

## Inbreeding does not reduce aggressiveness in wild male mice, *Mus musculus*

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**A b s t r a c t.** Inbreeding reduces quality and survival of offspring due to increased homozygosity and the expression of recessive deleterious mutations. However, there are only few studies examining how inbreeding affects behavior in adults. We aimed to replicate an earlier study in wild house mice by inducing a stress factor – infection with *Salmonella*. To examine whether less inbred males are more aggressive and have a higher probability to win brief encounters, we confronted full-sib inbred and outbred males in a neutral arena and recorded aggressive as well as defensive behaviors. Contrary to our expectations, any effects of inbreeding on aggressive and defensive behaviors were not dependent on infection status. Furthermore, neither infection treatment nor inbreeding affected the amount of aggressive and defensive behaviors displayed by males. Short-term aggression assays may be a useful tool for investigating certain aspects of aggressive behavior; however, long-term aggression assays might be more suitable to monitor all aspects of competitive ability and antagonistic interactions as well as effects of certain treatments on competitive ability and aggressiveness. These results may have important implications for opposed selection pressures arising from female choice and male-male competition.

**Key words:** aggression, *Salmonella*, wild house mouse

### Introduction

Inbreeding causes reduced fitness due to increasing homozygosity and the expression of deleterious recessive alleles (“inbreeding depression”; Charlesworth & Charlesworth 1987, 1999). Inbreeding reduces the quality and survival of young (Britten 1996, Hansson & Westerberg 2002, Wang et al. 2002, Reed & Frankham 2003), though there are surprisingly few studies on how inbreeding affects behavior or other traits during the adult part of life cycle (but see Meagher et al. 2000, Charpentier et al. 2008). The detrimental effects of inbreeding are usually greater in stressful conditions than in the laboratory, indicating that inbreeding depression needs to be measured under realistic ecological conditions (Miller 1994, Meagher et al. 2000, Keller et al. 2002, Keller & Waller 2002, Armbruster & Reed 2005, Marr et al. 2006). For example, a large study designed to measure the fitness consequences of close inbreeding (from full sib matings) in wild-derived mice (*Mus domesticus*) found a large (58%) fitness decline among mice living in seminatural enclosures, whereas the laboratory controls showed a relatively small (20%) effect (Meagher et al. 2000). Inbred males had lower survival than outbred ones in the enclosures, but sexual selection also played a role: in the enclosures, inbred males were less likely to obtain and defend territories than outbred males, which significantly reduced their reproductive success. In a follow-up study, it was shown that even moderate inbreeding (from first cousin matings) results in a significant reduction of male reproductive success (Ilmonen et al. 2008). It is unclear how inbreeding

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reduces males' competitive ability, and our aim was to determine whether inbreeding reduces males' aggressive behavior during dyadic interactions with unfamiliar males.

In house mice (*Mus domesticus*), males compete over territories through aggressive interactions and competitive scent-marking (Rich & Hurst 1998, 1999, Gosling & Roberts 2001, Hurst & Beynon 2004) and dominant, territorial males achieve more matings (Horn 1974, Kuse & DeFries 1976, Singleton & Hay 1983, but see Qvarnström & Forsgren 1998) as well as more access to food and shelter than subordinates (Crowcroft & Rowe 1962, Bronson 1979, Miczek et al. 2001). It is often assumed that males achieve social dominance and territorial success due to being more aggressive, and Eklund (1996) found that wild-derived male mice (*Mus domesticus*) are more likely to win male-male encounters when competing against inbred males. In her study, males of different inbreeding level competed in brief, agonistic encounters and were scored for various aggressive and defensive behaviors. According to these scores, males were determined winner or loser of the interaction. Eklund (l. c.) showed that less inbred males are more aggressive and have a higher probability to win short encounters. This finding could explain why inbred males are less able to become socially dominant (Meagher et al. 2000), but while such brief assays are useful for measuring aggressiveness, it is unclear whether they can predict a males' ability to become socially dominant in a more natural and complex social setting. Cairns and coworkers (1983) found that mouse lines selected for high and low male aggression cease to differ in aggression scores after repeated testing. Moreover, fighting has high fitness costs, and being more aggressive does not necessarily increase social status or fitness (Marler & Moore 1988). A variety of studies find that inbreeding reduces male competitive ability and mating success, but Eklund (1996) is one of few studies to show that inbreeding reduces male aggression.

