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DISSERTATION

Sexual selection and heterozygosity
in wild house mice (*Mus musculus musculus*)

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I dedicate this work to my beloved Dad

Michael Arthur Thoß

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You will always be in my heart.

Table of Content

Author contributions

	Chapter I	Chapter II	Chapter III	Chapter IV
Conceived and Designed the study	KM DP	KM DP	PI DP	PI GS DP
Run the experiment	**MT** KM	**MT** KM	MP	GS **MT** PI
Performed Statistical analyses	**MT** PI	**MT** PI	**MT** PI	PI
Wrote the Paper	**MT** PI DP	**MT** PI DP	**MT** PI DP	PI DP
Final approval	**All authors**	**All authors**	**All authors**	**All authors**

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General introduction

1. Introduction

Genetic heterozygosity is often correlated with improved individual fitness (heterozygosity-fitness correlations, HFCs) as it enhances survival (Richardson et al. 2004; Townsend et al. 2009) and reproductive success (Kempenaers 2007). Inbreeding depression is the most dramatic example of the importance of heterozygosity (Keller and Waller 2002) and increasing evidence suggests that benefits of heterozygosity are generally due to dominance (masking of deleterious alleles) rather than overdominance effects (Penn 2002; Roff 2002). However, it is unclear whether HFCs arise through detrimental effects of inbreeding, and no studies so far have examined the influence of genome-wide heterozygosity on fitness while controlling for inbreeding. We aimed to investigate effects of genome-wide heterozygosity on different fitness components, especially reproductive and mating success, survival and male social dominance in populations of systematically outbred wild-derived house mice (Mus musculus musculus) in semi-natural enclosures (Chapters I and II).

The role of heterozygosity in sexual selection is controversial. Male mating and reproductive success are reduced by inbreeding as it impairs males' competitive ability (Meagher et al. 2000); however it remains unclear whether this is due to inbreeding reducing male aggressiveness. We competed full-sib inbred and outbred males in an arena setting and investigated effects of inbreeding on males' competitive ability (Chapter III). According to Brown (1997), females should prefer to mate with outbred, heterozygous males to improve offspring heterozygosity; however, the evidence is mixed (Widdig et al. 2004; Charpentier et al. 2005; Richardson et al. 2005; Beltran 2008). One possible explanation for these equivocal results is that none of these studies have examined whether females' own inbreeding level affects their mate preferences. Especially, inbred females are expected to gain more direct and indirect benefits from mating with heterozygotes than outbred females; however inbred females might not be able to afford the costs of being choosy. We investigated in a y-maze

setting whether female preferences for the scents of experimentally inbred vs. outbred males are dependent on female's own inbreeding level (Chapter IV).

2. Does heterozygosity increase reproductive success?

Genome-wide heterozygosity enhances fitness through improving survival (Meagher et al. 2000; Keller and Waller 2002; Ilmonen et al. 2008) and reproduction (Höglund et al. 2002; Ilmonen et al. 2008; Fitzpatrick and Evans 2009; Zajitschek et al. 2009) by masking recessive deleterious mutation that otherwise impair important functions such as disease resistance (Coltman et al. 1999b; Ilmonen et al. 2007; Charpentier et al. 2008b; Ilmonen et al. 2008). Heterozygosity-fitness correlations may be due to small effects of many loci (general effects) or large effects of few loci (direct effects) (Grueber et al. 2008), such as the major histocompatibility complex, MHC. Indeed, numerous studies show that both genome-wide and MHC heterozygosity enhance survival (Arkush et al. 2002) and reproductive success (Schwensow et al. 2008a; Schwensow et al. 2008b; Setchell et al. 2010; Smith et al. 2010). None of these studies report whether genome-wide or MHC heterozygosity had greater effects, but for determining whether and how MHC heterozygosity affects fitness, it is crucial to control for potential confounding effects from background genes.

MHC genes are among the most polymorphic loci in vertebrates and control immune response to pathogens and parasites (Apanius et al. 1997). MHC heterozygosity increases host survival by enhancing resistance to infectious agents in wild outbred species (Arkush et al. 2002; Penn 2002; Penn et al. 2002; Oliver et al. 2009). Interestingly, there is increasing evidence that MHC heterozygosity can also enhance reproductive success. In female hares (Lepus europaeus), MHC heterozygosity was correlated with increased fecundity for females, indicated by the number of placental scars (Smith et al. 2010). In humans, heterozygosity at MHC-linked microsatellites predicted the number of sexual partners for women, but not men (Lie et al. 2010). It remains unclear how MHC heterozygosity enhances reproductive success.

One possible explanation is that MHC heterozygosity improves health which subsequently enhances social status and attractiveness of pheromones (Freeland 1981; Penn and Potts 1998; Charpentier et al. 2008a). Another potential explanation is that MHC heterozygotes are favoured in mate choice independent of their health as females should prefer to mate with heterozygous males to increase offspring heterozygosity (Brown 1997). MHC heterozygous mice differ in their urinary volatile composition from homozygotes (Willse et al. 2005) and female mice are more attracted to the scent of males heterozygous genome-wide (Ilmonen et al. 2009) and at major urinary protein (MUP) loci (Thom et al. 2008).

In Chapter I, we examined the relative effects of heterozygosity at MHC and background loci on fitness of house mice (Mus musculus musculus) living in large, semi-natural enclosures. We used wild-derived mice systematically outbred for two generations to ensure that MHC heterozygotes and homozygotes were equally outbred. We first examined the effects of genome-wide heterozygosity (comprising both neutral microsatellite markers and markers at MHC loci) on fitness, and subsequently the relative effects of MHC and background loci. We provide first evidence that heterozygosity at MHC loci significantly enhanced reproductive success (while controlling for background heterozygosity) without affecting survival. Successful breeders were more MHC heterozygous than non-breeders and MHC heterozygosity increased reproductive and mating success among dominant males, but not among subordinate males.

3. Is heterozygosity heritable, and if yes, how?

Brown (1997) suggested that females should prefer to mate with heterozygous, high quality males, in order to produce heterozygous offspring (heterozygosity-as-good-genes or HGG hypothesis). Heterozygosity plays a significant role in intra-sexual selection, improving males' social status, competitive ability, mating and reproductive success (Eklund 1996; Meagher et al. 2000; Kempenaers 2007). However, it remains unclear whether females prefer

to mate with heterozygous males (inter-sexual selection), or how such preferences might benefit their offspring. Several studies have found female preferences for heterozygous males (genome-wide: (Widdig et al. 2004; Charpentier et al. 2005; García-Navas et al. 2009; Ryder et al. 2010) and at MHC: (Reusch et al. 2001; Sauermann et al. 2001; Thornhill et al. 2003; Roberts et al. 2005; Bonneaud et al. 2006), whereas others do not (Westerdahl 2004; Richardson et al. 2005; Beltran et al. 2008). Furthermore, the HGG hypothesis states that mating with heterozygous mates increases heterozygosity and/or allelic diversity in the offspring which would require heritability of heterozygosity from parents to offspring. Three studies in birds have found parent-offspring correlations in heterozygosity suggesting heritability of heterozygosity in the broad sense (Reid et al. 2005; García-Navas et al. 2009; Ortego et al. 2009). None of these earlier studies suggest a mechanism for heritability of heterozygosity; however there are two possible explanations for heritability of heterozygosity to arise. First, heterozygous females might be less related to the population and therefore more likely to mate with non-kin males carrying dissimilar alleles which, in turn, results in more heterozygous offspring (Roberts et al. 2006). Second, heterozygous individuals are more likely to carry rare alleles (Apanius et al. 1997) and consequently, mating with heterozygotes confers increased heterozygosity to offspring.

Recent models find that preferences for heterozygotes can evolve in fluctuating environments, despite significant fitness costs and in the absence of additive genetic benefits (Charlesworth 1988; Mitton et al. 1993; Neff and Pitcher 2008). In these models, preferences for heterozygotes can evolve assuming unequal allele frequencies (Lehmann 2007; Fromhage et al. 2009) and correlations in heterozygosity between parents and their offspring (Fromhage et al. 2009). Female preferences for heterozygous males maintain higher genetic diversity at the population level than random mating (Neff and Pitcher 2008) which could provide a potential resolution to the 'lek-paradox' as it might explain how genetic diversity can be maintained despite directional selection resulting from mate choice.

In Chapter II, we further analysed data from our study on wild-derived house mice in semi-natural enclosures (see Chapter I). We examined mating biases with respect to genome-wide and MHC heterozygosity. We tested for parent-offspring correlations in heterozygosity and provide a potential mechanism involving rare alleles to explain broad sense heritability of heterozygosity. We found assortative mating for MHC heterozygosity and a strong parent-offspring correlation in genome-wide heterozygosity which provides first evidence for heritability of heterozygosity in mammals. Heterozygous individuals are more likely to carry rare alleles and heterozygous parents produce more heterozygous offspring than homozygotes. We suggest that passing on rare alleles from parents to offspring has the potential to explain heritability of heterozygosity.

4. Does heterozygosity affect male-male competition?

Inbreeding depression is an informative example to illustrate the importance of heterozygosity in sexual selection (Keller and Waller 2002). Increased homozygosity resulting from inbreeding reduces juvenile quality and survival (Britten 1996; Hansson and Westerberg 2002; Wang 2002); however, little is known about how inbreeding affects behaviour and other traits in adults. Two studies on wild-derived house mice (*Mus domesticus*) found that both moderate (first cousin matings) and close (full-sib matings) inbreeding significantly reduces male fitness through impairing male competitive ability (Meagher et al. 2000; Ilmonen et al. 2008). Inbred males were less likely to obtain and defend territories than outbred males which resulted in reduced reproductive success in inbred males.

We have shown in Chapter II that heterozygosity increases male reproductive success which might be due to male-male competition. Eklund (1996) found that outbred wild-derived male mice (*Mus domesticus*) are more likely to win male-male encounters than inbred males. Outbred males were more aggressive and, in turn, had 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), however, it is unclear whether aggressiveness can predict a males' competitive ability in a more natural and complex social setting. Furthermore, detrimental effects of inbreeding might become apparent only under ecologically realistic conditions (Meagher et al. 2000; Keller and Waller 2002; Armbruster and Reed 2005; Marr et al. 2006) and studies show that infection can magnify the harmful effects of inbreeding (Coltman et al. 1999a; Keller and Waller 2002; Spielman et al. 2004; Ilmonen et al. 2008).

In Chapter III, we aimed at replicating Eklund's (1996) study due to ambiguities in the methods. We used an arena setting for the dyadic agonistic encounters and tested each male pair (consisting of one full-sib inbred and one outbred male each) twice to ensure a consistent dominance relationship. In an attempt to make our laboratory experiment more ecologically relevant, we introduced pathogen infection with *Salmonella* as stress treatment. We found that inbreeding did not affect males' ability to win short, agonistic encounters, regardless of whether both males were infected or not. Our results do not support the idea that the lower reproductive and territorial success of inbred males is due to reduced aggressiveness and ability to win agonistic encounters (Eklund 1996; Meagher et al. 2000).

5. Do females prefer heterozygous males?

Male mating and reproductive success is reduced due to detrimental effects of inbreeding on male's competitive ability (Eklund 1996; Meagher et al. 2000; Ilmonen et al. 2008). Inbreeding also affects sperm competition through impaired testis descent (Mansfield and Land 2002), lower sperm concentration (Margulis and Walsh 2002) and reduced sperm quality (Gage et al. 2006). However, male reproductive success might also be influenced by female preferences for heterozygous males (Kempenaers 2007). As outlined above, some studies find preferences for heterozygous males (Widdig et al. 2004; Charpentier et al. 2005; García-Navas et al. 2009; Ryder et al. 2010), whereas others do not (Westerdahl 2004; Richardson et al. 2005; Beltran et al. 2008). None of these studies investigate whether

female's own heterozygosity affects the preference for heterozygous males which might explain equivocal results. Only two studies in fish considered whether inbreeding affects female mating preferences (Mazzi et al. 2004; Frommen and Bakker 2006). Whereas one study found that inbred females are choosier than outbred ones (Mazzi et al. 2004); the second study found no effects of inbreeding on inbreeding avoidance in females (Frommen and Bakker 2006). On the one hand, inbred females are expected to be choosier than outbred females as they are expected to gain more direct (parental care) and indirect benefits by mating with heterozygous, high quality males. On the other hand, inbred females are more likely to be in poor condition and therefore cannot afford the costs of being choosy.

In Chapter IV, we investigated whether female's own inbreeding level influences their odour preference for full-sib inbred vs. outbred males. Full-sib inbred and outbred females were presented with scent marks, a testosterone-dependent secondary sexual trait in mice, of one full-sib inbred and one outbred male in a y-maze setting. Again, to achieve an ecologically relevant setting and test whether infection results in more apparent female preferences, we experimentally infected half of the males with Salmonella and females were always presented with odour of two healthy or two infected males. We provide first evidence that females' own inbreeding level affects mate preferences. In general, females preferred the scent of outbred males and this preference was especially pronounced in inbred females; however, infection did not increase the relative attractiveness of outbred vs. inbred males.

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MHC heterozygosity enhances reproductive success

Submitted

by

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MHC heterozygosity enhances reproductive success

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We investigated how heterozygosity at the major histocompatibility complex (MHC) affects fitness in wild-derived (F2) house mice (*Mus musculus musculus*). To compare and control for potential confounding effects from overall or genome-wide heterozygosity, we used mice that were systematically outbred. We measured heterozygosity at MHC and background loci using 15 microsatellite markers on 11 different chromosomes, and measured individual survival and reproductive success in large, semi-natural population enclosures. We found that overall heterozygosity significantly increased reproductive success, as expected, but surprisingly, this effect was entirely due to heterozygosity at two MHC loci. This result was not due to MHC heterozygosity increasing survival, and though MHC heterozygosity was correlated with increased body mass, body mass did not correlate with reproductive success when heterozygosity is controlled. Breeders were more MHC heterozygous than non-breeders for both sexes, indicating that MHC heterozygosity enhanced fecundity, mating success, or both. MHC heterozygosity increased the number of mates and offspring for males, yet only among socially dominant males. These findings suggest that MHC heterozygosity increased mating success among dominant males, either through intra- or inter-sexual selection. Our results show that (1) heterozygosity-fitness correlations can potentially be explained by few loci, such as MHC; (2) MHC heterozygosity can increase fitness, even without affecting survival; (3) MHC heterozygosity enhances fitness among outbred as well as laboratory mice;

and (4) they support recent studies that also found that MHC heterozygosity enhances mating and reproductive success.

1. INTRODUCTION

Heterozygosity is often correlated with increased individual fitness, including reproductive success (Kempenaers, 2007), as well as survival (Richardson *et al.*, 2004; Townsend *et al.*, 2009) and fitness components, such as disease resistance (Charpentier *et al.*, 2008c; Coltman *et al.*, 1999; Reid *et al.*, 2005) (heterozygosity fitness correlations or HFCs). Inbreeding depression is the most dramatic example of the importance of heterozygosity (Keller & Waller, 2002), though it is unclear whether HFCs are due to inbreeding, because no studies to our knowledge have examined effects of genome-wide heterozygosity on fitness while controlling for inbreeding. Increasing evidence shows that the benefits of heterozygosity are generally due to dominance (masking deleterious alleles) rather than overdominance (Penn, 2002; Roff, 2002). HFCs may be due to small effects of many loci (general effects) or large effects due to one or few loci (direct effects), and either of these may be due to non-random associations with other functional loci affecting fitness (local effects) (Grueber *et al.*, 2008). In this study, we aimed to compare the effects of heterozygosity at the major histocompatibility complex (MHC) with overall heterozygosity – while controlling for close inbreeding – in populations of wild-derived house mice (*Mus musculus musculus*).

