Scandinavian Journal of Laboratory Animal Science

2016, Volume 42, Number 7
ISSN 2002-0112

Who is a compatible partner for a male mouse?

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Summary

The complex issue of social housing of laboratory mice was addressed by studying the housing compatibility of male mice with castrated males, or with ovariectomized females, for a period of up to seven weeks. Sexually mature males were shown to be socially incompatible with castrated males of the same age but to be more compatible with same age or older ovariectomized females (results varied according to the mouse strains used). These ovariectomized females could also be repeatedly housed with different sets of younger male mice, even after being briefly separated and again re-paired. Our data suggest that ovariectomized females could be used to establish a long-term companion group fully compatible for male mice group housing.

INTRODUCTION

Unless scientifically or medically justified, socially compatible animals should be housed in groups in accordance with legal requirements and AAALAC guidance (*Directive 2010/63/EU, 2010; National Research Council (US), 2011*). While most laboratory animals can be group housed, several studies have shown that group housing of male mice may result in systemic stress and produce biased research (*Karolewicz et al., 2001; Van Loo et al., 2001; Spani et al., 2003; Voikar et al., 2005; Rettich et al., 2006; Nicholson et al., 2009; Meakin et al., 2013*). This social incompatibility is also present between wild male mice but not observed in males paired with females (*Palanza et al. 2001*). Diverse strategies have been implemented to successfully address this challenge, varying from different group formation to housing and environmental enrichment provisions (*Van Loo et al., 2001; Emond et al., 2003; Van Loo et al., 2003; Vaughan et al., 2014*).

In order to identify adequate social partners for group housing of male mice, we individually paired sexually mature male mice with either a castrated male or an ovariectomized female and observed for signs of aggressive behavior among the pairings for up to 7 weeks.

MATERIAL AND METHODS

Animals and housing

The experimental protocol was approved by the regional animal welfare committee according to Swedish regulatory requirements (Gothenburg Ethical Review Board number 156-2014).

Altogether 50 intact males, 10 castrated males and 20 ovariectomized female mice were used in the experimental study. Male mice from two different strains were chosen: C57BL/6N (Charles River, Germany) due to their common use in animal exper-

iments and a gene manipulated in-house strain B6-GT(Rosa)26Sor$^{tm22(OS\ Luc\ Reporter)Aztc}$ (abbreviated to B6-Luc WT, Astrazenca) which exhibits accentuated aggressive behavior. Castration of ten 4 week-old B6-Luc WT mice was performed at our surgical facilities under isoflurane anaesthesia through a small midline abdominal incision. Ketoprofen (Comforion Vet® - 3mg/kg) was used for pain relief on the day of surgery and thereafter for as long as required. After the surgery, the mice were left to recover for a minimum of five days before the experiment.

The females used were C57BL/6J (Charles River, France) and BALB/cJ female (Charles River, France), ovariectomized at the age of three weeks by the vendor and transported 24 hours later.

Prior to the experiment, all experimental male and female mice were distributed and housed in groups of five of the same strain and sex in open top cages (425x265x150mm; Macrolon Eurostandard type 3.0, Tecniplast, Italy). All mice, either transported from vendors or transferred from our breeding facility, were moved for acclimatization to the experimental room one week prior to the start of the experimental study.

Cages used during both the acclimatization period and experimental study contained hardwood bedding (J. Rettenmaier & Söhne GMBH, Germany) with nesting material (Ancare, USA), shredded paper (Papyrus AB, Sweden), gnawing sticks (Tapvei, Estonia), and a shelter (LBSServing Biotechnology, United Kingdom). Pelleted feed (R70, Lantmännen, Sweden) and water were available *ad libitum*. Cages and cage lids were changed weekly and nests were partly (if soiled) or completely (if unsoiled) moved to the new cage supplemented with new nesting material as required. The temperature in the animal room was between 20°C and 23°C, relative humidity from 40% to 60% with 20 air changes/hour. The light/dark cycle had a duration of 12 hours with the light starting at 7AM.