Our aim here was to replicate Eklund's (1996) finding that outbred males are indeed better able to win short-term agonistic encounters than inbred males in wild-derived male mice (*Mus musculus*). Replication is an important aspect of science, though surprisingly rare in behavioral ecology (Kelly 2006); however, true replication of the Eklund (1996) study is impossible due to ambiguity in the methods (e.g., the sample size and the number of interactions for each male are unclear, and each male was tested between one and five times). This study is an attempt to partially replicate Eklund's study, avoid pseudo-replication and employing clear statistical analyses. To measure the aggressiveness of inbred versus outbred males, we used an agonistic behavior assay conducted in a plastic testing arena that was divided into two equal-sized compartments by a removable divider. We assigned males to pairs and observed male-male interactions for ten minutes while scoring aggressive and defensive behaviors. Each male pair was tested twice to ensure a consistent outcome of the dominance interaction. Repeatability was calculated by the number of male pairs with inconsistent outcome in both trials divided by the number of male pairs for which data of both trails was available multiplied by one hundred. This value was subtracted from one hundred. In our study, repeatability was 85%. We attempted to make our laboratory experiment more ecologically realistic by introducing a stress treatment. It is possible that the detrimental effects of inbreeding only become apparent after males are challenged with a stressor such as infection and indeed infection can magnify the harmful effects of inbreeding in wild house mice (Ilmonen et al. 2008) and other species (O'Brien & Evermann 1988, Coltman et al. 1999, Keller & Waller 2002, Spielman et al. 2004). Therefore, before competing males in the agonistic

behavior assay, we experimentally infected half of the male pairs with an avirulent pathogen (*Salmonella enterica*) and sham-infected the other half as controls. This treatment allowed us to determine whether infection magnifies any differences between inbred versus outbred males. Contrary to Eklund (1996), we found no evidence that inbreeding influences males' ability to win short-term aggressive interactions, regardless of interacting inbred and outbred males are both healthy or both stressed with an experimental infection.

## Material and Methods

### Animals and housing

We trapped wild house mice (*Mus musculus*) near Vienna, Austria and bred the F2 generation to produce full-sib inbred males (sister-brother mating; Wright's inbreeding coefficient:  $f = 0.25$ ) and outbred males (matings between non-relatives:  $f = 0.00$ ). Mice were weaned at 21 days of age and experimental males were housed singly in acrylic cages. The cages contained pine bedding and wood-wool for environmental enrichment. The mice were kept under a 12:12 h dark:light cycle and provided with food (Altromin rodent diet 1324) and water *ad libitum*. Males were individually marked with distinctive fur-cuts on their back one day prior to the interaction.

### Infection procedure

In total, 52 inbred and 52 outbred male mice were grouped into dyads consisting each of one inbred and one outbred male. Males were matched for age and weight. The dyads were randomly assigned to infection or sham-treatment group. The males in the infection group (26 dyads) were orally infected with *Salmonella enterica* serovar Typhimurium; strain SRI-11 (30  $\mu$ l,  $10^7$  colony forming units per ml) (Mittrecker & Kaufmann 2000, Humphries et al. 2005), whereas the males in the sham-treatment group received the same volume of phosphate-buffered saline. Males were restricted from food and water four hours prior to inoculation to rule out variation in systemic infection due to variation in food in the gut. Male-male interaction experiments were carried out eleven days post-inoculation.

