal corticosterone (Hunt and Hambly 2006) and plasma corticosterone level (Ieraci et al. 2016; Martin and Brown 2010). Also, single-housed males have a lower adrenal gland mass, another indicator for reduced stress (Benton et al. 1978). Spleen size or rather splenomegaly is another parameter to determine the level of social stress and the activation of the immune system (Blanchard et al. 1993; Blanchard et al. 1995; Kaliste-Korhonen and Eskola 2000). We found that co-housed mice had bigger spleens compared to single-housed mice in both strains. This result could be explained by the social stress associated to co-housing, as also other studies found that both, dominant and subordinate males, showed splenomegaly and changes of haematological parameters compared to the single-housed control groups (Turney and Harmsen 1984; Blanchard et al. 1993; Mucignat-Caretta et al. 2014). Similarly, splenomegaly can be more distinct in subordinate males (Turney and Harmsen 1984) and in cages with a disruption in social hierarchies (Avitsur et al. 2007), potentially as such mice face higher stress levels (Blanchard et al. 1993; Blanchard et al. 1995). We found that experimental mice with high CM levels also tended to have bigger spleens, supporting the link between individual stress levels and spleen size. Hence, one could argue that individual housing should generally be preferred for male mice, however, this conclusion would be prematurely made. Short term individual housing as executed in our experiment is not comparable to long-term isolation without acoustic, visual and olfactory inputs from other mice. Such keeping conditions can cause several welfare problems and induce the development of stereotypies (Kappel et al. 2017; Ieraci et al. 2016), thereby diminishing animal welfare.

CCI had no effect on the overall level of aggression within cages. This is somehow surprising, because attack rates were significantly higher on days with cage change than without cage change and mice with short CCI underwent cage changing more often. It has been shown that handling during cage change and the disruption of odour cues can lead to a brief increase in

aggression (Gray and Hurst 1995; Van Loo et al. 2000). However, in our study, such peaks in aggression seemed to have no significant impact on the overall level of attacks within groups. Furthermore, CCI had no effect on mean faecal CM levels. We performed faeces collection immediately before cage change to catch the overall stress levels of experimental mice, rather than to assess their immediate stress levels related to the experimental manipulations. Our samples therefore depicted basal stress and stressful events of the previous 4-12 h, depending on the lag time, which can vary with mouse activity pattern and metabolism (Touma et al. 2003). Even if a shorter CCI did not significantly affect basal stress and aggression levels in this study, frequent cage change should still be avoided, as our observational data reveal that it leads to a short peak in intra-group aggression and previous studies showed that it results in an increased serum corticosterone levels - even if this effects already disappears after 60 minutes (Rasmussen et al. 2011). Stress related to cage change could further be reduced using a gentle passive transfer technique (Rasmussen et al. 2011) and transferring nesting material from the dirty cage (Van Loo et al. 2000). The downside of longer CCIs are a disturbed microenvironment and too high ammonia levels, which are depending on the cage ventilation system, bedding material, animal density and sex and all these factors must be considered when choosing the optimal CCI. Urine spot characteristics are, for example, valid and practicable indicators when to clean cages (Washington and Payton 2016) and our applied CCIs were in the range of normal regarding urine spot characteristics.

Different from what we expected, there was no positive relationship between active attack rates and individual TM levels. This is in contrast to the widespread assumption that high testosterone levels lead to increased aggression. This has experimentally been shown in castrated and intact male mice, where both enhanced their aggressive behaviour after an exogenous androgen therapy (Lee and Naranjo 1974; Martínez-Sanchis et al. 1998) and where endogenous testosterone levels were positively correlated with aggression levels (Leshner and Moyer 1975). However, we are the first to investigate this correlation using faecal TM measurement and there is no reason to believe that faecal samples provide different results than serum measurements (Auer et al. 2020). Interestingly, we found that males with higher TM levels received more attacks compared to males with low TM levels, suggesting that high individual testosterone levels are indeed costly as they potentially trigger attacks from conspecifics. This has never been reported before and we hypothesise that increased TM levels in males lead to more attacks

from conspecifics as males with high testosterone levels are perceived as potent rivals that need to be kept in rein. Overall, our study confirms that there is no straight forward relationship between particular hormone levels and male aggression (Haug et al. 1986; Johnson and Whalen 1988) and further studies are required to better understand their relationships.