MHC genes are highly polymorphic loci that control immune recognition of pathogens and parasites (Apanius *et al.*, 1997), and MHC heterozygosity increases host survival by enhancing resistance to infectious agents (Arkush *et al.*, 2002; Oliver *et al.*, 2009; Penn *et al.*, 2002; reviewed in Penn, 2002). Studies on MHC heterozygosity have mostly been conducted with congenic and other inbred strains of laboratory mice, which controls for background heterozygosity, but on the other hand, the positive findings are potentially an artifact of using mice with a homogenous genetic background (e.g., interactions with background genes can influence disease resistance (Vidal *et al.*, 1993; Vukusic *et al.*, 1995) and may change the relative resistance of different genotypes (Apanius *et al.*, 1997)). In fact, a study with semi-wild mice found no evidence that MHC heterozygosity increases disease resistance (Ilmonen

et al., 2007). On the contrary, MHC heterozygotes showed *reduced* fitness when experimentally infected with virulent *Salmonella* strains, which may be due to selection favoring intermediate or 'optimal' rather than maximal MHC heterozygosity (Ilmonen *et al.*, 2007), as shown with stickleback fish (*Gasterosteus aculeatus*) (Reusch *et al.*, 2001). Therefore, studies on wild, outbred mice and other species are needed, and especially ones to compare functional MHC loci versus background genes and measure their relative effects on fitness.

Studies on wild, outbred species find that MHC-heterozygosity protects against many (though not all) infectious diseases; however, few studies have controlled for potentially confounding effects of background heterozygosity or measured the consequences of MHC heterozygosity for host fitness (Penn, 2002). In chinook salmon (*Oncorhynchus tshawytscha)*, multilocus heterozygosity (MLH) and individual MHC heterozygosity are associated with increased pathogen resistance and survival (Arkush *et al.*, 2002), though the study does not report whether MLH or MHC heterozygosity had greater effects. In free-ranging brushtailed possums (*Trichosurus cunninghami*), heterozygosity at a subset of one MHC-linked and another microsatellite marker (though not at the other 6 neutral markers) was associated with reduced parasite load and increased survival (Banks *et al.*, 2010). In humans, MHC heterozygosity is associated with increased resistance to viral infections (hepatitis, (Thursz *et al.*, 1997); HIV, (Carrington *et al.*, 1999; Jeffery *et al.*, 2000)). As MHC heterozygosity appears to be most important for protecting against multiple pathogens (McClelland *et al.*, 2003; Penn *et al.*, 2002), more studies are needed to assess fitness, rather than merely testing resistance to a particular pathogen.

Interestingly, recent studies report that MHC heterozygosity can enhance reproductive success, either through enhanced fecundity, mating success (sexual selection), or both. In fat-tailed dwarf lemurs (*Cheirogaleus medius)* and grey mouse lemur (*Microcebus murinus*), MLH and MHC individual diversity (see below) were associated with increased reproductive success in males (Schwensow *et al.*, 2008a; Schwensow *et al.*, 2008b). In both studies, social and genetic fathers had higher numbers of MHC alleles, MHC supertypes and higher MLH than randomly chosen males. In male mandrills (*Mandrillus sphinx*), both MLH and MHC

amino acid diversity were correlated with reproductive success (Setchell *et al.*, 2010). In female hares (*Lepus europaeus*), MHC heterozygosity was correlated with increased fecundity for females, indicated by the number of placental scars (Smith *et al.*, 2010). Heterozygosity at MHC-linked microsatellites (though not other markers) predicted the number of sexual partners for women, but not men (Lie *et al.*, 2010) (although it is unclear that having more partners increases women's reproductive success). These findings raise the possibility that the fitness consequences of MHC heterozygosity may be underestimated or even undetected unless reproductive success is measured, as shown with studies on genome-wide heterozygosity (Meagher *et al.*, 2000).

It is unclear how MHC heterozygosity enhances reproductive success, and these recent findings may be explained by heterozygosity enhancing disease resistance, which then subsequently improves social status (Charpentier *et al.*, 2008b; Freeland, 1981; Rau, 1983; Rau, 1984), the development of weapons (Ditchkoff *et al.*, 2001), attractiveness of pheromones (Penn & Potts, 1998) and other secondary sexual traits (Garamszegi *et al.*, 2003; Loyau *et al.*, 2005). Another possibility is that MHC heterozygotes are favored in mate choice regardless of their health. During the breeding season, male lemurs (*Lemur catta*) advertise genetic heterozygosity through the semiochemical diversity of their gland secretions (Charpentier *et al.*, 2008a). Female mice are more attracted to the scent of more heterozygous males (genome-wide (Ilmonen *et al.*, 2009) and major urinary protein loci (Thom *et al.*, 2008)), and MHC heterozygous mice have different volatile compounds in their urine than homozygotes (Willse *et al.*, 2005). Also, women are more attracted to the faces of MHC heterozygous men (Roberts *et al.*, 2005). Nevertheless, such findings are not necessarily due to mating preferences for heterozygosity *per se*, contrary to some claims (Thom *et al.*, 2008), as health benefits would be difficult, if not impossible, to rule out.

Perhaps the most difficult challenge for determining whether and how MHC heterozygosity affects fitness is controlling for potential confounding effects from background genes. This is crucial because MHC heterozygosity can be correlated with overall (genome-wide or background) heterozygosity (Roberts *et al.*, 2006) – which is known to enhance survival (Ilmonen *et al.*, 2008; Keller & Waller, 2002; Meagher *et al.*, 2000), resistance to

pathogens (Charpentier *et al.*, 2008c; Coltman *et al.*, 1999; Ilmonen *et al.*, 2008), reproduction (Fitzpatrick & Evans, 2009; Höglund *et al.*, 2002; Ilmonen *et al.*, 2008; Zajitschek *et al.*, 2009), males' social status (Eklund, 1996; Meagher *et al.*, 2000; Nevison, 2001) and sexual attractiveness (Ilmonen *et al.*, 2009; Miller, 1993). To compare the fitness consequences of MHC and genome-wide heterozygosity, Potts and colleagues (1994) studied semi-wild mice (wild mice crossed with domesticated mice) in semi-natural enclosures. They found that MHC heterozygous males had a fourfold advantage in gaining territories compared to MHC homozygotes. However, this advantage was only detected when MHC heterozygosity correlated with genome-wide heterozygosity; not when both measures were uncorrelated within a population. Therefore, the fitness benefits from MHC heterozygosity were confounded by genome-wide heterozygosity. To our knowledge, only two studies on the fitness of MHC heterozygotes with outbred species have ruled out potential confounds from background heterozygosity. First, a study on rhesus macaques (*Macaca mulatta*) found that MHC heterozygous males had 1.5 times higher reproductive success than homozygotes, but detected no effects from background heterozygosity (10 microsatellite markers) of MHC heterozygotes versus homozygotes (Sauermann *et al.*, 2001). Second, a recent study in red jungle fowl (*Gallus gallus*) found that MHC heterozygotes had better survival during a coccidiosis epidemic than homozygotes, whereas no effect of genome-wide heterozygosity was detected (27 microsatellite markers) (Worley *et al.*, 2010). Several other studies also provide evidence that MHC heterozygosity enhances fitness in wild, outbred species, but they measured *individual allelic diversity* (Alcaide *et al.*, 2008; Consuegra & Garcia de Leaniz, 2008; Jäger *et al.*, 2007; Kalbe *et al.*, 2009; Kurtz *et al.*, 2004; Paterson *et al.*, 1998), rather than heterozygosity *per se* because it is difficult to measure heterozygosity of particular loci in species that have duplications of closely related genes. Consequently, it is unclear if the observed benefits are due to having greater heterozygosity, copy number, or both. The problem with these previous studies, however, is that large numbers of molecular markers are required to detect differences in inbreeding, other than close inbreeding (Balloux *et al.*, 2004; Slate *et al.*, 2004), and therefore, studies are needed that examine the effects of MHC heterozygosity among equally outbred individuals.

In this study, we examined how heterozygosity at MHC and background loci affects the fitness of house mice living in large, semi-natural population enclosures over six weeks. Such competition experiments have revealed significant fitness differences due to deleterious genes that are missed in laboratory conditions (Carroll *et al.*, 2004; Meagher *et al.*, 2000). We used wild-derived mice (F2 offspring from wild mice) that were systematically outbred for two generations, which ensures that MHC heterozygotes and homozygotes were equally outbred. We first analyzed the effects of genome-wide heterozygosity (comprising both neutral microsatellite markers and markers at MHC loci) on fitness, and subsequently the relative effects of MHC and background loci.

2. MATERIALS AND METHODS

Mice and Colony conditions

We trapped wild house mice at four different sites in or near Vienna, Austria, "KLIVV" (48° 12' 38'' N, 16° 16' 54'' E), "Safaripark" (48° 18' 22'' N, 16° 43' 48'' E), "Schottenhof" (48° 14' 54'' N, 16° 15' 32'' E) and "VetUni" (48° 15' 22'' N, 16° 25' 55'' E). A colony was subsequently maintained through outbreeding (i.e., no matings between relatives) and mice from the different sites were not interbred to prevent artificially elevating allelic diversity. Offspring were weaned at 21 d, and ear punches were made for individual identification and tissues were stored at -20°C. Cages contained wood shavings (ABEDD) and nesting material for environmental enrichment. Mice were kept under a 12:12 h dark:light cycle and provided with food (Altromin rodent diet 1324) and water *ad libitum*. Study females (F2 generation) were housed in pairs with a sister (Type IIL cages, 32.5 x 16 x 14 cm) and males were kept singly (Type I cages, 22 x 16 x 14 cm). For each replicate population, we chose 12 mice to allow for related and unrelated individuals within each enclosure population (i.e., two sisters and one brother from three different families and three unrelated, unfamiliar males). Males and females were closely age and weight-matched within each replicate population.

Population enclosures

Mature mice were released into population enclosures, which were large indoor arenas (4.5 x 4.5 m), and each of which was subdivided into six equally sized compartments by 40-cm-high wire mesh (1-cm-grids). These fences were used as territorial boundaries by dominant males; subordinate males as well as females used to climb the mesh to escape harassment by dominant, territorial males. Each compartment contained several shelters, and food, water, nesting material and wood shavings were provided *ad libitum*. The mice were identified by individual ear marks for both sexes and individual fur cuts for males. The study was conducted over six weeks and checks were made twice a week for litters born and mortality. New born pups were removed immediately, euthanized and tissue samples stored at -20°C for paternity analysis. In total, we ran 10 population replicates, four "VetUni", three "KLIVV", two "Schottenhof" and one "Safaripark" population.

Behavioral observations

Mice were observed six times per wk for one hour per d around dusk, and observations were made with binoculars from outside the enclosure. Aggressive (attack, chase, fight), defensive (flee, upright defensive) and other social and sexual behaviors (grooming, anogenital sniffing) were recorded, though copulation was never observed, and the individuals involved and their locations were also noted. These data were used to determine males' social status, territory ownership, territory boundaries and females' location within these territories, as well as general activity of mice.

Genotyping

DNA was extracted from ear punches and other tissue samples using a standard protocol (Sambrook *et al.*, 1989). A total of 120 adults and 133 offspring were genotyped at 15 microsatellite loci in two different multiplex runs (Set 1: D9Mit34, D9Mit135, D10Mit20, D11Mit150, D17Saha, D17Mit28; Set 2: D1Mit404, D1Mit456, D2Mit252, D2Mit380, D5Mit25, D6Mit138, D7Mit227, D15Mit16, D19Mit39; see Mouse Microsatellite Data Base of Japan) using a Multiplex-PCR MasterMix (Qiagen Multiplex PCR kit). The markers are

located on 11 different chromosomes and were previously screened to confirm that they were polymorphic. Mean allele number per locus was 3.92 (ranging from 2.13 to 6.50) and mean observed heterozygosity per locus was 0.49 (ranging from 0.08 to 0.68). Multilocus heterozygosity estimated from Set 1 and Set 2 separately correlated strongly (Pearson correlation: $r = 0.28$, $N = 118$, $p = 0.002$). Marker D17Saha is located within the MHC class II locus; Marker D17Mit28 is adjacent to MHC class I K locus. Amplification mixes were subjected to a denaturation step at 94°C for 15 min followed by 30 cycles at 94°C for 30 s, 55°C for 90 s and 72°C for 60 s, followed by an elongation step at 72°C for 10 min. Amplification products were analyzed using an automated sequencer (Beckman Coulter CEQ 800). Allele scoring was made using Beckman Coulter CEQ 8000 System software and allele sizes were determined with SLS+400 as size standard. Individuals genotyped for fewer than 10 microsatellite loci, 2 adults (1.7%) and one litter of 5 offspring (3.8%), were excluded from any further analysis.

Paternity assignment and heterozygosity measures

Paternity assignment was made by complete exclusion. Each population enclosure contained six males and mothers were known for all litters; paternity was assigned to a male by excluding the other five potential sires. Paternity results were confirmed using the program CERVUS 3.0.3 (Marshall *et al.*, 1998). Observed heterozygosity (H_o, number of heterozygous loci divided by total number of loci) and standardized multilocus heterozygosity (stMLH, number of heterozygous loci divided by observed heterozygosity for those loci within the population) were calculated using IRmacroN4, a macro for Microsoft Excel written by W. Amos (downloadable from: http://www.zoo.cam.ac.uk/zoostaff/amos/#ComputerPrograms). We report results using standardized multilocus heterozygosity to control for potential non-independence of data within each population and allow for comparisons among populations.

Statistical analyses

We examined the data for normality assumptions before conducting parametric tests (SPSS 17.0) by examining unstandardized residuals and P-P plots. Additionally, we conducted

Kolmogorov-Smirnov tests for each dataset. We used a univariate generalized linear model (GZLM) to test whether the individual number of heterozygous loci at both MHC and background loci affected reproductive success. We also tested whether individual heterozygosity affected reproductive success and calculated effect sizes as we were interested in which of our three measures of heterozygosity (multilocus, MHC or background) explained most of the variation in reproductive success. MHC and background heterozygosity were correlated (Spearman rank correlation, $r_s = 0.18$, $N = 117$, $p = 0.054$), and we checked for multicollinearity by calculating the variance inflation factor (VIF) for both predictors. We can rule out potential bias from multicollinearity in the regression models as the index lies within the critical limits (VIF = 1.04 for both predictors (critical value for multicollinearity: VIF ≥ 4 (Bowerman & O'Connell, 1990)). To determine how heterozygosity affected survival, we used a Cox regression with heterozygosity as a covariate. Using a multivariate GLM, we investigated the effects of MHC heterozygosity (fixed factor) on weight at beginning and weight at termination of the experiment (dependent variables). Furthermore, we used partial correlations to test the effect of weight at the beginning of the experiment on subsequent reproductive success when MHC heterozygosity is controlled. We employed independent samples t-test to test for differences in heterozygosity between reproducing males and non-reproducing males as well as between reproducing females and non-reproducing females. We ran a generalized linear model (GZLM) with Poisson distribution using reproductive success as dependent variable, and MHC heterozygosity and dominance as fixed factors. We employed non-parametric Spearman rank correlations to investigate the effects of MHC heterozygosity on dominant and subordinate males separately. We used non-parametric statistic in this test since data for dominants and subordinates separately could not be transformed to normality. Subsequently, we ran a GZLM with Poisson distribution using mating success as dependent variable, and MHC heterozygosity and dominance as fixed factors. Finally, we employed a non-parametric Mann-Whitney U test to compare heterozygosity between dominant males versus subordinate males, as the data could not be normalized.