### Competitive, agonistic behavior assay

To test the hypothesis that outbred males win short-term encounters more often than inbred males, we used a plastic testing arena (100 x 60 x 60 cm) that was divided into two equal-sized compartments by a removable opaque plastic divider. At the beginning of the trial, the dyads were introduced into the testing arena with one male in each of the compartments. The males were allowed to habituate for five minutes. Then, the opaque divider was removed and males could interact for 10 minutes. Interactions were videotaped using a video camera installed above the testing arena. The males were separated as soon as one mouse got seriously injured including repeated attacks towards the head or visible wounds. At the end of the trial, the males were separated and returned to their home cages. After four hours the trial was repeated for each dyad.

We analyzed the last five minutes of the males' interactions using OBSERVER XT (Noldus Information Technology, The Netherlands). We incorporated only the second half

of the interaction into the analysis, since then the mice might have already established a social dominance hierarchy. We scored standard aggressive behaviors including attacking, engaging in a fight, and chasing the opponent for both males. Fleeing from an opponent was scored as defensive behavior. Additionally, we recorded the time the males spent exploring the test arena, allo-grooming or staying immobile (Table 1, all behaviors as defined in *Benus et al. 1992*). According to these scores, males were determined to win or lose the interaction. A winner of an interaction scored more than twice as much aggressive behavior as his opponent that was then determined to have lost this interaction. If both males exhibited approximately the same number of aggressive behavior, this interaction was considered a draw. We incorporated only those dyads into the further analysis in which both trials were consistent in their outcome or the outcome of the first interaction was unclear, but the second interaction resulted in a clear dominance relationship. Therefore, 5 dyads were excluded from the analysis. Another 21 dyads were excluded from further analysis because one or both experimental males died before the trial or because of missing videos.

### Statistical analysis

We used Spearman's rho Correlation test to determine correlations in between different male behaviors. We used Multivariate General Linear Model to test whether the male infection treatment influenced the effects of inbreeding on aggressive and defensive behaviors. We used Wilcoxon signed rank test to estimate the influence of inbreeding on aggressive and defensive behaviors as well as the time spent immobile. Furthermore, we used Mann-Whitney test to investigate effects of infection on aggressive and defensive behaviors as well as the time spent immobile. Statistical significance values were set to  $p = 0.05$ .

**Table 1.** Description of aggressive, defensive and other behavior scored during male encounters.

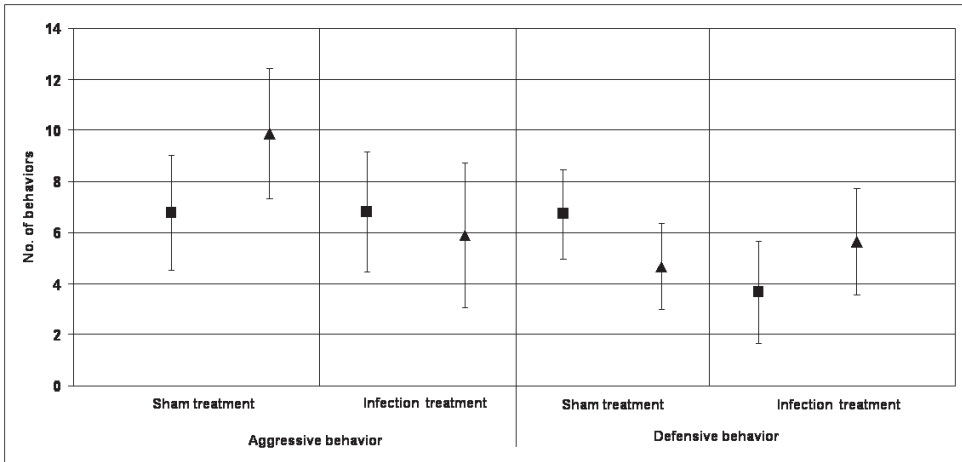
<b>Behavior</b>	<b>Description</b>
<b>Aggressive behavior</b>	
Attack	Rushing or leaping at the opponent with kicks and bites
Chase	Pursuing fleeing opponent
Fight	Both mice tumbling over eachother accompanied by squeaking, kicking and biting
<b>Defensive behavior</b>	
Flight	Rapid movement away form the opponent with sudden changes in direction
<b>Other behavior</b>	
Grooming	Wiping, licking and nibbeling the fur with forepaws and mouth
Exploration	Locomotion, no high speed, no apparent direction
Immobility	Absence of any movement