4.2.2. *Male wounds and barbering behaviour*

The incidence of severe fighting with visible injuries and wounds was low in our study occurring in only 3 % of co-housed mice. Other studies report rates between 25 % (Nicholson et al. 2009) to up to 100 % (van Loo et al. 2003) and it has been shown that fighting induced wounds occur more often in subordinate mice, in bigger groups and in larger cages (van Loo et al. 2001b). We did not observe any link between the incidence of wounds and our specific experimental conditions, suggesting that neither our applied group sizes, nor the chosen cage change intervals elevate severe fighting rates. The likelihood of injurious aggression seems to further depend on mouse strain and their level of aggressive behaviour (van Loo et al. 2003), which could explain why mice in other studies had a much higher incidence of fighting induced wounds (van Loo et al. 2003). Surprisingly, we observed the less aggressive inbred strain to show more fighting induced wounds than the more aggressive hybrid strain in our study. However, we could not statistically compare their rates due to the low incidence of this behaviour. In summary, all refinement methods which can reduce injuries from aggression should be considered and applied in mouse colonies, as this is the second leading cause of unplanned euthanasia in male mice (Gaskill et al. 2017).

Overall, 20% of the experimental mice showed barbering (fur and whisker trimming) or self-barbering (which we did not discriminate in our study). We found that group size had a significant effect on barbering prevalence: single-housed mice showed almost no self-barbering (4%), pair-housed mice had a barbering prevalence of 11% and group-housed mice had a barbering prevalence of 35%. Given that barbering can be regarded as abnormal behaviour with consequences for animal welfare and research quality (Garner et al. 2004a; Sarna 2000), we would recommend pair housing rather than group housing based on our observations. However, a previous study using a cross-sectional epidemiological survey design (as a result mice varied not only in group size, but also age, sex, strain, breeding status, cage design and stocking density) did not find any correlation in the number of cage mates and the prevalence of

barbering (Garner et al. 2004a) and future studies are required to analyse strain and sex specific biases on barbering behaviour in groups. We could further show that barbered and non-barbered males had similar stress hormone levels and did not differ in their attack rates, which is in line with a previous study, that also found no relationship between barbering and male aggressiveness (Kalueff and Tuohimaa 2005). Thus, barbered mice seem to have no elevated basal stress hormone level, although barbering is painful for the recipient (Sarna 2000) and it is known to occur in a context of social stress (Garner et al. 2004a). It might also be that getting barbered is a process of reducing stress, as *Van de Broek et al.* (1993) showed that barbered mice choose to be barbered, maybe to reduce aggression or to release endorphins. The final function of this behaviour is still not clear and further studies are required on that topic. In our study, B6D2F1 hybrid mice did not differ in their barbering prevalence from C57BL/6N inbred mice, though differences between other strains have revealed that genetic background can influence barbering prevalence (Garner et al. 2004b; Kalueff and Tuohimaa 2005). Furthermore, barbering prevalence can be affected by other factors including sex, age, cage design and position, sibling-only cages and the presence of another barbering group mate (Garner et al. 2004a; Garner et al. 2004b). Interestingly, CCI had no effect on barbering prevalence. We are the first to show that differences in CCI have no influence on male barbering behaviour and this finding further suggests that a more frequent CCI does not impact on animal welfare beyond the short-term aggression effects.

4.2.3. *Co-sleeping as a predictor of animal welfare*

We could show that there was a negative relationship between co-sleeping (or "nest-sharing") and intra-group aggression and stress hormone levels. Thus, co-sleeping can be used as an indicator for inter-male aggression and animal welfare. Another study has reported that co-housed male mice usually sleep in close body contact and always in the same sleeping area (Van Loo et al. 2004), but to our knowledge we are the first to show the direct relationship between co-sleeping and male stress levels and rates of aggression. Separate sleeping occurred in our experiment mainly in groups with high levels of aggression and males from the comparatively more aggressive B6D2F1 hybrid strain co-slept less than the C57BL/6N inbred males. The genetic background seems to strongly influence resting behaviour, whereby more aggressive strains show a higher prevalence of separate sleeping (Mondragón et al. 1987a;

Bisazza 1981b). Interestingly, one study found an influence of social status on male resting behaviour and that only subordinate mice among themselves shared nests (Sandnabba 1997). We did not observe any differences in nest sharing of mice of different social status, but it is an interesting aspect for further research, also regarding dominance determination.