3. RESULTS

We examined how overall heterozygosity affected fitness, and found that the number of heterozygous loci (for all 15 loci, both MHC and background loci) was significantly correlated with reproductive success in both sexes (GZLM number of offspring: N = 117: all 15 loci: Wald $X^2 = 10.6$, df = 1, $R^2 = 0.031$, $P = 0.001$; MHC loci: Wald $X^2 = 51.69$, df = 2, $R^2 = 0.115$, $P < 0.001$; background loci: Wald $X^2 = 10.6$, df = 1, $R^2 = 0.031$, $P = 0.001$; **Figure 1**). We also analyzed the data using standardized multilocus heterozygosity (individual heterozygosity standardized by the population mean) to compare between replicate populations and ensure that results are not just driven by population differences in heterozygosity. We found that overall heterozygosity was still positively and significantly correlated with reproductive success for both sexes (GZLM number of offspring: Wald $X^2 = 9.04$, df = 1, $R^2 = 0.06$, $P = 0.003$).

We compared the relative effects of both MHC-linked loci versus background loci, and unexpectedly we found that this relationship was entirely explained by MHC: MHC heterozygosity was significantly correlated with reproductive success, whereas background heterozygosity had no effect on fitness (MHC: Wald $X^2 = 11.71$, df = 2, $R^2 = 0.115$, $P = 0.003$; background: Wald $X^2 = 0.44$, df = 1, $R^2 = 0.00$, $P = 0.51$; MHC*background: Wald $X^2 = 8.72$, df = 2, $P = 0.013$). The significant interaction shows that the effects of MHC on fitness were greater than background heterozygosity (which explained virtually nothing). Our subsequent analyses therefore focused on determining how MHC heterozygosity enhanced reproductive success.

There are two types of selective explanations for how MHC heterozygosity increased reproductive success, which are not necessarily mutually exclusive. First, MHC heterozygosity may have enhanced survival (natural selection); however, we found no such effect (Cox regression, MHC heterozygosity: $X^2 = 2.2$, df = 1, $P = 0.14$). Second, MHC heterozygosity may have enhanced fecundity or mating success (intra- or inter-sexual selection), and we found evidence consistent with both processes. Individuals of both sexes that successfully reproduced (‘breeders’) were significantly more heterozygous at MHC loci than those that failed to reproduce (‘non-breeders’) (Independent samples t-test, Males: Sires:

N = 29, Non-sires: N = 30, t = 2.02, *P* = 0.048; Females: Dams: N = 22, Non-dams: N = 36, t = 3.30, *P* = 0.002; **Figure 2**). In contrast, background heterozygosity had no effect on becoming a breeder (Independent samples t-test, Males: Sires: N = 29, Non-sires: N = 30, t = 0.58, *P* = 0.56; Females: Dams: N = 22, Non-dams: N = 36, t = 1.28, *P* = 0.21). Among males, we found their reproductive success was affected by their social status and by a significant interaction of MHC heterozygosity and social dominance (GZLM, N = 59, MHC heterozygosity: Wald X^2 = 3.87, df = 2, *P* = 0.144; Dominance: Wald X^2 = 6.43, df = 1, *P* = 0.011; MHC heterozygosity*dominance: Wald X^2 = 6.49, df = 2, *P* = 0.039). Dominant, territorial males tended to sire more offspring than subordinate males (Independent samples t-test: t = 1.98, df = 56, *P* = 0.052), as expected, however, dominant and subordinate males did not differ in heterozygosity at MHC (Mann-Whitney U test, *Z* = -1.59, *P* = 0.11) or background loci (*Z* = -0.01, *P* = 0.99). MHC heterozygosity was correlated with increased reproductive success among dominant (Spearman rank correlation, N = 29, r_s = 0.50, *P* = 0.007), but not among subordinate males (Spearman rank correlation, N = 30, r_s = 0.14, *P* = 0.48). MHC heterozygosity, but not social dominance, was significantly correlated with the number of mates males obtained (GZLM, N = 59, MHC heterozygosity: Wald X^2 = 14.04, df = 2, *P* = 0.001; Dominance: Wald X^2 = 0.73, df = 1, *P* = 0.39; Dominance*MHC heterozygosity: Wald X^2 = 0.56, df = 2, *P* = 0.75). Thus, breeders had higher levels of MHC heterozygosity than non-breeders and for males this effect was not explained by their social status. MHC heterozygosity did not improve males' social status, though it was correlated with increased mating success among dominant males.

Finally, the benefits for reproductive success may be due to MHC heterozygosity enhancing individual health, but we found mixed evidence for this mechanism. MHC heterozygosity was significantly correlated with greater body mass at the beginning and termination of the experiment (Multivariate GLM: weight at beginning: F = 14.7, N = 90, R^2 = 0.25, *P* < 0.001; at termination: F = 17.2, N = 90, R^2 = 0.28, *P* < 0.001). This result holds after excluding all females that were visibly pregnant at the termination of the experiment (F = 10.24, N = 84, R^2 = 0.184, *P* < 0.001). Yet, we were unable to detect any association between MHC heterozygosity and weight gain during the experiment (Pearson correlation: r =

0.13, N = 90, $P = 0.23$). Moreover, increased body mass at the start of the experiment did not correlate with reproductive success when controlling for heterozygosity (Partial correlation, N = 117, r = 0.11, df = 114, $P = 0.24$). Thus, MHC heterozygosity was correlated with greater body mass, but this does not explain the greater reproductive success of MHC heterozygotes.

4. DISCUSSION

We found that overall multilocus heterozygosity (15 microsatellite loci) was associated with increased reproductive success for both sexes in the population enclosures, and surprisingly, this result was entirely explained by heterozygosity at two MHC loci. We can rule out potential confounds from genome-wide heterozygosity, not only because we could not detect any fitness effects from background heterozygosity, but mainly because these mice were systematically outbred for two generations so that MHC heterozygotes were not more outbred than homozygotes. This result provides compelling evidence that heterozygosity at functional MHC loci can enhance reproductive success when inbreeding is controlled, and to our knowledge, this is the first such evidence. In fact, there is only one study that examined effects of genome-wide heterozygosity on fitness (over-winter survival and subsequent recruitment to the natal population) while controlling for close inbreeding. In great reed warblers (*Acrocephalus arundinaceus*) recruited siblings were significantly more heterozygous than non-recruited ones at five (Hansson *et al.*, 2001) and 14 additional microsatellite markers (Hansson *et al.*, 2004). In this previous study, multilocus heterozygosity measured by the two sets of microsatellites were not correlated, also within-dyad difference in multilocus heterozygosity between siblings were not correlated between the two sets. In contrast, in our study, heterozygosity estimated separately from two panels of microsatellite markers was closely correlated (see Methods), and therefore, provide a consistent measurement of individual genetic heterozygosity.

The enhanced reproductive success of MHC heterozygous mice in our study was not due to improved survival (which is not surprising for a six-week study), but rather to improved fertility, sexual selection, or both. MHC heterozygotes in both sexes were more likely to breed successfully than homozygotes, though it is not entirely clear why. Dominant,

territorial males tended to sire more offspring than subordinates, as expected; however, dominant males were not more heterozygous at MHC loci than subordinates. This finding might seem to rule out male-male competition (intrasexual selection) for explaining the success of heterozygotes, but we cannot rule out competition among the territorial males. MHC heterozygous males sired more offspring than homozygotes, and interestingly, this effect was only among the dominant males (MHC heterozygosity did not improve the reproductive success of subordinate males). Female house mice can move freely among the territories of different dominant males, and thereby they might be able to preferentially mate with more MHC heterozygotes among the dominant males. Thus, we found no evidence that MHC heterozygosity improves males' social status, and though mate choice might play a role, we cannot rule out the role of intra-sexual selection among dominant individuals. Experiments are needed to determine if mice, like humans (Roberts *et al.*, 2005) and other primates (Sauermann *et al.*, 2001), show preferences for MHC heterozygous individuals.

We stress that although there was no survival advantage, the enhanced reproductive success of MHC heterozygotes may have been due to enhanced health (Penn, 2002; Penn *et al.*, 2002). We found significant effects of MHC heterozygosity on condition (weight), but not on weight gain during the experiment; and weight did not predict reproductive success when heterozygosity is statistically controlled. Wild house mice harbor a wide variety of pathogens and parasites (Becker *et al.*, 2007), and we verified that the wild-derived mice in our colony are parasitized (*Aspicularis* pin worms). By improving overall health, MHC heterozygosity can potentially enhance social status (intra-sexual selection) and sexual attractiveness (inter-sexual selection), as well as fecundity, which can potentially explain ours as well as previous findings (Charpentier *et al.*, 2008b; Husak *et al.*, 2006; Roberts *et al.*, 2005; Thornhill *et al.*, 2003). Alternatively, MHC heterozygotes could be more sexually attractive than homozygotes (Roberts *et al.*, 2005), regardless of their health. MHC heterozygotes may be more likely to carry rare or dissimilar allelic variants (Mitton *et al.*, 1993), and therefore, mating with MHC heterozygotes might increase offspring MHC dissimilarity and heterozygosity and provide an advantage in pathogen resistance and survival (Penn & Potts, 1999).

Our study indicates the importance of investigating HFCs not only on the level of genome-wide heterozygosity (general effects), but also local effects due to linkage with functional loci. Under the inbreeding hypothesis (Charlesworth & Charlesworth, 1987), all loci should be equally negatively affected by inbreeding. Therefore, in the case of the general effect hypothesis, fitness measures are expected to show a positive correlation with heterozygosity at each single locus. Under the local effects hypothesis, the effect of heterozygosity on fitness or fitness components at each particular locus is independent (David, 1998) and can be positive, negative or non-existing, depending on allelic interactions (dominance/overdominance) and functional importance (Lieutenant-Gosselin & Bernatchez, 2006). Consequently, we conclude that our study shows large effects of a few loci and provides evidence for the local effects hypothesis. Since the importance of local HFCs and the number and proportion of loci presenting HFCs remains to be determined, the significance of their effects on fitness traits still needs further investigation. Recent studies provide evidence for HFCs due to local effects in a variety of taxa (Bean *et al.*, 2004; Markert *et al.*, 2004; Spielman *et al.*, 2004; Tiira *et al.*, 2006); however, small numbers of loci have been investigated and unclear chromosomal location of markers limit the conclusiveness of these results (Lieutenant-Gosselin & Bernatchez, 2006).

Taken together, our findings show that MHC heterozygosity enhances fitness even in wild, outbred mice. To our knowledge, ours is the first study to report this finding and, simultaneously, control for background heterozygosity by systematic outbreeding of experimental animals. Our findings are consistent with several recent studies (Sauermann *et al.*, 2001; Schwensow *et al.*, 2008a; Schwensow *et al.*, 2008b; Setchell *et al.*, 2010; Smith *et al.*, 2010; Widdig *et al.*, 2004) and further experimental tests are needed to determine whether MHC heterozygosity enhances reproductive success through intersexual, intrasexual selection, or both. Furthermore, our results provide the first evidence that although MLH can be significantly correlated with fitness, this finding can be entirely explained by only two MHC loci. MHC heterozygosity is unlikely to explain all HFCs in all species, and more studies are needed to compare the relative importance of MHC and background heterozygosity. Finally, it should be noted that we found no effects on survival (and we had

virtually no deaths in our colony during our six-week study), and therefore, our findings provide further evidence that fitness effects from small genetic differences are more readily detected in competitive, semi-natural conditions than conventional laboratory studies. Such findings have important implications for ecological, evolutionary, and functional genomics.

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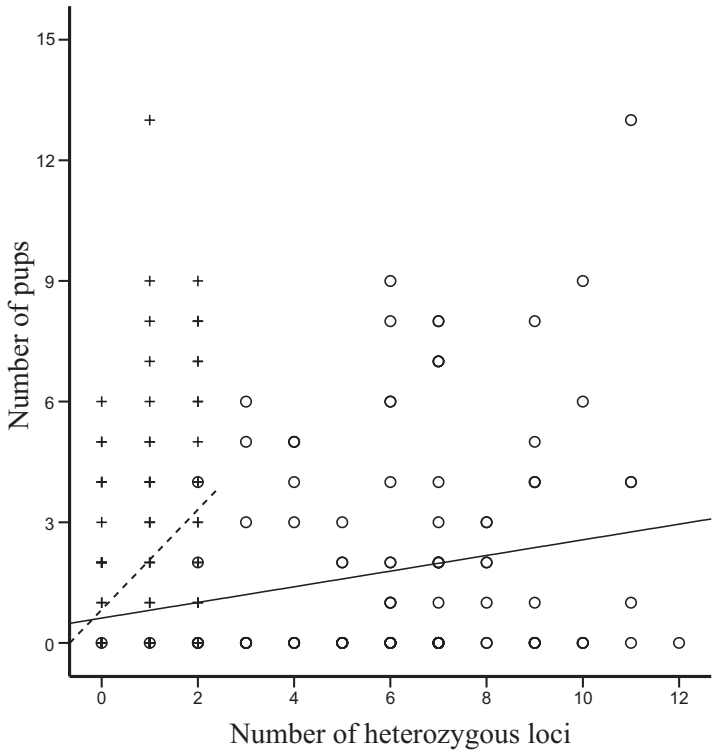

Figure 1: Correlation of number of heterozygous loci at MHC-linked microsatellites (plus symbols) and at background microsatellites (circles) with number of pups. Dotted line represents regression slope for MHC-linked microsatellites. Solid line indicates regression slope for background microsatellites.

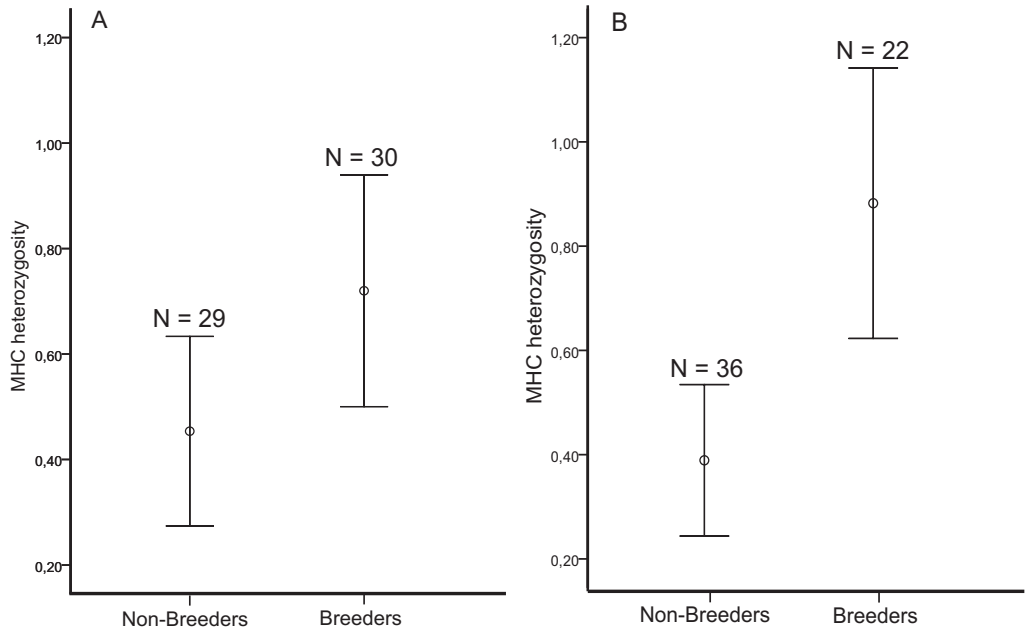

Figure 2: MHC heterozygosity in breeders versus non-breeders for (A) males and (B) females. Error bars indicate means ± 1SE. Samples sizes are indicated above the error bars.