Experimental design

After the adaptation period, each mouse was randomly chosen and assigned into one of six different types of pair groups (Figure 1). Initial mice pairings (five cages per pair combination) were initiated when the sexually mature males were five weeks old and assigned as:

1. C57BL/6N male paired with castrated B6-Luc WT male (five weeks old)
2. B6-Luc WT male paired with castrated B6-Luc WT male (five weeks old)
3. C57BL/6N male paired with ovariectomized C57BL/6N female (five weeks old)
4. B6-Luc WT male paired with ovariectomized C57BL/6N female (five weeks old)
5. C57BL/6N male paired with ovariectomized BALB/cJ female (four weeks old)
6. B6-Luc WT male paired with ovariectomized BALB/cJ female (four weeks old)

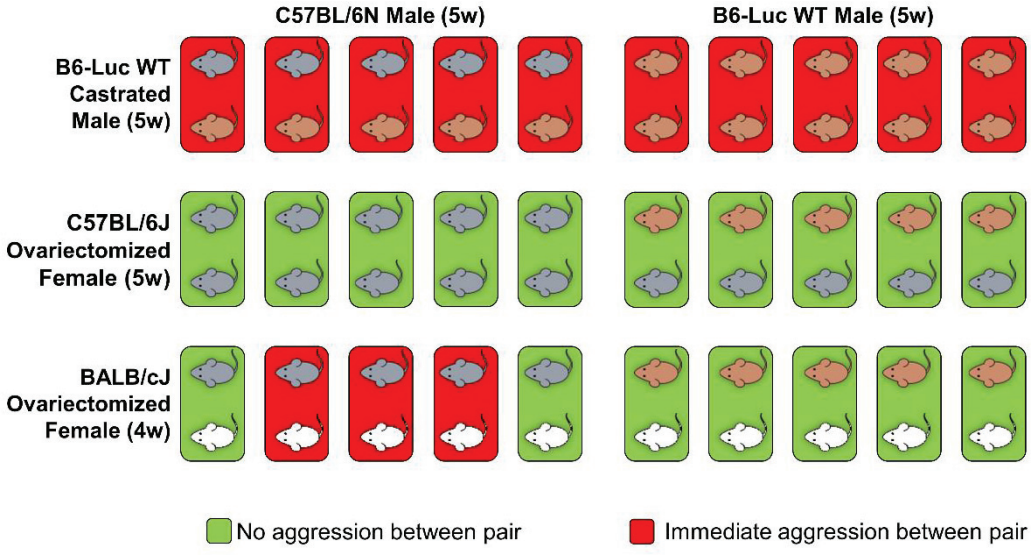

Figure 1. Assessment of aggressive behavior among pairs of sexually mature male mice and castrated males and ovariectomized females. Visual assessment of aggression between pairings consisting of one C57BL/6N or B6-Luc WT male mouse with either one castrated male B6-Luc WT or one ovariectomized C57BL/6J or BALB/cJ female of similar age or older.

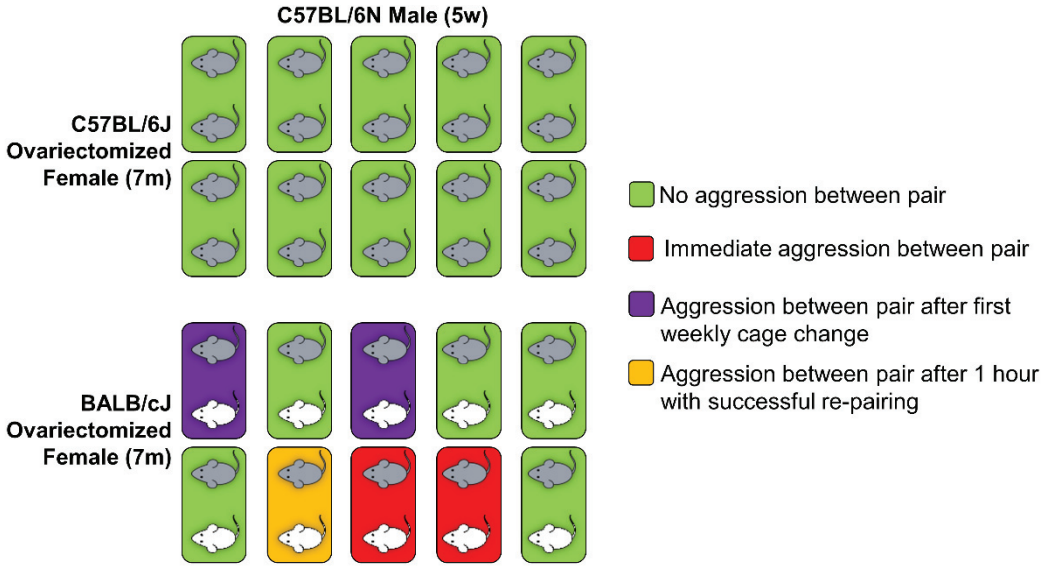

Figure 2. Assessment of aggressive behavior among pairs of sexually mature male mice and older ovariectomized females. Visual assessment of aggression between pairings consisting of one C57BL/6N male mouse with either one ovariectomized C57BL/6J or ovariectomized BALB/cJ female (7 months old).