4.3. Plasticity in male sperm traits and reproductive organs

4.3.1. *The role of social status*

We did not find any differences in male sperm traits depending on their social status. This is surprising, as there are several studies showing that social dominance can affect sperm quality and quantity in various species including domestic fowls (*gallus gallus domesticus*) (Froman et al. 2002), arctic char (*salvelinus alpinus*) (Rudolfsen et al. 2006), bluegill sunfish (*lepomis macrochirus*) (Neff 2003), soay sheeps (*ovis aries*) (Preston et al. 2001) and even more closely related species like bank voles (*myodes glareolus*) (Kruczek and Styrna 2009a). In lab mice, it has also been reported that dominant males have higher sperm motility compared to subordinate males (Koyama and Kamimura 1998; Koyama and Kamimura 2000; Koyama and Kamimura 2003). *Koyama and Kamimura* (1998) actively manipulated male social status in applying a "modified resident-intruder tests", whereas we did solely observe the formation of social hierarchies within groups and related male social status to their sperm traits. Thus, it could be that specific factors that helped males to become dominant in our study were not related to their sperm traits. Alternatively, and not mutually exclusive, it could be that the link between dominance and sperm traits in male mice is not as straight forward as assumed. Furthermore we used mice from a different strain than *Koyama and Kamimura* (1998) and different strains are known to vary in their dominance behaviour (Nevison et al. 2000) and also in their sperm traits (Sztein et al. 2000). Subordination in male mice can be associated with a down-regulation of the hypothalamic–pituitary–gonadal (HPG) axis and with decreased testes and seminal vesicles weights (McKinney and Desjardins 1973a; Bronson and Eleftheriou 1964). However, we and other studies could not find any relationship between male social status and relative testes or seminal vesicles mass (Van Loo et al. 2000; van Loo et al. 2001b; Bronson 1973). Furthermore, in our study subordinate and dominant males did not differ in their body mass, which has also been shown in previous experiments (van Loo et al. 2001a; Williamson et al. 2017a; Kaliste-Korhonen and Eskola 2000). In summary, we did not find strong differences

between dominant and subordinate mice in our study. We are confident that our dominance classification was correct as we applied two different protocols to assess male social status, which showed highly consistent results. Thus, one reason why we did not find strong differences between dominant and subordinate males could be that the housing conditions in our experiment, (i.e. moderate group size and CCI, *ad libitum* access to food and sufficient nesting material) did not impose any constraints on subordinate males so that these males did not face a trade-off in which traits to invest and thus could keep up with dominant males (Reznick et al. 2000).

We did not find any difference in male TM levels depending on their social status and the often presumed relation between social rank and testosterone level is controversially discussed (Williamson et al. 2017a). Several studies show that serum or plasma testosterone levels do not differ between dominant and subordinate males (Koyama and Kamimura 1998; Selmanoff et al. 1977; Barnard et al. 1996; Hilakivi et al. 1989; van Loo et al. 2003), whereas other studies found that dominant males tended to have higher testosterone levels (Machida et al. 1981; Ely 1981; Koyama and Kamimura 2000). These studies were performed with different strains, group sizes, cage sizes, and the time spent together varied, as did female presence. In summary, it seems that whether social rank is associated with testosterone levels is context dependent (Williamson et al. 2017a) and further investigation is needed to better understand this relationship. We are the only one who investigated male testosterone levels over an extended period, and we did not observe any changes in their TM levels that would help to understand when and how testosterone affects social hierarchy formation.