II

Heritability of heterozygosity explained by rare alleles

Submitted

by

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Heritability of heterozygosity explained by rare alleles

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It has been suggested that females might evolve preferences to mate with heterozygous males to enhance offspring genetic diversity, and that such preferences can help to resolve the lek paradox (heterozygosity-as-good-genes or HGG hypothesis). We examined the role of heterozygosity and sexual selection in populations of wild-derived house mice (Mus musculus musculus) living in semi-natural enclosures. We previously found that heterozygosity at major histocompatibility (MHC) loci enhanced individual mating and reproductive success (Thoß et al. Submitted). Here, we further show that there was an assortative pattern of mating for heterozygosity at two MHC-linked loci, but not overall heterozygosity at 15 other microsatellite markers, which may be due to intra- or inter-sexual selection. Interestingly, parental heterozygosity was associated with overall individual heterozygosity of offspring, and increased genetic diversity of litters (mean number of alleles and allelic richness). We also found that more heterozygous parents carry more rare alleles and confer more rare alleles to their offspring than homozygous parents. We suggest that this later finding provides a novel explanation for how heterozygosity can be heritable, and it also suggests a mechanism through which non-random mating can enhance rather than erode offspring genetic diversity.

1. INTRODUCTION

It has been suggested that females should mate disassortatively to enhance offspring heterozygosity (Brown 1997; Tregenza and Wedell 2000; Penn 2002). Brown (1997) suggested another version of this idea, in which females should prefer to mate with high quality, heterozygous males, as a mechanism to produce heterozygous offspring. This version of the heterozygosity-as-good-genes or HGG hypothesis, however, has been rather controversial. On one hand, there is much evidence that heterozygosity increases individual fitness (Miller et al. 1993; Höglund et al. 2002; Keller and Waller 2002; Fitzpatrick and Evans 2009). For example, resistance to infectious diseases is enhanced by outbreeding (Coltman et al. 1999; Charpentier et al. 2008; Ilmonen et al. 2008), and even by heterozygosity at only some loci, such as the major histocompatibility complex (MHC) (Penn 2002; Penn et al. 2002). Moreover, a large number of studies have found that heterozygosity plays a role in intra-sexual selection, improving males' social status, competitive ability, mating and reproductive success (Eklund 1996; Meagher et al. 2000; Kempenaers 2007). However, one the other hand, it is unclear whether females prefer to mate with heterozygous males (inter-sexual selection), or how they might benefit by such preferences. Some studies provide evidence that females show a bias for males with high levels of overall heterozygosity (Widdig et al. 2004; Charpentier et al. 2005; García-Navas et al. 2009; Ryder et al. 2010), including MHC heterozygosity (Reusch et al. 2001; Sauermann et al. 2001; Thornhill et al. 2003; Roberts et al. 2005; Bonneaud et al. 2006). Other studies do not find such preferences (Westerdahl 2004; Richardson et al. 2005; Beltran et al. 2008), though negative results based on only a few genetic markers to measure heterozygosity are arguably inconclusive (Slate 2002; Balloux et al. 2004). Another potential explanation for the disparate results is that only certain females, such as inbred, homozygous ones, express a bias for heterozygous males (Ilmonen et al. 2009). Our aims in this study were to investigate the role of heterozygosity and non-random mating in house mice (*Mus musculus musculus*) and test whether heterozygous parents

produce more heterozygous offspring than homozygous parents.

The HGG hypothesis assumes that by mating with heterozygous males, females will increase the heterozygosity or allelic diversity of their offspring (Brown 1997); however, this notion has seemed implausible because heterozygosity is not heritable in the *narrow sense* (variation in additive (allelic) gene effects divided by the total phenotypic variance) (Kotiaho et al. 2008). Yet, recent studies have found significant parent-offspring correlations in inbreeding coefficient *f* (song sparrows, *Melospiza melodia,* (Reid et al. 2006)) or multilocus heterozygosity (blue tits, *Cyanistes caeruleus*, (García-Navas et al. 2009) and lesser kestrels, *Falco naumanni*, (Ortego et al. 2009)), which indicate that heterozygosity can be heritable, at least in a *broad sense*. None of these previous studies to our knowledge, however, have suggested a potential mechanism to explain how offspring can inherit their parents' heterozygosity.

There are at least two ways to explain how parents might confer heterozygosity to their offspring. First, if heterozygous females are more likely than homozygous females to mate with non-kin carrying dissimilar alleles, their offspring will be more heterozygous (Roberts et al. 2006). It is unclear, however, why heterozygous females should be more likely to avoid inbreeding than homozygous ones, and the only test of this idea to our knowledge supports the contrary ((Ilmonen et al. 2009), but see (Reid et al. 2007)). Second, heterozygous individuals may be more likely to carry *rare alleles*, because they occur in a heterozygous state more frequently than common ones (Apanius et al. 1997), and consequently mating with heterozygous individuals may confer increased heterozygosity. Thus, by mating with high quality males, females can potentially increase the genetic quality (allelic or additive genetic benefits) and heterozygosity (non-additive genetic benefits) of offspring.

Brown (1997) suggested that the HGG hypothesis could help to resolve the so-called 'lek paradox,' which assumes that sexual selection erodes the genetic diversity necessary to confer any genetic benefits to choosy females (Borgia 1979). Recent

theoretical models provide support for Brown's suggestion, at least under certain conditions (Lehmann et al. 2007; Neff and Pitcher 2008; Fromhage et al. 2009). One model finds that populations with females expressing a preference for heterozygous males maintain higher genetic diversity than populations with randomly mating females (Neff and Pitcher 2008). Other models find that preferences for heterozygotes can evolve in fluctuating environments, despite significant fitness costs and in the absence of additive genetic benefits (Charlesworth 1988; Mitton et al. 1993; Neff and Pitcher 2008), but require unequal allele frequencies in the population (resulting in rare and common alleles). More recent models support the idea that mating preferences for heterozygotes can evolve assuming (1) unequal allele frequencies (Lehmann et al. 2007, Fromhage et al. 2009) and (2) correlations in heterozygosity between parents and their offspring (Fromhage et al. 2009). However, these models assume overdominant selection, which is supported by only few examples; even with MHC genes, which are widely assumed to be under overdominant selection (Penn 2002). This assumption might be met by other forms of balancing selection, such as negative frequency-dependent selection that maintain diversity in the population.

In this study, we examined heterozygosity and sexual selection in wild-derived (F2 of wild-caught mice) house mice in large, population enclosures. We found that increased heterozygosity at MHC loci resulted in enhanced mating and reproductive success in these populations (Thoß et al., submitted). Here, we report that these mice showed an assortative mating pattern for overall heterozygosity (at 15 microsatellite loci), and that this result can be entirely explained by non-random mating at MHC loci. We found that overall heterozygosity of parents is correlated with the heterozygosity of their offspring, which provides evidence that heterozygosity can be heritable in mammals (as in birds (Reid et al. 2005; García-Navas et al. 2009; Ortego et al. 2009)). Moreover, we found that more heterozygous individuals carried more rare alleles than homozygous parents, and parents with more rare alleles also have offspring with more rare alleles. Our

findings are consistent with the idea that heterozygous parents confer heterozygosity to offspring and their litters show greater diversity than homozygous ones (Brown 1997), which may help explain why sexually selected traits show greater levels of genetic diversity than other traits (Pomiankowski and Møller 1995).

2. MATERIAL AND METHODS

Mice and Colony conditions

We trapped wild house mice (*Mus musculus musculus*) at four different sites in or near Vienna, Austria. Details of the trapping sites are provided elsewhere (Thoß et al., submitted). A colony was subsequently maintained through outbreeding (i.e., no matings between relatives), which controlled for inbreeding (background heterozygosity) to study effects of MHC-heterozygosity. Mice from different sites were not interbred, either in the colony or in the enclosures, to avoid artificially elevating allelic diversity. At weaning (21d), ear punches were made for individual identification and tissues were stored at -20°C. Cages contained wood shavings (ABEDD) and wood-wool for environmental enrichment. Mice were kept under a 12:12 h dark:light cycle and provided with food (Altromin rodent diet 1324) and water *ad libitum*. Study males (F2 generation) were kept singly (Type I cages, 22 x 16 x 14 cm) and females were housed in pairs with a sister (Type IIL cages, 32.5 x 16 x 14 cm). For each replicate population, we chose 12 age- and weight-matched mice (i.e., two sisters and one brother from three different families and three unrelated, unfamiliar males).

Population enclosures

Mature mice were released into population enclosures, which were large indoor arenas (4.5 x 4.5 m), subdivided into six equally sized compartments by 40-cm-high wire mesh (1-cm-grids). Each compartment contained several shelters, and food, water, nesting material and wood shavings were provided *ad libitum*. The mice were identified by

individual ear marks for both sexes and individual fur cuts for males. The study was conducted over six weeks and we checked twice a week for litters born and mortality. New born pups were removed immediately, euthanized and tissue samples were stored at -20°C for paternity analysis.

Genotyping

DNA was extracted from ear punches and other tissue samples using a standard protocol (Sambrook et al. 1989). A total of 120 adults and 133 offspring were genotyped at 15 microsatellite loci in two different multiplex PCR reactions as described elsewhere (Thoß et al. submitted). The markers are located on 11 different chromosomes and were confirmed to be polymorphic: Mean number of alleles per locus was 3.92 (ranging from 2.13 to 6.50) and mean observed heterozygosity per locus was 0.49 (ranging from 0.08 to 0.68). We confirmed that we can detect inbreeding since offspring heterozygosity decreased with increasing parental relatedness estimated at these 15 markers. Details of the amplification are given elsewhere (Thoß et al, submitted). Amplification products were analyzed using an automated sequencer (Beckman Coulter CEQ 800) and allele scoring was made using Beckman Coulter CEQ 8000 System software. Individuals genotyped for fewer than 10 microsatellite loci, 2 adults (1.7%) and one litter of 5 offspring (3.8%), were excluded from any further analysis.

Paternity assignment, heterozygosity, allele frequencies, genetic diversity

Paternity assignment was made by complete exclusion. Mothers were known for all litters as we closely followed visibly pregnant females until birth. Half of the litters were born in a cage after termination of the experiment. Each population enclosure contained six males and paternity was assigned to a male by excluding the other five potential sires. Paternity results were confirmed using the program CERVUS 3.0.3 (Marshall et al. 1998). Standardized multilocus heterozygosity (stMLH, number of heterozygous loci

divided by observed heterozygosity for those loci within the population) was calculated using IRmacroN4, a macro for Microsoft Excel written by William Amos (downloadable from: http://www.zoo.cam.ac.uk/zoostaff/amos/#ComputerPrograms). We report results using standardized multilocus heterozygosity to allow for comparisons among populations. For multiply sired litters, sire heterozygosity was corrected using the proportion of sired offspring within the litter. We summed the corrected heterozygosity for all sires in a litter and obtained mean sire heterozygosity. For example, consider a litter of six pups in which male 1 (standardized multilocus heterozygosity, MLH = 0.75) sired three pups (50% or 0.5), male 2 (MLH = 0.45) sired two pups (33% or 0.33) and male 3 (MLH = 0.62) sired one pup (17% or 0.17). In this case, mean sire heterozygosity was calculated as (0.75x0.5) + (0.45x0.33) + (0.62x0.17) = 0.59. Allele frequencies, mean number of alleles per locus and allelic richness were calculated separately for each replicate population using the program FSTAT developed by Jérôme Goudet (downloadable from: http://www2.unil.ch/popgen/softwares/fstat.htm). Taking a conservative criterion, an allele was defined as 'rare' if it was found in less than 10% of the individuals and in a more liberal criterion we defined an allele as 'rare' if found in less than 20% of the individuals of each replicate. We report results from the conservative approach throughout and from the liberal criterion only, when the results differed.

Statistical analyses

We inspected the data for normality assumptions (SPSS 17.0) by examining unstandardized residuals and P-P plots and ran Kolmogorov-Smirnov tests for each dataset. If data violated normality assumption we used non-parametric tests. We used non-parametric Spearman's correlation to examine correlations in heterozygosity between parents (heterozygosity-based mate choice) and between parents and their offspring. We used linear regressions to test effects of sire and dam heterozygosity on offspring genetic diversity (mean number of alleles per locus in a litter and allelic

richness in a litter) separately. We employed Pearson's correlation to test for correlations between individual heterozygosity and individual number of rare alleles for both parents and offspring. For parents, we investigated correlations between individual number of rare alleles and individual number of rare alleles passed to offspring and individual heterozygosity and individual number of rare alleles passed to offspring.

3. RESULTS

We tested whether the mice showed any patterns of non-random mating with respect to individual heterozygosity, and found a significant correlation in the overall heterozygosity between dams and sires (Spearman correlation: $N = 45$, $r_s = 0.34$, $R^2 = 0.28$, $p = 0.025$). Unexpectedly, this pattern of assortative mating for overall heterozygosity can be entirely explained by heterozygosity at MHC loci ($r_s = 0.42$, $R^2 = 0.20$, $p = 0.004$); background (non-MHC) heterozygosity does not correlate between dams and sires ($r_s = 0.13$, $R^2 = 0.04$, $p = 0.40$) (**Figure 1).** As expected, parents with higher levels of overall heterozygosity carried more rare alleles than homozygous parents (Pearson correlation, $N = 117$, $r = 0.34$, $R^2 = 0.10$, $p < 0.001$). Therefore, we tested for assortative mating with respect to rare alleles, and found a strong, positive correlation in number of rare alleles carried by dams versus sires ($r_s = 0.59$, $R^2 = 0.27$, $p < 0.001$) (**Figure 2).**

We examined whether heterozygous parents confer heterozygosity to their offspring, and found that both overall and MHC heterozygosity of parents strongly correlated with (1) offspring overall heterozygosity (Spearman's correlation, $N = 109$, overall heterozygosity: $r_s = 0.46$, $R^2 = 0.29$, $p < 0.001$; MHC heterozygosity: $r_s = 0.34$, $R^2 = 0.15$, $p < 0.001$) (**Figure 3**) and (2) within litter heterozygosity (Pearson's correlation, $N = 22$, overall heterozygosity: $r = 0.79$, $R^2 = 0.62$, $p < 0.001$; MHC heterozygosity: $r = 0.63$, $R^2 = 0.39$, $p = 0.002$). There was neither a parent-offspring correlation for MHC heterozygosity ($N = 109$, $r_s = 0.09$, $R^2 = 0.15$, $p = 0.36$), nor did parental MHC

heterozygosity predict within litter MHC diversity (N = 22, r = 0.24, R^2 = 0.06, p = 0.28). As expected, offspring overall heterozygosity decreased with increasing relatedness of dam and sire (r_s = -0.2, R^2 = 0.10, p < 0.001).

In addition to heterozygosity, we examined two other measures of offspring genetic diversity. We found strong effects of parental heterozygosity on the *mean number of alleles* per locus within a litter and litter *allelic richness*. Sire heterozygosity was significantly correlated with the mean number of alleles in a litter (Linear regression, N = 22, t = 2.87, R^2 = 0.29, p = 0.01), though dam heterozygosity was not (t = 1.79, R^2 = 0.14, p = 0.09). Furthermore, allelic richness within litters increased with both sire and dam heterozygosity (sire: N = 22, t = 3.27, R^2 = 0.35, p = 0.004; dam: N = 22, t = 2.63, R^2 = 0.26, p = 0.016). Surprisingly, neither mean number of alleles within litters nor allelic richness increased with number of sires in a litter (mean number of alleles: t = 1.35, R^2 = 0.08, p = 0.19; allelic richness: t = 0.67, R^2 = 0.02, p = 0.51).