The second set of the pairings were started when the previously paired ovariectomized females were seven months old (Figure 2) and re-paired with new five weeks old intact male mice (10 cages per pairing combination):

1. C57BL/6N male with ovariectomized C57BL/6N female (seven months)
2. C57BL/6N male with ovariectomized BALB/cJ female (seven months)

Animal behavior and statistical analyses

Live visual assessment of aggressive behavior of the newly formed pair was performed for 20 minutes post-pairing, re-evaluated three hours later and weekly before cage changes, always by the same observer, for a period of seven weeks. Observations focused on the presence of aggressive engagement such as threatening postures, chasing or biting between the two paired mice.

Binomial logistic regression was run on the presence or absence of aggression in individual cage pairings with Firth bias-adjusted estimates in JMP 11 (JMP statistical software, SAS) using a simple two factor model to test whether mice were paired with the same or different strain and whether their partner was a castrated male or ovariectomized female.

RESULTS

In all intact male/castrated male pairings a strong display of aggressive behavior (*e.g.* chasing, biting) was observed within the first 20 minutes, always initiated by the intact male and independent of the genetic background ($\chi 2 = 0.89$; $P = 0.35$). Castrated B6-Luc WT mice were immediately removed due to serious fighting and, when housed together in their pre-experimental groups, showed no aggressive behavior against the other co-housed castrated males for the duration of the study.

Aggression was not observed in either strain of intact males paired with ovariectomized C57BL/6J females or between intact B6-Luc WT males and ovariectomized BALB/cJ females (Figure 1). Our analysis showed that being housed with a castrated female regardless of strain reduced the likelihood of being separated due to aggression by 76% (F = 21.4%; M = 97.3%; $\chi 2 = 23.8$; $P < 0.001$).

In most cases initial contacts between intact males and ovariectomized females were exploratory with friendly sniffing and followed either by mating attempts or chasing (if the female would not readily mate). Between intact C57BL/6N males and ovariectomized BALB/cJ females, aggression was observed in three out of five pairs within the initial assessment period which forced the separation of the pair.

Based on these observations we further explored the possibility of using ovariectomized females as a companion to be housed throughout their life with different sets of male mouse partners. We paired older female ovariectomized C57BL/6J or ovariectomized BALB/cJ (previously paired with intact males when they were 4 or 5 weeks old) with younger intact

C57BL/6N mice and assessed for aggression (Figure 2). Data were analyzed by binomial logistic regression with Firth bias-adjusted estimates to test whether being housed with a castrated female of the same strain affected the likelihood of being separated. We found that if an intact male C57BL/6J is housed with another ovariectomized female C57BL/6 mouse this reduces the likelihood of being separated by approximately 45% compared with being housed with an ovariectomized female BALB/cJ mouse (C57BL/6J = 4.5% and BALB/cJ = 50%; $\chi 2 = 7.71$; $P = 0.005$). No aggression was observed between the intact males and ovariectomized C57BL/6J females. However, pairing with BALB/cJ females led to aggression in five of the pairings. In three cages, aggression was immediately observed and initiated by the older female (one female was successfully re-paired with a different intact male subsequently). In the remaining two pairs aggression followed the first weekly cage change. After seven weeks, the pairs of older ovariectomized C57BL/6J females and male mice were separated by removing the male to an adjacent new cage for a period of five days to simulate a scenario of animal surgery requiring isolated recovery. The cage housing of the pair was left unchanged during this separation and males were subsequently re-introduced in the same pairs. In all ten cases, aggression was not observed after separation and re-pairing for a subsequent period of five days. This data could not be analyzed statistically due to lack of variability.

DISCUSSION

Overall, our results show that co-housing of intact male mice with castrated males is infeasible and that pairing with similar-age or older ovariectomized females is more successful, although with some variation in aggression based on strain background. Moreover, successful pairings allowed for short-term separation and re-introduction, useful in studies that require isolation due to experimental design. However, the proposed co-housing approach might lead not only to an increase in experimental animal numbers but also the need of surgical intervention to generate ovariectomized females. While seemingly contradicting the 3Rs principles for animal experimentation, we believe that it provides a valid alternative for research scientists and Animal Welfare and Ethical Review Bodies in very specific scenarios for which the animal's welfare gained by social housing of male mice might be considered to outweigh these issues. Additional data are needed to assess the experimental impact of co-housing of male mice with ovariectomized females within our own experimental animal models following current published recommendations *(Van Loo et al., 2001; Spani et al., 2003; Voikar et al., 2005; Nicholson et al., 2009*).

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