4.3.2. *Housing conditions and hormone levels*

We found no effects of CCI on sperm quantity, motility, longevity or swimming velocity. To our knowledge, we are the first to investigate the effects of different cage change regimes on sperm traits. Since cage change has been shown to affect male stress hormone levels (Rasmussen et al. 2011; Gray and Hurst 1995; Van Loo et al. 2000), which in turn can impair sperm production (Arun et al. 2016a; Arun et al. 2016b), we expected to find inferior sperm traits in males of the short CCI group. Surprisingly though, we did not even find any effects of CCI on male CM levels. In addition, other parameters, which are related to animal stress levels like spleen and body mass, were not affected by CCI. Therefore, our data indicate that our

chosen cage change intervals are both in a range where animals can cope with well. Even if cage change leads to some short-term increase in male aggression that might potentially negatively affect the animals, we did not find any evidence that our chosen intervals differentially affect animal reproduction or cause any negative long-term effects on welfare.

Group size affected some but not other sperm traits in our experiment. Single-housed males had lower sperm motility than group-housed mice. This result is in line with sperm competition theory and it could be explained by a strategic up-regulation of sperm traits under a higher risk of sperm competition in co-housed males. Other studies could show that mice under a high risk of sperm competition produced more sperm than mice under a low risk (Ramm et al. 2015; Ramm and Stockley 2009). We did not find an effect of group size on sperm number, swimming velocity or longevity, though we found higher intra-cage variation in sperm number and sperm motility in group-housed compared to pair-housed males, suggesting that more intense sperm competition leads to more variation in sperm traits. Sperm traits are known to depend on testosterone levels, as testosterone is the main factor to stimulate spermatogenesis and sperm maturation (McLachlan et al. 1995; Sharpe 1987). However, the exact pathway has not been explored, and we did not find any correlations between TM levels and sperm number or sperm motility. Interestingly though, TM correlated with sperm velocity, supporting the hypothesis that testosterone plays a key role in the production of high-quality sperm (Liu et al. 2013; Koyama 2004). Similar to other studies we could not show an effect of group size on TM levels (van Loo et al. 2001b; Williamson et al. 2017a), which is somehow surprising, as single-housed mice are usually dominant (Brain 1975b) and thus should have on average higher testosterone levels than co-housed mice (van Loo et al. 2001b). However, as already mentioned before, a general correlation between testosterone and social rank is doubtful and further studies are required to better understand this relationship.

Male testes mass was bigger in group-housed than in pair-housed mice. This result could potentially also be explained by an adaption to a higher risk of sperm competition since *Ramm et al.* found that mice can increase testis mass by 12 % when exposed to a high sperm competition treatment (Ramm et al. 2015). However, in order to concur with sperm competition theory, single-housed mice from our study should have had comparatively smaller testes, which was not the case. On the other hand, testis size has been shown to depend on individual stress

levels and males with chronic stress are known to have smaller reproductive organs (Arun et al. 2016a; Arun et al. 2016b). Thus, it could be that we did not find a difference in male testes mass between single and co-housed males, as single-housed males could grow larger testes due to their lower stress levels. In line with this argument, we found seminal vesicles mass to negatively correlate with CM levels and the less stressed single-housed mice had bigger seminal vesicles than co-housed mice.

4.3.3. *Male genetic background*

We found that C57BL/6N inbred males tended to have more, but slower sperm. This is surprising, because B6D2F1 hybrid mice had bigger testes and testes size is known to positively correlate with sperm number (Chubb 1992; Hunt and Mittwoch 1987). However, testes size is not the only factor influencing sperm number, as sperm number can further depend on the efficiency and density of the sperm-producing tissue (i.e. seminiferous tubuli) (Firman et al. 2015). Genetic differences in sperm production efficiency could also be responsible for the lower sperm number in hybrid mice despite their greater testes mass. An alternative and not mutually exclusive explanation for the lower sperm quantity in hybrid males could be the consequences of epididymal sperm extraction: a study in boars found a heterosis effect in sperm number, but only in the caput epididymis, whereas the sperm number in the caudae epididymis was lower compared to the parental strain (Neely et al. 1980). The authors from this study suggested that crossbred boars were eliminating more sperm either through increased frequency of masturbation or increased sperm output per masturbation. Similar effects could also account for our findings. To our knowledge, masturbation (or "inter-male copulatory-like behaviour" or "mounting") is known (Sarna 2000) but barely described in mice, though such behaviours were often observed in our experiment and should therefore be mentioned in future research. Male genetic background had no influence on sperm motility or longevity, which is surprising, as hybrids are known to be more fertile (Sztein et al. 2000) and we expected those males to show superior sperm traits due to hybrid vigour. In summary, hybrid mice have faster sperm, but sperm motility and longevity were not influenced by hybrid vigour and sperm number actually tended to be lower. Thus, the higher fertility rates of hybrid mice (Sztein et al. 2000) are likely be explained by their increased sperm velocity, or other sperm traits, which we have not investigated, like sperm fertilization ability.