Finally, we examined the genetic consequences of mating with individuals carrying rare alleles for the offspring. We found that individual overall heterozygosity strongly correlated with the individual number of rare alleles for both parents and offspring (Pearson correlation, parents: N = 117, r = 0.34, R^2 = 0.10, p <0.001; offspring: N = 109, r = 0.34, R^2 = 0.09, p < 0.001)). Individual parental heterozygosity correlated strongly with the number of rare alleles that parents conveyed to their offspring (N = 52, r = 0.33, R^2 = 0.29, p < 0.001) and tended to correlate with the actual number of rare alleles in their offspring (N = 117, r = 0.17, R^2 = 0.01, p = 0.07), though this result is significant with the less stringent classification of 'rare' (i.e., when an allele was found in less than 20% of the individuals) (N = 117, r = 0.29, R^2 = 0.08, p = 0.001) (**Figure 4).**

4. DISCUSSION

We found genetic evidence for non-random, assortative mating for overall heterozygosity, and surprisingly, this correlation was entirely explained by heterozygosity

at MHC loci. Two previous studies have also found assortative mating for heterozygosity in birds (García-Navas et al. 2009; Ortego et al. 2009), though this study is, to our knowledge, the first to examine mammals and the first such findings with MHC genes. We also found that parents with high overall heterozygosity produced the most heterozygous offspring, as found in three studies on birds (Reid et al. 2005; García-Navas et al. 2009; Ortego et al. 2009), and moreover, they produced the most genetically diverse litters, which supports the HGG hypothesis (Brown 1997). To explain how parent-offspring correlations in heterozygosity arise, we analyzed the number of rare alleles carried by parents, and the number of alleles and allelic richness in litters (measures of offspring diversity). We found that more heterozygous parents carried more rare alleles than homozygous parents, parental heterozygosity increased both the number of alleles and allelic richness in the offspring, and parents with more rare alleles have offspring with more rare alleles. This is the first such evidence to our knowledge, and we suggest that our findings may help explain (broad sense) heritability of heterozygosity. Below we address each of our main findings in more detail.

Our findings indicate that heterozygosity plays a role in sexual selection, though it is unclear whether the pattern of assortative mating for MHC-heterozygosity was due to inter- or intra-sexual selection. Our previous analyses show that MHC-heterozygosity increased males' mating and reproductive success – though only among the dominant, territorial males – suggesting a role for female preferences among winners of male-male competition (Thoß et al. submitted). Females can discriminate MHC-heterozygotes from homozygotes based on odor cues (Yamazaki et al. 1984; Willse et al. 2005), and evidence from other species suggests that females show preferences for males with greater MHC heterozygosity (Sauermann et al. 2001; Thornhill et al. 2003; Roberts et al. 2005; Bonneaud et al. 2006). The assortative mating in our study is not necessarily due to direct preferences for MHC heterozygosity *per se*, as this bias may have arisen through competition (intra- and inter-sexual selection) for mates that are healthy and in good

condition due to MHC-heterozygosity (Arkush et al. 2002; Penn 2002; Penn et al. 2002; Oliver et al. 2009). We expected that females with lower overall heterozygosity might mate preferentially with more heterozygous males, as suggested in a previous study with experimentally inbred wild mice (Ilmonen et al. 2009); however, we cannot rule out the results of the previous study since none of the mice in the present study were inbred. When taken together, our results indicate that MHC-heterozygosity enhanced mating and reproductive success (Thoß et al. submitted), and there was a pattern of assortative mating for MHC-heterozygosity in these populations. Neither of these findings is explained by overall background heterozygosity.

Our findings provide support for the suggestion that mating with more heterozygous individuals can increase offspring heterozygosity and genetic diversity (Brown 1997). Parents with high overall heterozygosity produced more heterozygous offspring (and more homozygous parents produced homozygous offspring), which supports the idea that heterozygosity can be heritable, at least in a broad sense. Furthermore, parental heterozygosity increased offspring diversity measured as number of alleles and allelic richness in the litter which further supports the HGG hypothesis. We aimed to examine the genetic consequences for offspring when females mated with more versus less heterozygous males; however, this was impossible since females in our study only rarely mated with homozygous males (and vice versa) (Thoß et al. submitted). To adequately test this assumption, studies are needed that compare offspring heterozygosity from females (and males) experimentally assigned to mate with more versus less heterozygous individuals.

We found that heterozygous individuals carried more rare alleles than homozygous ones, which helps to explain how parent-offspring correlations in heterozygosity arise. Heterozygous parents produced heterozygous offspring that carried more rare alleles than offspring of homozygous parents. In other words, more heterozygous parents carry more rare alleles and therefore mating with such individuals

increases offspring heterozygosity and within-litter diversity. This is the first evidence to our knowledge that more heterozygous individuals carry more rare alleles than homozygotes, and that heterozygous parents confer a higher number of alleles – including rare alleles – to their offspring than less heterozygous individuals. Therefore, mate preferences for heterozygotes could provide non-additive genetic benefits in terms of more fit (more heterozygous) offspring. Furthermore, mating with heterozygotes resulted in higher within-litter diversity which might play an important role for the maintenance of genetic diversity in populations.

Our findings may also provide a potential resolution to the lek paradox. MHC-heterozygosity increased mating and reproductive success (Thoß et al. submitted) – but we find no evidence that this heterozygosity-fitness is explained by rare allele advantage (Apanius et al. 1997). The assortative mating for MHC-heterozygosity in this study may have arisen as a by-product of non-random mating for individuals carrying rare alleles, and parent-offspring correlations for heterozygosity as well as increased offspring diversity can be explained by rare alleles. This interpretation is consistent with the Hamilton-Zuk hypothesis (Hamilton and Zuk 1982), which suggests that sexual selection reinforces negative frequency-dependent selection from pathogens, which was proposed as a way to resolve the lek paradox. We suggest that the above mentioned frequency-dependent selection combined with condition-dependent expression of sexually selected traits (Houle 1998) and genic capture of variance in those traits (Rowe and Houle 1996; Tomkins et al. 2004) could work in concert to maintain high additive genetic variation in sexually selected traits.

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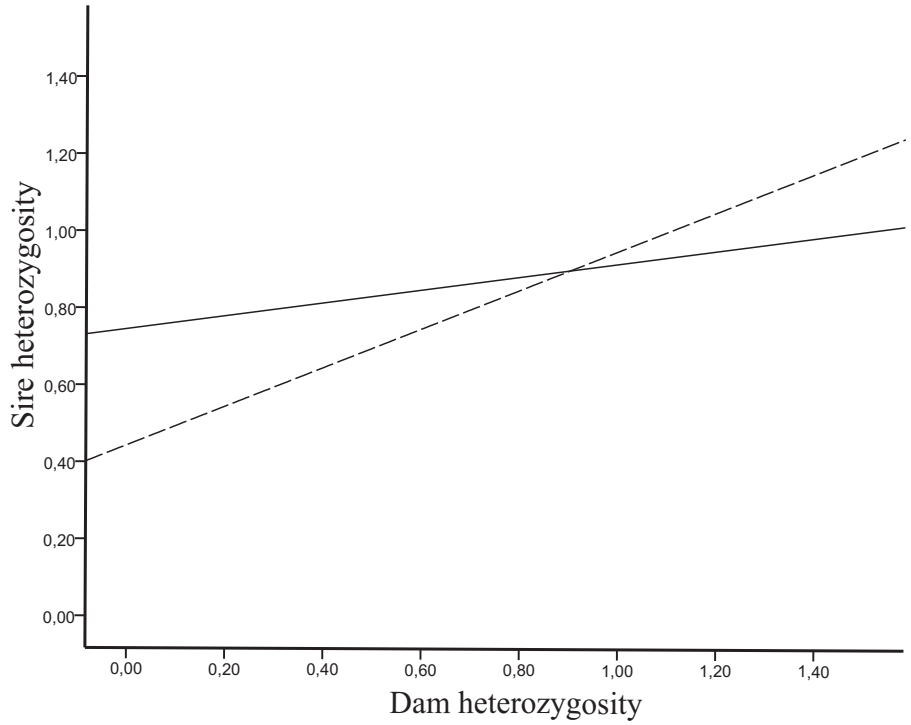

Figure 1: Relationship of the heterozygosity of dams and sires. Solid line indicates regression slope for background heterozygosity (13 non-linked microsatellite markers), and dashed line indicates slope for MHC heterozygosity (2 MHC-linked microsatellites).

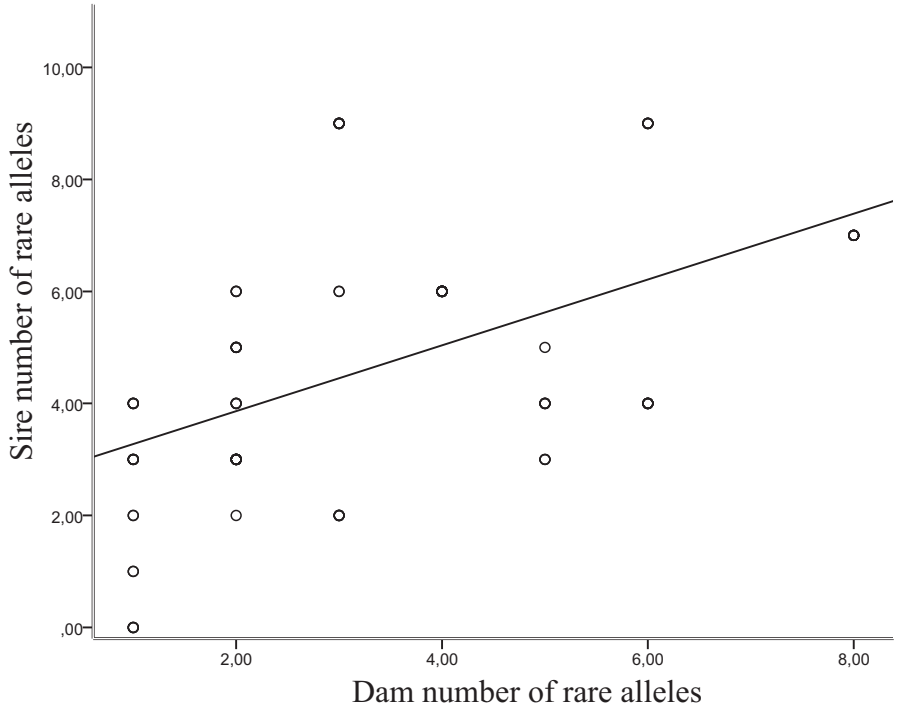

Figure 2: Correlation in number of rare alleles at 15 microsatellite loci for dam and sire.
Solid line indicates regression slope.

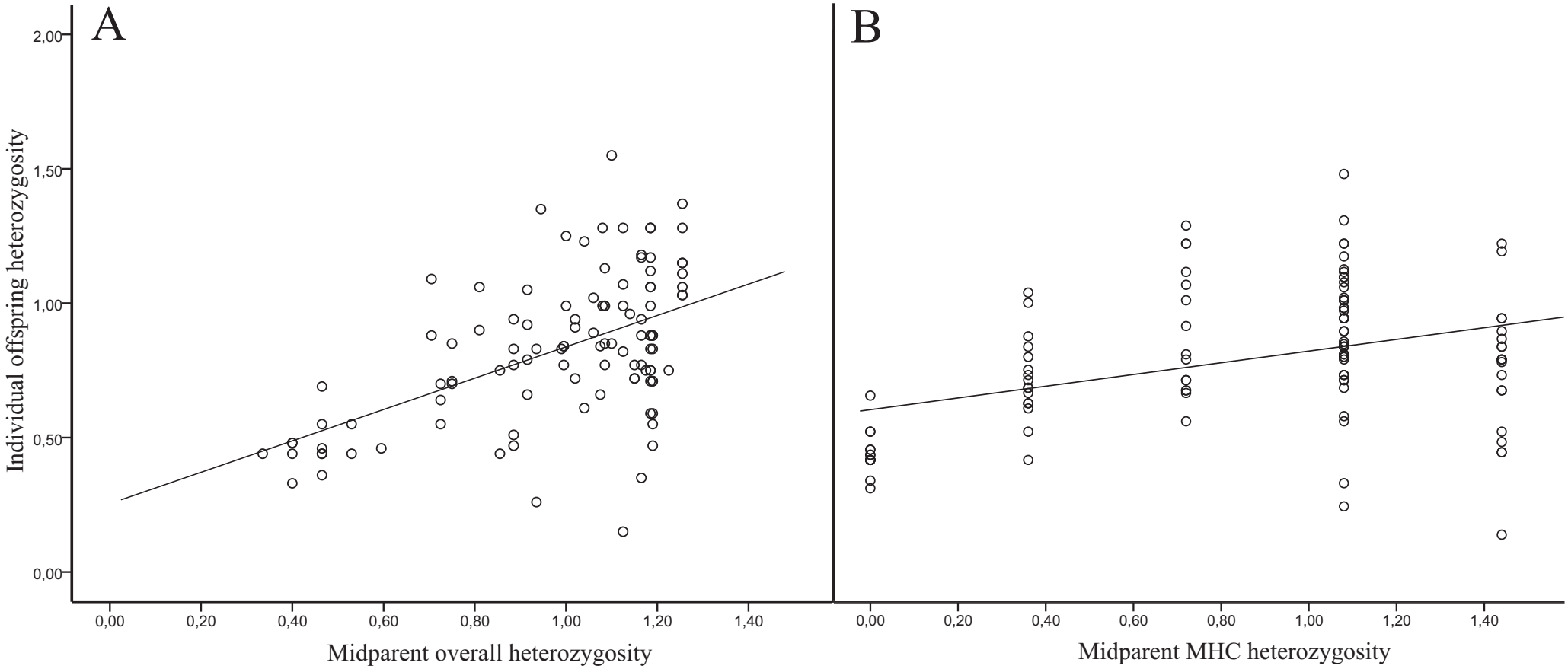

Figure 3: Relationship of parental heterozygosity, both overall (15 microsatellite markers) (A) and at MHC loci (B), versus offspring heterozygosity. Solid lines indicate regression slopes.