## Results

The number of aggressive and defensive behaviors exhibited by a male and the time a male spent immobile were highly correlated. The number of aggressive behaviors exhibited by a male in the encounter was negatively correlated with the number of defensive behaviors shown ( $N = 52$ , Spearman's rho, 2-tailed,  $\rho = -0.77$ ,  $p < 0.001$ ). Furthermore, the number of aggressive behavior displayed during an encounter was also negatively correlated with the time males spent immobile ( $\rho = -0.67$ ,  $p < 0.001$ ). As expected, the number of defensive behaviors exhibited by a male during the interaction was positively correlated with the time a male spent immobile ( $\rho = 0.84$ ,  $p < 0.001$ ).

Any effects of inbreeding on aggressive and defensive behaviors were not dependent on infection status. Neither inbreeding or infection alone, nor their interaction calculated for aggressive and defensive behaviors reached significance level ( $N = 26$ , Multivariate GLM, for all measured behaviors,  $df = 1$ ,  $p > 0.05$ ).

We found no evidence that inbreeding affected the amount of aggressive behaviors ( $N = 26$ , Wilcoxon signed rank,  $Z = -0.72$ ,  $p = 0.47$ ; Fig. 1) and defensive behaviors ( $Z = -0.29$ ,  $p = 0.77$ ; Fig. 1) displayed by males. Furthermore, inbreeding had no effect on freezing duration ( $Z = -0.93$ ,  $p = 0.36$ ). Similarly, infection treatment did not affect the amount of aggressive behavior ( $N = 26$ , Mann-Whitney test,  $Z = -0.29$ ,  $p = 0.77$ ; Fig. 1) and defensive behavior ( $Z = -0.06$ ,  $p = 0.95$ ; Fig. 1) displayed by males during male-male encounters. Additionally, infection had no influence on freezing duration ( $Z = -0.62$ ,  $p = 0.54$ ).



**Fig. 1.** Number of aggressive (left hand side) and defensive (right hand side) behaviors shown by males during five minutes of male-male encounters (mean  $\pm$  SE). In each encounter, both males were either sham treated or infected with *Salmonella*. Inbred males are indicated with squares, outbred males are indicated with triangles.

## Discussion

We found no evidence that inbreeding reduced males' ability to win brief, agonistic encounters, regardless of whether both males were infected with *Salmonella* or not. Therefore, our results do not support the hypothesis that the reduced territorial success of inbred mice (M e a g h e r et al. 2000) is due to reduced aggression or ability to win brief, agonistic encounters (E k l u n d 1996). The reason that inbreeding reduces males' ability

to obtain and hold territories in population enclosures is most likely due to inbred males having poorer health and disease resistance compared to outbred males (I l m o n e n et al. 2008), which need not predict aggressiveness during brief male-male encounters. The number of aggressive and defensive behaviors as well as time a male spend immobile was inter-correlated. According to our expectations, the number of aggressive behavior was negatively correlated with the number of defensive behaviors and the time spent immobile. Furthermore, the amount of defensive behaviors was positively correlated with the time a male spent immobile. This result suggests that all behaviors measured in this study account of the same character feature of a male and that a highly aggressive male on average displays significantly less defensive behaviors. Inbreeding has been shown to affect males' ability to dominate other males and maintain a territory (M e a g h e r et al. 2000) and might influence males' stamina in general. Furthermore, condition-dependent life history traits are expected to be especially vulnerable to inbreeding, since male's overall condition and health is influenced by multiple genes, which provide a large mutational target. However, we found no evidence that full-sib inbreeding had an effect on males' competitive ability although statistically full-sib inbred males were homozygous at every fourth loci (W r i g h t 1922, M i t t o n 1994). Contrary to earlier findings (I l m o n e n et al. 2008), we found no evidence that *Salmonella* infection magnified the harmful effects of inbreeding, but this is probably because in this study we were not able to find any negative impact of inbreeding on *Salmonella* resistance.