B6D2F1 hybrid males were generally bigger had a greater relative testes mass and higher TM levels compared to C57BL/6N inbred males. In turn, C57BL/6N inbred males had a bigger seminal vesicle mass. The heterosis effect is known to have influence on body and organ weights in mice and our results are in line with another study that showed that hybrid mice are heavier, have bigger testes and smaller vesicle glands compared to their parental strain (Gregorová et al. 1977). The lower seminal vesicles mass in hybrid males can be explained by a possible trade-off between testes and seminal vesicles mass, or, as discussed before, by the theory of a more frequent emptying of seminal fluid or increased output in the hybrid strain through masturbation/mounting. As previously mentioned, the mean faecal TM levels were also higher in B6D2F1 hybrid males than in C57BL/6N inbred males, probably because hybrid mice have bigger testes and testes size correlates positively with androgen levels (Francois et al. 1990; Carlier et al. 1990; McKinney and Desjardins 1973b).

4.3.4. *Male barbering behaviour*

Interestingly, we could show that barbered and non-barbered mice differed in some, but not all parameters of reproduction. Barbered mice had lower sperm quantity compared to non-barbered mice, whereas sperm motility and longevity was not affected by the prevalence of barbering. Interestingly, we found that faster sperm swimming velocity accompanied lower sperm number, indicating that there is a tread-off in sperm quality parameters in barbered mice (Reznick et al. 2000). Furthermore, barbered mice had a significantly lower relative seminal vesicle mass, suggesting that the stress associated with barbering can affect the size of reproductive organs. Thus, barbered mice should only be used with caution when used in studies related to reproduction. It has already been suggested that barbered mice are unrepresentative of "normal" mice in behavioural research, due to changes in brain function and the somatosensory cortex (Sarna 2000; Garner et al. 2004b), but we provide the first linkage between the incidence of male barbering and their reproductive traits.

5. Conclusion

5.1. Determination of dominance hierarchy

For social status determination in group-housed male mice, we recommend post cage change observations over evening observations. The results of both protocols differed just marginally, but considerably more agonistic behaviours were recorded in a shorter period and observations during the dark phase were more challenging and error prone. The barbering score did not proof as a reliable method to determine male social status. First, the prevalence of barbering was low and in some cases all mice within a group showed an equal level of barbering; second, the results of barbering marginally suited the results of the behavioural observations. However, further research should compare additional methods (tube test, resident intruder test, territory urine marking, etc.) to validate or disqualify the use of barbering as a reliable method to assess dominance behaviour.

5.2. Strain and housing effects on animal welfare

B6D2F1 hybrid males showed significantly more aggressive behaviour than C57BL/6N inbred males. A more frequent CCI did not cause higher aggression or stress levels over the course of the experiment, even though the manipulation lead to a short peak of aggressive behaviour. We would therefore recommend applying a longer cage change interval. Short-term individual housing seemed to have no negative impact on mice' welfare, as single-housed males even showed lower stress levels (measured by their faecal corticosterone level, their barbering behaviour and their spleen size) compared to co-housed males. Pair-housed and group-housed mice did not differ in these measurements, or in terms of the level of intra-group aggression, possibly as both group sizes favor the formation of stable social hierarchies. Thus, we can recommend both group sizes and individual housing as a short-term alternative in highly aggressive groups. Co-sleeping was associated with lower stress hormone and aggression levels and could thus be further used as an easy to observe and reliable indicator of animal welfare.