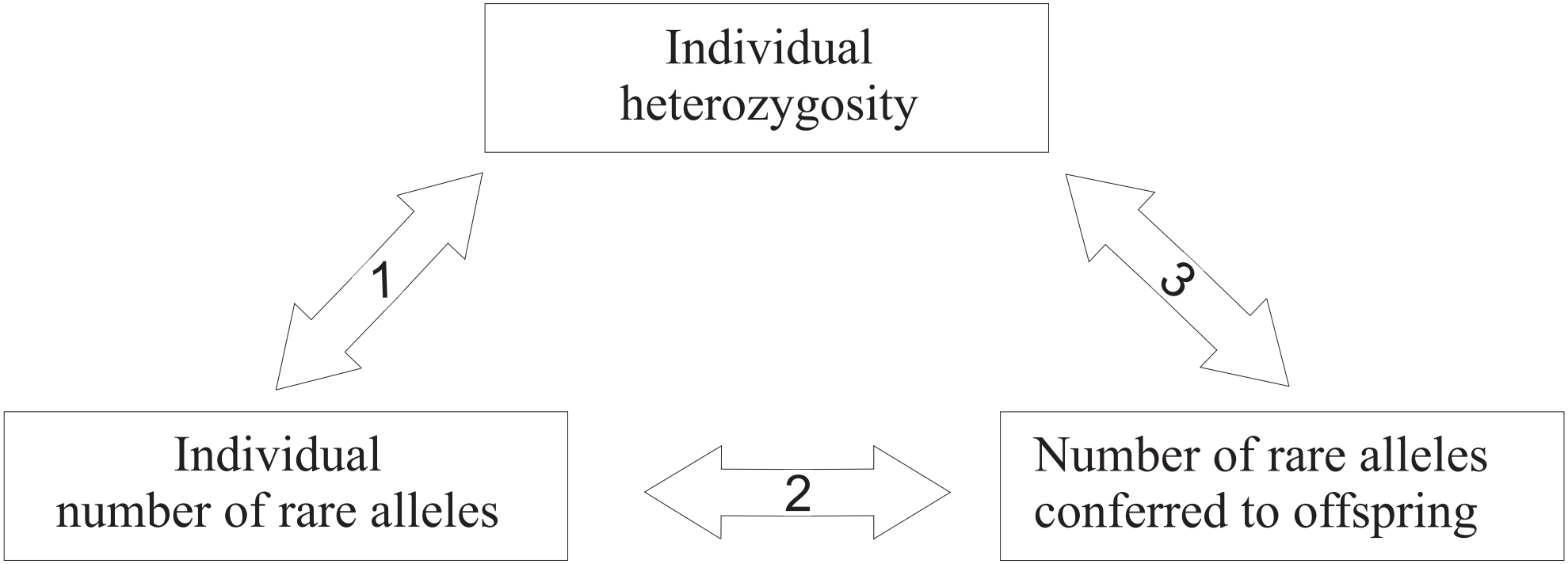

Figure 4: Relationship between individual heterozygosity and rare alleles, and the consequences of mating with heterozygous individuals. Heterozygous individuals (parents and offspring) carry more rare alleles than homozygous individuals (1). Number of rare alleles in the parents correlates strongly with those in the offspring (2). Parental heterozygosity correlates with number of rare alleles in the offspring, resulting in heterozygous offspring (3).

III

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

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by

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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"; C h a r l e s w o r t h & C h a r l e s w o r t h 1987, 1999). Inbreeding reduces the quality and survival of young (B r i t t e n 1996, H a n s s o n & W e s t e r b e r g 2002, W a n g et al. 2002, R e e d & F r a n k h a m 2003), though there are surprisingly few studies on how inbreeding affects behavior or other traits during the adult part of life cycle (but see M e a g h e r et al. 2000, C h a r p e n t i e r 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 (M i l l e r 1994, M e a g h e r et al. 2000, K e l l e r et al. 2002, K e l l e r & W a l l e r 2002, A r m b r u s t e r & R e e d 2005, M a r r 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 (M e a g h e r 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 (I l m o n e n 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 trails 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 μl, 10^7 colony forming units per ml) (Mittrücker & 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 B e n u s 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.

S t a t i s t i c a l a n a l y s i s

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.

Behavior	Description
Aggressive behavior	
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
Defensive behavior	
Flight	Rapid movement away form the opponent with sudden changes in direction
Other behavior	
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).

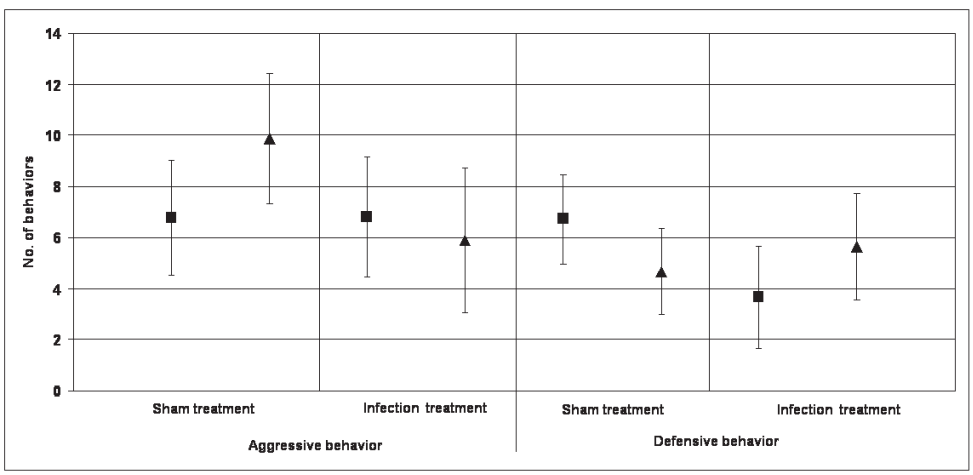

Fig. 1. Number of aggressive (left hand side) and defensive (right hand side) behaviors shown by males during five minutes of male-male encounters (mean ± SE). In each encounter, both males were either sham treated or infected with *Salmonella*. Inbred males are indicated with squares, outbred males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

Females prefer the scent of outbred males: Good-genes-as-heterozygosity?

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Females prefer the scent of outbred males: good-genes-as-heterozygosity?

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Abstract

Background: There is increasing interest to determine the relative importance of non-additive genetic benefits as opposed to additive ones for the evolution of mating preferences and maintenance of genetic variation in sexual ornaments. The 'good-genes-as-heterozygosity' hypothesis predicts that females should prefer to mate with more heterozygous males to gain more heterozygous (and less inbred) offspring. Heterozygosity increases males' sexual ornamentation, mating success and reproduction success, yet few experiments have tested whether females are preferentially attracted to heterozygous males, and none have tested whether females' own heterozygosity influences their preferences. Outbred females might have the luxury of being more choosey, but on the other hand, inbred females might have more to gain by mating with heterozygous males. We manipulated heterozygosity in wild-derived house mice (*Mus musculus musculus*) through inbreeding and tested whether the females are more attracted to the scent of outbred versus inbred males, and whether females' own inbreeding status affects their preferences. We also tested whether infecting *both* inbred and outbred males with *Salmonella* would magnify females' preferences for outbred males.

Results: Females showed a significant preference for outbred males, and this preference was more pronounced among inbred females. We found no evidence that *Salmonella* infection increased the relative attractiveness of outbred versus inbred males; however, we found no evidence that inbreeding affected males' disease resistance in this study.

Conclusion: Our findings support the idea that females are more attracted to outbred males, and they suggest that such preferences may be stronger among inbred than outbred females, which is consistent with the 'good-genes-as-heterozygosity' hypothesis. It is unclear whether this odour preference reflects females' actual mating preferences, though it suggests that future studies should consider females' as well as males' heterozygosity. Our study has implications for efforts to understand how mate choice can provide genetic benefits without eroding genetic diversity (lek paradox), and also conservation efforts to determine the fitness consequences of inbreeding and the maintenance of genetic diversity in small, inbred populations.

Background

After considering the potential benefits of mate choice, Jerram Brown [1] decided he would "put aside the idea that there is a best male and that he is best for every female," and instead concluded that females should prefer males that genetically complement themselves, as a way to increase offspring heterozygosity or genetic diversity, which he called the "heterozygosity theory" of mate choice. Actually, Trivers [2] first suggested that females should choose mates to enhance their genetic compatibility, and this hypothesis has been supported in a variety of species [3-6]. Mating preferences for genetic compatibility, however, cannot explain why in many species females prefer males with extravagant secondary sexual traits. In another version of this model, Brown also suggested that when a "best" male is found, his superiority may be due to heterozygosity at one or more loci, and females may prefer to mate with such males to increase their offspring heterozygosity or diversity [1]. This version of the "good-genes-as-heterozygosity" hypothesis [7] has received increasing theoretical [8-13] and empirical attention [reviewed in [14]].

The main problem has been trying to explain how mating with heterozygous males could possibility provide genetic benefits. Several studies have found positive correlations between parent and offspring heterozgosity (e.g. [15-17]) and inbreeding coefficient, f [18], and therefore, these findings suggest that apparent non-additive genetic variation can be heritable. Such correlations arise when the frequencies of the alternative homozygous male genotypes in a population are uneven, because in such conditions homozygous females are able to increase the proportion of heterozygous offspring by mating with heterozygous males [10,12,15]. However, previous theoretical models have not explicitly addressed how females' own heterozygosity might influence their mating preferences, and have overlooked the fact that only homozygous females can increase the heterozygosity of their offspring by mating with heterozygous males (e.g., see Table 2 in [12]). Thus, females' mating preferences need not be absolute and can be conditional, depending upon their own heterozygosity. Our aims were to manipulate heterozygosity in wild house mice (*Mus musculus musculus*) through inbreeding, as this reduces genome-wide heterozygosity [19,20] by increasing the proportion of homologous alleles that are identical by descent [21,22], and test whether females are more attracted to outbred versus inbred males, and whether females' preferences depend upon their being inbred versus outbred.

Several studies have shown that heterozygosity plays a role in sexual selection [reviewed in [14]]. Male mating and reproductive success are enhanced by heterozygosity and reduced by inbreeding due to direct male-male competition [23-28]. For example, inbreeding in house mice reduces male fitness partly because it impairs males' ability to become socially dominant and maintain territories necessary to obtain mates [28-30]. Also, inbreeding may affect sperm competition as it impairs males' testicle size and sperm concentration [31,32] and decreasing heterozygosity lowers sperm quality [33], but see [34] for criticisms.

Another way that heterozygosity influences male mating success is through female preferences for heterozygous males, and a few studies support this idea [reviewed in [14]]. Maynard Smith [35], for example, found that female fruitflies (*Drosophila subobscura*) are less likely to mate with inbred than outbred males due to poor performance of inbred males during courtship. Subsequent work confirms that inbreeding or homozygosity reduces male courtship behaviour [27,36-38] and the expression of other secondary sexual traits [7,39-42], although it is unclear how inbreeding or heterozygosity affects males' attractiveness to females. Female fur seals (*Arctocephalus gazelle*) appear to seek out more heterozygous males, which they might assess through males' body size, condition, dominance behaviors, or territory quality [16] but see also [43] for criticisms. One study in Arctic charr (*Salvelinus alpinus*) suggests that heterozygous males are favored by cryptic female choice (sperm selection) [44]. Most studies have failed to find statistically significant evidence that females prefer heterozygous males [reviewed in [14]], but it is unclear whether the number of genetic markers used in these studies are sufficient to accurately assess overall heterozygosity [45]. Therefore, studies are needed that experimentally manipulate males' overall heterozygosity to test how this affects their sexual attractiveness and mating success.

Although several studies have investigated the effect of heterozygosity on male secondary sexual traits and mating success, none to our knowledge have examined whether *females' own heterozygosity* affects their preferences for heterozygous males. Several studies suggest that females' mating preferences can be condition-dependent [46-48], but only two studies, both in fish, have considered whether inbreeding affects females' mating preferences in general: the first one found that inbred females were choosier than oubred ones regarding the fluctuating asymmetry of computer-animated males [49], whereas the second found no evidence that inbreeding affects females' inbreeding avoidance [50]. Thus, inbred females may be choosier also about males' heterozygosity than outbred ones, as one would expect if they gain genetic benefits by mating with heterozygous males (e.g. see Table 2 in [12]). Inbred females may also stand more to gain in terms of *direct benefits* than outbred females by mating with high quality, heterozygous males as a way to

compensate for their own poor parental quality [51-54]. On the other hand, inbred females in poor condition may not be able to afford the costs of being choosy [46-48]. Thus, studies are also needed that experimentally manipulate females' heterozygosity to test how this affects their preferences for heterozygous males.

We trapped wild house mice and inbred (sib-sib mating) the F2 generation to manipulate heterozygosity of males, and tested whether this treatment reduces their attractiveness to females in comparison to outbred males in an olfactory preference assay. Females were presented with males' scent-marks, which are a testosterone-mediated, condition-dependent secondary sexual trait used in courtship [55]. We also manipulated the heterozygosity of females through inbreeding to test whether this affects their preferences for outbred males. Often, the detrimental effects of inbreeding only become apparent after exposure to infectious agents, social competition, or other stressful conditions [27,28,30,56]. Therefore, in each trial, we tested females' preferences for inbred versus outbred males when *both males* had been experimentally infected with *Salmonella* or *both* were sham-infected. The infection treatment was performed to make a negative result more conclusive, and if inbreeding reduces males' attractiveness due to their relatively poor health and condition, then we predicted that infection would magnify the differences between males. In fact, *Salmonella* infection has been found to magnify the fitness differences between inbred versus outbred males [30]. We found that females show a strong and clear preference for the scent of outbred males, regardless of whether the both of the males were experimentally infected or not, and this preference was somewhat stronger among the inbred females.

Methods

Animals and housing

We trapped wild house mice from a single population (Safaripark, Gänserndorf) near Vienna, Austria and bred the F2 generation to produce full-sib inbred (sister-brother-mating; Wright's inbreeding coefficient; $f = 0.25$) and outbred mice (matings between unrelated individuals; $f = 0.00$). At weaning, we housed the offspring singly in acrylic cages, half of the inbred and outbred males in type I cages (22 × 16 × 14 cm) and the other half in type IIL cages (32.5 × 16 × 14 cm, IVC). The females were housed in type IIL cages. The cages contained pine bedding and wood-wool for environmental enrichment. All the mice were provided food (Altromin rodent diet 1324) and water *ad libitum* and kept under a 12:12 h dark:light cycle. For the odour preference test, we chose 52 triplets (one inbred male, one outbred male and one female) in which the three mice were closely age-matched, unrelated and unfamiliar to each other. All mice were sexually mature. Experimental protocol was approved by the Austrian Federal Ministry of Science and Research' Animal Care and Use Committee (BMWF-66.015/0023-c/GT/2007).

Experimental infections

The 52 males (26 inbred and 26 outbred males) of the infection group were experimentally infected with 30 μl of *Salmonella enterica* serovar Typhimurium [strain SRI – 11, 10^6 colony forming units (cfu)/ml] orally, which is a natural infection route. *S. enterica* serovar Typhimurium is an enteric mouse pathogen that becomes systemic by invading the intestinal mucosa and by replicating intracellularly within host macrophages [57]. Host resistance to *Salmonella* is under genetic control and influenced by nramp, major histocompatibility complex and other immune resistance loci [58], and requires both innate and acquired arms of the immune system [59]. We used *Salmonella* as an experimental pathogen as our previous work found that inbreeding increases the susceptibility of mice to *Salmonella* [30], and *Salmonella* infection reduces male scent-marking and the attractiveness of males' scent to females [55]. Therefore, we predicted that female preferences for outbred versus inbred males would be more pronounced when *both* of the males were experimentally infected with *Salmonella* (and if the results were negative, this treatment would allow us to conclude that this result was not an artefact of the males not being infected or otherwise challenged, as occurs in more normal ecological circumstances). The bacteria (stored as frozen stocks at -80°C) were cultured in 15 ml of heart-brain infusion at 37°C for 12 h while shaking at 170 rpm. The overnight solution was diluted to the desired concentration with sterile phosphate buffered saline (PBS) and the concentration of viable bacteria was verified by quantitative plate counts in duplicates. After infection, the mice were housed singly and euthanized 11-days post inoculation with CO_2. The mice were inspected on a daily basis and the individuals that showed clear symptoms of severe infection were euthanized immediately to avoid any unnecessary suffering. The spleens of the mice were dissected and homogenized in 1 ml of PBS under sterile conditions. 50 μl of each homogenate was cultured on selective agar plates and incubated overnight (37°C). The *Salmonella* loads per spleen were determined by calculating the number of cfu/ml of spleen homogenates on the plates (the mean of two replicate plates per mouse). The mice were restricted from food and water four hours prior to inoculation to rule out variation in systemic infection due to food in the gut. Three of the *Salmonella*-infected males died before the scent mark collection and therefore we could not perform any odour preference tests with these triplets. The 52 males (26 inbred and 26 outbred males) of the control group were sham-infected by given them equal volume of sterile PBS. We used a lower *Salmonella* dosage here than in a previous study, and therefore, we expected lower mor-

tality, especially since in a previous study most mortality occurred only after the mice had been repeatedly challenged with mixed strain infections over several months [60]; however, mortality was unexpectedly 10% higher in this study.