In this study, we tried to partially replicate a study by E k l u n d (1996) that showed that inbreeding negatively impacts aggression in wild house mice (*Mus domesticus*). Surprisingly, we found no evidence that full-sib inbreeding affects aggression scores in experimental males (*Mus musculus*). There are some differences in the experimental setup that might have caused these discrepancies. First, our experimental mice were grouped into pairs in our study and male-male interactions were only carried out within the pair. In E k l u n d ' s study (1996), male were tested between one and five times with varying partners. Repeated tests of the same male is pseudo-replication if not statistically controlled, and differences could be due to the "winner-effect", i.e., an increased probability of winning an aggressive encounter following previous victories (D u g a t k i n 1997). Winning encounters against intruding males may increase the probability of winning future encounters, as has been shown in deer mice (*Peromyscus californicus*) (O y e g b i l e & M a r l e r 2005). Second, in our study male pairs were tested twice to ensure a consistent dominance relationship which might not be apparent in one short encounter. Therefore, we used a conservative approach providing a more reliable picture of social dominance hierarchies. Third, we employed a much larger testing arena than E k l u n d (1996) leaving an opportunity for less aggressive males to escape repeated attacks of highly aggressive males in the short term. Finally, we videotaped all interactions allowing more detailed observations of male-male interactions which were impossible using direct observations, as in E k l u n d ' s study (1996).

The standard test for measuring aggressive behavior is the resident-intruder-paradigm, where resident males encounter different types of intruders in either a home cage or a neutral arena. This facilitates clear measures of aggressive behavior by eliminating undesirable side effects (T h u r m o n d 1975). In our study, we confronted two residents in a neutral arena and explored differences in aggression due to differing inbreeding levels. The need to perform repeated aggression tests has been suggested earlier (M i c z e k et al. 2001) and is highlighted by a study on highly aggressive  $\beta$ -estrogen receptor knock-out mice showing that wild-type mice increase their level of aggressiveness when repeatedly confronted with highly aggressive knock-out mice (O g a w a et al. 1998). One of the most common measurements

of aggressiveness is latency to attack, although this does not correlate with other measures of aggression (Miczek et al. 2001). A male with shorter attack latency exhibits more aggressive behaviors and is determined as winner of the dominance interaction. However, there is no evidence that winning short-term encounters and having short attack latency increases reproductive success (Oakeshott 1974, Shackleton et al. 2005). Moreover, Crabal and coworkers (2008) showed that aggressiveness and territoriality had no effect on mating success and females did not prefer males holding a territory in *Drosophila*. Three breeding lines with intermediate territoriality had some of the highest and the lowest levels of mating success. The authors conclude that male-male interactions might not define mating success. There is accumulating evidence in several species including wild house mice and birds that females avoid aggressive males and have higher reproductive success when housed with less aggressive brother pairs compared to more aggressive pairs of unrelated males (Ophir & Galef Jr. 2003, Ensminger & Meikle 2005, Ensminger & Crowley 2007).

To conclude, we found no evidence that inbreeding and infection affects aggressiveness and the probability to win short-term interactions in male house mice (*Mus musculus*). This study is an attempt to partially replicate Eklund (1996) by improving the methodology. Although we avoided pseudo-replication and employed clear statistical methods, we were not able to replicate Eklund's findings. Short-term aggression assays may be a useful tool for investigating certain aspects of aggressive behavior; however, long-term aggression assays might be more suitable to monitor all aspects of competitive ability and antagonistic interactions as well as effects of certain treatments on competitive ability and aggressiveness. Furthermore, aggressiveness might not be correlated with reproductive success. These results may have implications for opposed selection pressures arising from female choice and male-male competition. Females might avoid highly aggressive males (Qvarnström & Forsgren 1998, Spence & Smith 2006) and prefer males of intermediate aggressiveness as mates whereas male-male competition favors highly aggressive males.

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