5.3. Social housing effects on male reproduction

Dominant and subordinate mice differed neither in sperm quality traits, male testosterone levels, nor the size of their reproductive organs. Thus, the conventional housing conditions we applied potentially allowed subordinate males to compensate and keep up with dominant males in terms

of reproductive traits. Housing males under more ecologically relevant conditions for mice (e.g. bigger territories, limited access to food, natural parasite burdens, confrontation with intruders etc.) would likely enforce differences in reproductive traits. Surprisingly, hybrid males only showed faster swimming sperm compared to inbred mice and did not show any other superiority in reproduction traits. Thus, the well-known heterosis effect was only small in our study. Pair-housed and group-housed mice did not differ in their sperm traits, though single-housed mice had lower sperm motility and higher seminal vesicles mass, likely explained by the missing sperm competition risk and the lower stress level of single-housed males. The CCI had no effect on sperm traits or reproductive organ weights, suggesting that the cage change intervals that we have chosen are moderate and do not influence male reproductive traits. Barbered males differ significantly from non-barbered males in some reproduction traits. Thus, like in other regards, barbered mice should not be used in studies on animal reproduction.

6. Zusammenfassung

Ziel des Versuches war es die Auswirkungen des sozialen Status und der Haltungsbedingungen von männlichen Mäusen, explizit die Gruppengröße und das Intervall des Käfigwechselns, auf das Wohlbefinden der Tiere und ihre Spermienparameter in zwei Mausstämmen zu beobachten. Wir konnten zeigen, dass eine Beobachtung des agonistischen Verhaltens direkt anschließend an das Käfigwechseln schnell und zuverlässig Informationen über die Hierarchie innerhalb einer Gruppe liefert. Dem entgegengestellt eignet sich das „barbering"-Verhalten (das Trimmen von Fell und Tasthaaren) kaum zum Bestimmen der Dominanz, aber als Kriterium zur Evaluierung des Wohlbefindens der Tiere.

Männliche B6D2F1 Hybridmäuse waren deutlich aggressiver als C57BL/6N Inzuchtmäuse. Die unterschiedliche Gruppengröße und das unterschiedlich lange Käfigwechselintervall hatten keine Auswirkungen auf das langfristige Aggressionsverhalten und den Stresslevel der Tiere. Allerdings zeigten die Mäuse am Tag des Käfigwechsels ein höheres Aggressionslevel und diese kurzfristigen Anstiege sollten durch ein längeres Intervall minimiert werden. Allein gehaltene Tiere hatten ein geringeres Stresshormonlevel, zeigten weniger häufig „barbering" und kleinere Milzen (ebenfalls ein Merkmal für Stress). Trotzdem wird Einzelhaltung, auch in der Literatur, nicht generell empfohlen, sondern nur als Alternative im Falle, dass Kämpfe innerhalb einer Gruppe zu Wunden führen. Des Weiteren konnten wir erstmals zeigen, dass Gruppen die wenig bis kein Aggressionsverhalten und ein niedrigeres Stresshormonlevel zeigen häufiger gemeinsam als Gruppe schlafen. Damit kann häufiges Zusammenschlafen als Merkmal für Wohlbefinden gesehen werden.

Männliche Säugetiere zeigen häufig auch innerhalb einer Spezies große Schwankungen bezüglich ihres Reproduktionserfolgs und Spermienparameter, wobei die beeinflussenden Faktoren und physiologischen Abläufe kaum erforscht sind. Eine Auswirkung des sozialen Status der Tiere auf die Spermienparameter, das Testosteronlevel, sowie die Größe der Reproduktionsorgane konnten wir in unseren Mausstämmen nicht bestätigen. Auch das Käfigwechselintervall und die unterschiedliche Gruppengröße hatten keine Auswirkungen. Männchen in Einzelhaltung hatten allerdings eine geringere Anzahl motiler Spermien und stärker gefüllte Samenblasen, was einerseits auf das fehlende Konkurrenzverhalten („sperm competition risk") und andererseits auf ein niedrigeres Stresslevel zurückzuführen ist.

Hybridtiere und Inzuchttiere unterschieden sich erwartungsgemäß in vielen Parametern, zum einen aufgrund des genetischen Hintergrunds, zum anderen aufgrund des Heterosis-Effekts. Interessanterweise zeigten Tiere mit „barbering“ deutliche Unterschiede in Spermienparametern und sogar der Größe der Samenblase. Tiere, die „barbering“ zeigen, sollten bei diesbezüglichen Studien ausgeschlossen werden, da sie nicht die Gesamtpopulation repräsentieren.

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