Scent-mark collection

To collect the scent marks, we placed the males into a new small cage on a sterile filter paper (20.5 × 14.5 cm) for four hours eight days after inoculation. We collected scent marks in the morning (8:00–12:00 a.m.). During this time, males were provided food and water *ad libitum*. Males were stimulated with female urine because stimulated males show more scent-marking and females show a preference for scents of sexually stimulated males [55]. We placed a small piece of filter paper (2 × 2 cm) containing 10 μl of female urine into the males' cages. We used mixture of urine from 15 mature females (different from those used in the odour preference tests), which we collected by placing females on tinfoil, pipetting up the urine, and storing it at -80°C. The filter papers with male scent marks were stored individually in Ziploc® plastic bags (Toppits, Allround Zipper, 3 l) at -80°C until used in female odour preference tests. The cages of the males were filled with new bedding after the scent mark collection so that they felt comfortable in their cages (normal weekly animal care taking which we connected with the experiments). This way, 46 marked filter papers of infected males (23 inbred, 23 outbred) and 52 marked filter papers of sham-infected males (26 inbred, 26 outbred) could be generated.

Odour preference assays

We tested females during oestrus, determined by examining vaginal smears under a microscope [61], to ensure they were sexually active. The Y-maze apparatus for our odour preference tests was composed of acrylic, and contained a start chamber (5.5 × 12.5 × 5.5 cm), where the mice were first placed, and two arms of choice chambers. The start chamber was separated from the first section of the choice chambers or neutral zone, and the choice chambers (5.5 × 13.5 × 5.5 cm; without neutral zone) were separated from the chambers containing the filter papers (5.5 × 31.5 × 5.5 cm) with wire-mesh dividers. The dividers prevented the females from touching or chewing the filter papers. We placed an air pump (Sera Air 110) and the scent marked filter papers at the end of the chambers to ensure a constant airflow of volatiles through the maze. The pump was kept constantly on in the colony room to habituate the females to its sound.

The experiments were conducted in the morning beginning at 8:00 a.m. under dim light, recorded on videotape (Sony Handycam DCR-SR 30E) and the videos were later analysed using Observer software (Noldus, Version 3). At the start of each trial, a female was placed in the start chamber for 5 min to habituate to the maze, and after this time the scent-marked filter papers were placed in the maze. The air pump was turned on and the female was released into neutral zone of the choice chambers. Based on preliminary tests, we recorded the females for 5 min because thereafter they were less active. We recorded the following behaviours: (1) the number; and (2) the duration female actively investigated the dividers between the choice chambers and the chambers containing the filter papers; and (3) the number of visits; and (4) total time a female spent on each side of the Y-maze. We predicted *a priori* that the two investigatory behaviours (1 and 2) would be the most informative for female preferences, because the females actively gather information and show interest in the odour. The other two behaviours (3 and 4) were recorded because these are commonly used in preference tests. We considered any side biases females showed to indicate an odour preference. After each trial, the Y-maze was cleaned with ethanol (to remove scents from previous trial), and we alternated the sides of the maze in which the filter papers were placed (between inbred versus outbred males, infected versus sham-infected pairs of males, and inbred versus outbred females) to avoid biases due to possible side-preferences. Each filter paper and each female was tested only once. To avoid possible experimenter biases, there was only one observer who recorded the data (videotape playbacks) and she was blind to the inbreeding and infection status of the animals.

Statistical analyses

We tested the data for assumptions of normality and equality of variances before conducting parametric tests (SPSS version 15.0). For statistical analyses, we used General Linear Model (GLM), repeated measures. The tests of within-subjects effects was used to test whether there was a general preference for outbred versus inbred males, and whether the female inbreeding status or male infection status (both *Salmonella*-infected or sham-infected males) had any influence on female preference. The between-subjects effect was used to test whether the female inbreeding status or male infection status influenced female behaviours. We ran paired samples *t*-test separately for inbred and outbred females, but only for the number of investigations, because the interaction term with female inbreeding status was statistically significant only for this variable. Furthermore, all of the four female behaviours were highly inter-correlated ($R > 0.47$, $N = 49$, $P < 0.001$, for all pair-wise correlations). We used directed tests instead of one- or two-tailed tests [62] for the overall female preference for outbred males over inbred males, because we had a clear *a priori*-prediction that females would prefer the outbred males over the inbred ones, which is consistent with previous results [14]. We also

used directed test for testing the effect of male infection status on female preference because we predicted *a priori* that female preference for outbred male is more pronounced when both males are experimentally infected. To test for differences in the *Salmonella* loads we used a *t*-test ($\log_{10}$-transformed data) and to test for differences in the prevalence (infected or non-infected) and the mortality between inbred and outbred males we used Chi-square tests. We used directed tests because in a previous study it was found that the outbred males are more resistant to *Salmonella* than the inbred ones [30]. We obtained the critical values for each directed test from the *P*-values of the corresponding one-tailed test by using $\gamma/\alpha = 0.8$ as a pragmatic conventional value [62]. Using two-tailed tests instead of directed tests does not change the interpretation of our results, except that the observed female preference for outbred versus inbred males measured by duration of investigations becomes only marginally significant ($P = 0.05$).

Results

The results of GLM multivariate analysis showed that females preferred significantly outbred males over the inbred ones [Within-subjects effects, outbred (OB) versus inbred (IB) male: F = 3.0, d.f. = 4, $P_{dir} = 0.02$] measured by average of the four female preference behaviours, whereas neither the female inbreeding status (interaction term: OB versus IB male × female inbreeding status: F = 1.6, d.f. = 4, $P = 0.19$) or experimental infection (interaction term: OB vs IB × male infection status: F = 0.2, d.f. = 4, $P_{dir} = 0.59$) had no significant effects on female preference for outbred males. When using univariate models we found that females significantly preferred outbred compared to inbred males, measured by number of investigations (Table 1, Fig. 1a), duration of investigations (Table 2, Fig. 1b) and number of visits (Table 3, Fig. 1c), but not by total duration (Table 4, Fig. 1d). Interestingly, we found that preference for outbred males was somewhat stronger in inbred females versus outbred females (Figs 1a–d). This difference between inbred and outbred females was statistically significant for number of investigations (Table 1; Within-subjects contrasts, interaction term: OB versus IB male × female inbreeding status), and there was a similar, but non-significant trend for duration of investigations. Females' inbreeding status did not influence their preferences for number of visits (Table 3) or total duration

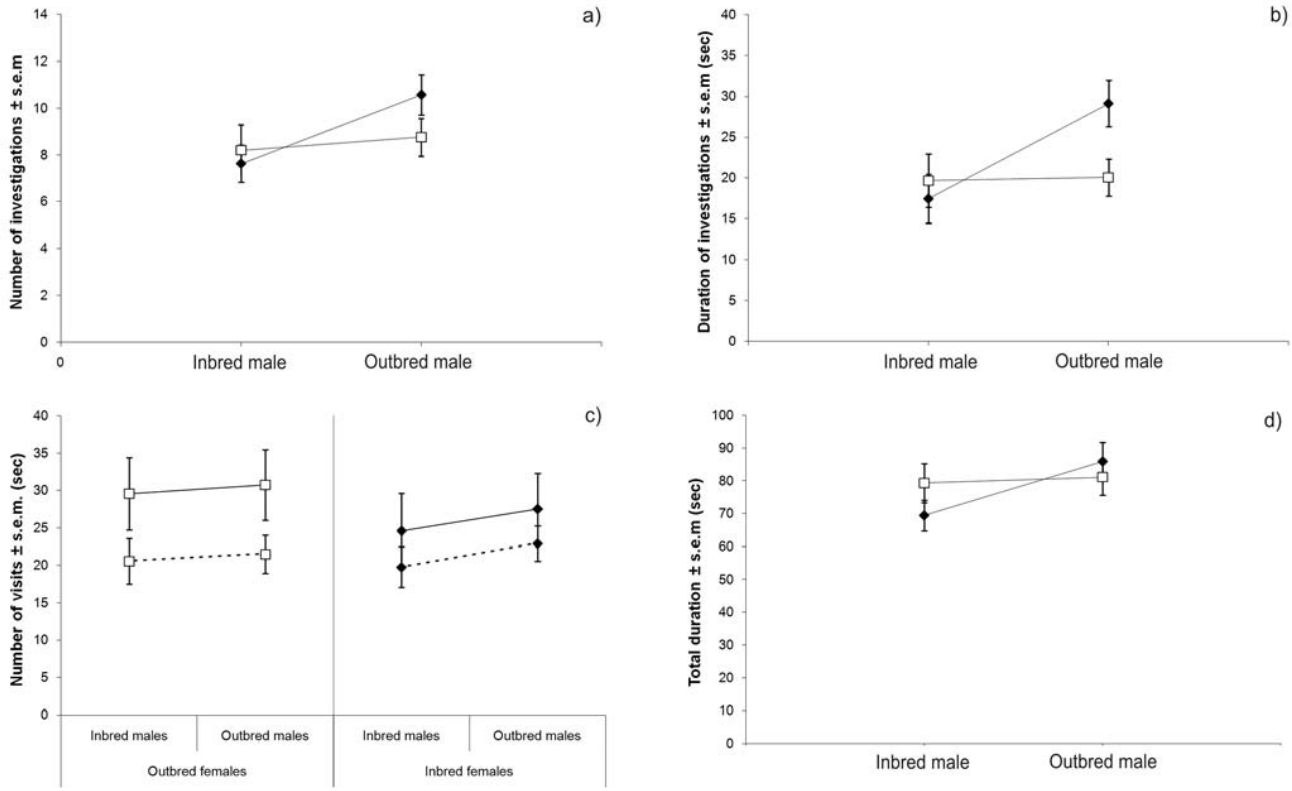

Figure 1

Female preferences measured as a) number of investigations, b) duration of investigations, c) number of visits and d) total duration separately for outbred (white symbol, n = 25) and inbred females (black symbol, n = 24). Data is pooled for trials in which both of the males were sham-infected or both infected, except for 1 c, in which the data is shown separately for trials with two sham-infected males (dashed line) and two infected males (solid line).

Table 1: Summary table for the results of GLM repeated measurements analyses for number of investigations.

Tests of Within-Subjects Contrasts	**d.f.**	***F***	***P***
preference OB vs IB (directed)	1	12.5	**0.0006**
preference OB vs IB × female inbreeding status	1	6.1	**0.017**
preference OB vs IB × male infection status (directed)	1	0.0	0.56
preference OB vs IB × female inbreeding status × male infection status	1	0.7	0.42
Error (OB vs IB)	45		
Tests of Between-Subjects Effects	**d.f.**	***F***	***P***
female inbreeding status	1	0.3	0.57
male infection status	1	0.1	0.78
female inbreeding status × male infection status	1	1.0	0.32
Error	45		

(OB = outbred; IB = inbred).

(Table 4). When testing the inbred and outbred females separately, inbred females investigated the scent marks of outbred males significantly more often compared to the scent marks of inbred males (paired samples *t*-test, t = 4.50, d.f. = 23, P_{dir} = 0.0001), but outbred females did not show any clear preference (paired samples *t*-test, t = 0.79, d.f. = 24, P_{dir} = 0.44, Fig. 1a).

We found no significant evidence that infecting both the inbred and outbred males influenced their relative attractiveness: the females still preferred outbred males, but contrary to our expectation this preference was not magnified when both of the males were experimentally infected (Tables 1, 2, 3, 4). Interestingly, the females showed a tendency to shift the sides within the Y-maze more often (number of visits, Fig. 1c) when they were presented with the scents from two infected compared to two sham-infected males; however, this difference is not significant (the between-subjects effects: male infection status; Table 3). One possible reason we did not find that infection would magnify females' preferences for outbred males is that inbreeding did not appear to affect the males' resistance to Salmonella infection in this experiment. There was a statistically non-significant trend for lower mortality in the outbred males compared to the inbred ones (27% and 46%, respectively; Chi-square test, $\chi 2$ = 2.07, d.f. = 1, Pdir = 0.09). However, among the survivors, there were no statistically significant differences in Salmonella loads between inbred and outbred males after eleven days (log10 Salmonella load: 3.00 ± 0.83 and 2.64 ± 0.72, respectively; Independent samples t-test, t = 0.33, d.f. = 31, Pdir = 0.47). Although many mice completely cleared the infection, there was no difference in Salmonella prevalence (57% and 47%, respectively; Chi-square test, $\chi 2$ = 0.31, d.f. = 1, Pdir = 0.36). Thus, our experimental infection did not increase the females' preferences for outbred males.

Discussion

We found that female mice were more attracted to the scent marks of outbred compared to inbred males, as predicted, and this preference appeared to be more pro-

Table 2: Summary table for the results of GLM repeated measurements analyses for duration of investigations.

Tests of Within-Subjects Contrasts	**d.f.**	***F***	***P***
preference OB vs IB (directed)	1	4.0	**0.033**
preference OB vs IB × female inbreeding status	1	3.3	0.08
preference OB vs IB × male infection status (directed)	1	0.0	0.53
preference OB vs IB × female inbreeding status × male infection status	1	0.1	0.75
Error (OB vs IB)	45		
Tests of Between-Subjects Effects	**d.f.**	***F***	***P***
female inbreeding status	1	1.7	0.20
male infection status	1	0.8	0.39
female inbreeding status × male infection status	1	0.2	0.63
Error	45		

(OB = outbred; IB = inbred).

Table 3: Summary table for the results of GLM repeated measurements analyses for number of visits.

Tests of Within-Subjects Contrasts	**d.f.**	***F***	***P***
preference OB vs IB (directed)	1	8.0	**0.004**
preference OB vs IB × female inbreeding status	1	1.9	0.18
preference OB vs IB × male infection status (directed)	1	0.0	0.62
preference OB vs IB × female inbreeding status × male infection status	1	0.0	0.86
Error (OB vs IB)	45		
Tests of Between-Subjects Effects	**d.f.**	***F***	***P***
female inbreeding status	1	0.3	0.62
male infection status	1	3.4	0.07
female inbreeding status × male infection status	1	0.3	0.56
Error	45		

(OB = outbred; IB = inbred).

nounced among the inbred females compared to outbred ones. Our findings suggest that female house mice may prefer to mate with heterozygous males, and especially so if they have reduced heterozygosity themselves, which suggests a novel version of the 'heterozygosity-as-good-genes' hypothesis. Since we controlled for male-male interactions, our results cannot be due to outbred males being more socially dominant, and females simply preferring dominant males [63]. We suspect that inbreeding reduced the health and condition of the males, but we found no evidence that an experimental *Salmonella* infection increased the relative attractiveness of the outbred males. However, we cannot rule out the possibility that infection or other stressors would magnify the differences because, surprisingly, inbreeding had no detectable effect on the males' pathogen clearance in this study. When the two males in a trial were both infected (experimental infection-group), we found that the females tended to move between the males more frequently than when the males were both uninfected (sham-controls). This difference was not statistically significant ($P = 0.07$), but it suggests that the females may have more difficulty distinguishing the quality of the males when they are both infected, which is the opposite of what we assumed.

Our findings raise the possibility that inbred, homozygous females may gain more genetic (fitness) benefits by mating with heterozygous males compared to outbred, heterozygous females (see Table 2 in [12]). Most previous theoretical models do not support the idea that mating with heterozygous males will increase female fitness, or contribute to maintaining genetic variation in male traits or female preferences (the so-called 'lek paradox') (reviewed in [10]). Some have suggested that the model might work in fluctuating environments [1,8,9], or in small populations with genetic drift [10,11]. These conditions might be more realistic than often assumed, and especially so for species like house mice that live in small demes consisting of related individuals [64]. Two recent papers that incorporated finite population size and genetic drift [12] or populations with spatial genetic structure [13] found that inbreeding co-efficient (*f*) or hetero-

Table 4: Summary table for the results of GLM repeated measurements analyses for total duration

Tests of Within-Subjects Contrasts	**d.f.**	***F***	***P***
preference OB vs IB (directed)	1	2.3	0.08
preference OB vs IB × female inbreeding status	1	1.3	0.27
preference OB vs IB × male infection status (directed)	1	0.4	0.32
preference OB vs IB × female inbreeding status × male infection status	1	0.1	0.80
Error (OB vs IB)	45		
Tests of Between-Subjects Effects	**d.f.**	***F***	***P***
female inbreeding status	1	0.1	0.72
male infection status	1	1.8	0.19
female inbreeding status × male infection status	1	2.3	0.14
Error	45		

(OB = outbred; IB = inbred).

zygosity can be inherited, that female mate choice for outbred or heterozygous males can evolve, and that the "heritability" of *f* or heterozygosity, and hence the non-additive benefits for females, are highest in small populations. However, like [10], these models assume that heterozygotes have higher fitness due to overdominance, which is extremely rare (and observations of heterozygote advantage can be due to dominance rather than overdominance, and experiments support this interpretation [65]), and therefore, unlikely to provide a general solution. Our findings suggest that future models should incorporate the possibility that female preferences may be conditional depending upon their own heterozygosity. In genetically structured populations, heterozygous males may be more likely to carry locally rare and dissimilar alleles, which could be particularly important for homozygous females to increase offspring heterozygosity and reduce inbreeding (see also [66] and [18]). Females may gain other types of genetic benefits by mating with heterozygous males, such as increasing the within-brood genetic diversity of offspring [1,67,68], or optimizing the heterozygosity of offspring [69-71].

On the other hand, mating with heterozygous males may provide no genetic benefits for females; however, as previously mentioned, it may provide *direct benefits*. For example, in house mice, outbred males defend territories more effectively than inbred ones [28], which should reduce the risks of infanticide and sexual harassment by other males, and in other species, improve parental care. Such direct benefits might be relatively more important to inbred females since they are poorer parents than outbred females [51-54].

Our findings also raise questions about the proximate mechanisms controlling males' scent-marking behaviour and females' odour preferences. They indicate that inbreeding alters males' scent-marks, either by reducing the quantity or quality of marks they produce. Condition-dependent sexually selected traits are thought to be especially vulnerable to negative inbreeding effects, because male's overall condition and health is influenced by multiple genes, and hence provide a large mutational target [72], which is why outbred, heterozygous, males are expected to be able to invest more into costly secondary sexual traits [73]. We suspect that inbred males have lower androgens than outbred males, and subsequently reduced scent-marking, androgen-dependent Major Urinary Proteins (MUPs) and sexual pheromones in their urine. This seems likely since inbreeding impairs males' testicle size and function [31-33], courtship behaviour [27,36-38] and the expression of other secondary sexual traits [7,39-42]. It is difficult to understand why low quality males do not 'cheat' and produce more attractive scent-marks, unless scent-marking is costly and low quality males cannot afford the costs (handicap or costly signalling hypothesis), or unless it is physiologically impossible for males to produce compounds in their urine that would disguise poor health or condition [55,74-76]. The idea that quality of males' scent marks is influenced by heterozygosity, and thus potentially allow females to distinguish males with different levels of genetic diversity and relatedness, is supported by a recent study in ring-tailed lemurs (*Lemur catta*) that found that the chemical composition of males' odour reflects marker-based heterozygosity [77]. Moreover, inbred females may be more likely to recognize heterozygous males because, as we previously pointed out, such males may carry dissimilar and unfamiliar (locally rare) alleles, at least in genetically structured populations, such as found in house mice [64]. If female odour preferences are based on phenotypic matching, it should be an easier olfactory task for homozygous females to recognize novel and dissimilar alleles carried by heterozygous males than for heterozygous females. A recent study found that wild-derived females prefer to associate with male mice derived from crosses of laboratory strains that were heterozygous at markers linked to MUP genes [78], but it is unclear whether this is due to differences in males' *scent*. Also, the males in this study were allowed to interact before the trials, which might explain the results, as females prefer the scent of dominant males [63]. We would expect that male-male interactions would magnify differences in the attractiveness of homozygous versus heterozygous males [28], but this idea has not been tested. It would be interesting to know if females' preferences are influenced by their own MUP heterozygosity, and whether such preferences are affected by their own condition.

Conclusion

To conclude, our findings provide experimental support for the 'good-genes-as-heterozygosity'-hypothesis by showing that female mice prefer outbred males over the inbred ones. Furthermore, our results imply that this preference could be stronger among inbred females, which is in good agreement with predictions that inbred females have more to gain by preferring heterozygous males. Thus, there appears to be no 'best' strategy for every female when choosing among males with different heterozygosity levels. It is unclear from our study whether the females' preferences for scent-marks predict their actual mating preferences in the wild, but if so, our results have important implications for several issues in behavioural ecology, evolutionary biology, and conservation biology. Firstly, our findings suggest that mating preferences could help explain why inbred males have such a low reproductive success when they must compete for mates [27,28,30]. Secondly, our results contribute to the current debate on sexual selection theory and, in particular, how the non-additive genetic benefits could maintain additive genetic variance in male secondary sexual traits and con-

sequently directional mating preferences in females, which has been advocated at least as a partial resolution to the lek-paradox ([13,16,12,66], but see [43,10] and [11] for criticisms). Lastly, our results suggest that female preferences for heterozygous males may provide a selective factor against inbred males expressing deleterious, recessive mutations, and thus could help to maintain genetic diversity in endangered small populations (see also [17,10,12,79]).

Authors' contributions

PI, GS and DJP conceived and designed the experiments. GS, MT and PI carried out the experiments, and PI ran the statistical analyses. PI and DJP wrote the paper. All authors read and approved the final manuscript.

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General discussion

1. Main findings

Our data support the idea that MHC heterozygosity plays a role in sexual selection of wild house mice. We provide first evidence that MHC heterozygosity can enhance mating and reproductive success when inbreeding is controlled (Chapter I). Furthermore, we find assortative mating for MHC heterozygosity and provide first evidence for parent-offspring correlations in heterozygosity in a mammal (Chapter II). We find poor mating and reproductive success in homozygous males, however this cannot be attributed to reduced aggressiveness as inbreeding did not reduce the ability of males to win short, agonistic encounters (Chapter III). Poor mating and reproductive success in homozygous males is rather due to female preferences as we show that females, in general, prefer the scent of outbred, heterozygous males and this preference is especially pronounced in inbred, homozygous females (Chapter IV). Below we address the main findings in more detail.

2. MHC heterozygosity increases reproductive success

We show that multilocus heterozygosity (15 microsatellite loci) was associated with increased mating and reproductive success for both sexes in semi-natural enclosures; however this result can be entirely explained by heterozygosity at two MHC loci. We can rule out potential confounds from background heterozygosity as MHC heterozygotes and homozygotes were systematically outbred for two generations. This is the first evidence showing that heterozygosity at functional MHC loci can enhance reproductive success when inbreeding is controlled.

This finding is not due to MHC heterozygotes having higher survival than homozygotes, but rather MHC heterozygotes have improved fertility, an advantage in sexual selection or both. MHC heterozygotes were more likely to breed successfully than homozygotes, though it is not entirely clear why. We find no evidence that MHC heterozygosity enhances males' social status, as socially dominant and subordinate males did not differ in MHC heterozygosity. However, we cannot rule out a potential role of intra-sexual selection among socially dominant males since MHC heterozygosity enhances reproductive success only among socially dominant males, but not among subordinates.

MHC heterozygosity might improve health and, in turn, enhance social status, sexual attractiveness and fecundity which can explain previous findings (Thornhill et al. 2003; Roberts et al. 2005; Husak et al. 2006; Charpentier et al. 2008b). Alternatively, females might prefer MHC heterozygotes, regardless of their health (Roberts et al. 2005). Females can discriminate MHC-heterozygotes from homozygotes based on odor cues (Yamazaki et al. 1984; Willse et al. 2005), and evidence from other species suggests that females prefer MHC-heterozygous males (Reusch et al. 2001; Sauermann et al. 2001; Thornhill et al. 2003; Roberts et al. 2005; Bonneaud et al. 2006). MHC heterozygotes might carry rare or dissimilar alleles (Mitton et al. 1993) and mating with MHC heterozygotes might enhance offspring genetic diversity and provide an advantage in survival and pathogen resistance (Penn and Potts 1998).

3. Heterozygosity is heritable: A role for rare alleles

We investigated non-random mating patterns with respect to heterozygosity and find assortative mating for MHC heterozygosity in our semi-natural enclosures. This pattern of assortative mating is not necessarily due to direct female preferences for MHC heterozygotes as this bias might have arisen through intra- and intersexual competition for healthy, high quality mates. Our findings provide support for the heterozygosity-as-good-genes hypothesis or HGG hypothesis (Brown 1997) that females should prefer to mate with heterozygous males to increase offspring heterozygosity. We find that parents with high overall heterozygosity produced more heterozygous offspring and more homozygous parents produced homozygous offspring. This is the first evidence for a parent-offspring correlation in heterozygosity in mammals and supports the idea that heterozygosity can be heritable, at least in a broad sense. Further support for the HGG hypothesis comes from our finding that parental heterozygosity increases two other measures of offspring diversity: number of alleles and allelic richness in the litter.

Our analysis of rare alleles provides a potential mechanism to explain broad sense heritability of heterozygosity. We experimentally show, for the first time, that heterozygous individuals carry more rare alleles than homozygotes, and that heterozygous parents confer a higher number of rare alleles to their offspring than homozygous parents. Offspring of heterozygous parents are heterozygous themselves, as they possess more rare alleles than offspring of homozygous parents and litters of heterozygous parents are more genetically diverse. This mechanism can also help to resolve the lek paradox (Borgia 1979) as it is consistent with the interpretation that sexual

selection reinforces some sort of balancing selection such as negative frequency-dependent selection from pathogens (Hamilton and Zuk 1982).

4. Heterozygosity does not affect male competitive ability

We tested whether the poor mating and reproductive success of less heterozygous males in semi-natural enclosures is due to detrimental effects of inbreeding on competitive ability. We find no evidence that one generation of full-sib inbreeding negatively affected males' competitive ability. Therefore, our results do not support the hypothesis that lower reproductive success of inbred males (Meagher et al. 2000) is due to reduced aggression or competitive ability (Eklund 1996). Additionally and contrary to earlier findings (Ilmonen et al. 2008), we find no evidence that *Salmonella* infection magnifies the harmful effects of inbreeding. Statistically, full-sib inbred males are homozygous at every fourth locus (Wright 1922; Mitton 1994); however we do not find any negative impact of inbreeding on *Salmonella* resistance. We aimed at replicating an earlier study (Eklund 1996); however we were not able to replicate the findings although we avoided pseudo-replication and employed clear statistical methods.

We want to stress the point that short-term aggression assays (as the one used in this study) may be useful tools for examining certain aspects of aggressive behavior. However, long-term assays do seem more suitable to investigate all aspects of competitive ability as well as effects of certain treatments on competitive ability and aggressiveness. Furthermore, aggressiveness and the ability to win short-term interaction might be neither correlated with reproductive success nor with the probability to become socially dominant in a more natural setting (Oakeshott 1974; Shackleton 2005). Females

might avoid highly aggressive males (Qvarnström and Forsgren 1998; Spence and Smith 2006) and prefer males of intermediate aggressiveness as mates whereas male-male competition favors highly aggressive males. These results may hint to opposed selection pressures arising from female choice and male-male competition.

5. Females, especially homozygous females, prefer heterozygous males

The poor mating and reproductive success of less heterozygous males in semi-natural enclosures could also be due to female mate preferences. We found that, in general, females preferred the scent of outbred, heterozygous males and this preference was more pronounced in inbred, homozygous females. This finding suggests that female house mice may prefer to mate with heterozygous males, especially if they are themselves homozygous. It is therefore possible that inbred, homozygous females gain more indirect (genetic) benefits by mating with heterozygous males compared to outbred, heterozygous females (Neff and Pitcher 2008). We present a novel interpretation of the heterozygosity-as-good-genes hypothesis and suggest future studies should incorporate female preferences as conditional dependent on their own heterozygosity.

Females should prefer to mate with heterozygous males as they are more likely to carry locally rare alleles (see Chapter II) which are especially important for inbred, homozygous females to enhance within-brood genetic diversity (Charlesworth 1988; Brown 1997) or optimize offspring heterozygosity (Penn and Potts 1999; Reusch et al. 2001; Aeschlimann et al. 2003). Furthermore, scent marks are a condition-dependent sexually selection trait (Zala et al. 2004) and therefore particularly vulnerable to inbreeding (Rowe and Houle 1996). We suggest that inbred males have lower androgen

levels and therefore show reduced scent marking and reduced concentrations of androgen-dependent major urinary proteins (MUPs) and sexual pheromones in their urine. Consequently, females might assess the genetic diversity of potential mates through the quality of males' scent marks which, in turn, is influenced by heterozygosity. This idea is supported by a recent study in ring-tailed lemurs (Lemur catta) showing that the chemical composition of males' odor reflects marker-based heterozygosity (Charpentier et al. 2008a). Our findings propose that female mate preferences for heterozygotes could explain the poor mating and reproductive success of homozygous males when they must compete for mates in a semi-natural setting.

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General Summary

Heterozygosity is often associated with improved individual fitness as it enhances survival and reproductive success. Inbreeding depression is the most remarkable example of the importance of heterozygosity. However, the role of heterozygosity in sexual selection is controversial. Homozygous males show reduced mating and reproductive success as inbreeding impairs males' competitive ability. According to the heterozygosity-as-good-genes hypothesis, females should prefer to mate with outbred, heterozygous males to improve offspring heterozygosity; however, the evidence is mixed. We investigated effects of genome-wide heterozygosity on different fitness components, especially reproductive and mating success, survival and male social dominance in populations of wild-derived house mice (*Mus musculus musculus*) living in semi-natural enclosures (Chapters I and II) and provide first evidence that heterozygosity at major histocompatibility complex (MHC) can enhance mating and reproductive success when inbreeding is controlled. We found assortative mating for MHC heterozygosity and provide first evidence for parent-offspring correlations in heterozygosity in a mammal. Furthermore, we suggest that rare alleles can help to explain broad sense heritability of heterozygosity. We also find poor mating and reproductive success in homozygous males; however, this cannot be attributed to reduced aggressiveness in inbred males as inbreeding did not reduce the ability of males to win short, agonistic encounters in an arena setting (Chapter III). Reduced reproductive success in homozygous males might rather be due to female preferences for heterozygous males as we show that females prefer the scent of outbred, heterozygous males and this preference is especially pronounced in inbred, homozygous females (Chapter IV).

Allgemeine Zusammenfassung

Genetische Heterozygotie ist häufig mit verbesserter individueller Fitness verbunden, da sie die Überlebenswahrscheinlichkeit und den Reproduktionserfolg erhöht. Inzucht-depression ist ein aussagekräftiges Beispiel für die Wichtigkeit genetischer Heterozygotie. Die Rolle von Heterzygotie in sexueller Selektion hingegen ist umstritten. Homozygote Männchen zeigen einen reduzierten Verpaarungs- und Reproduktionserfolg, da Inzucht die männliche Konkurrenzfähigkeit beeinträchtigt. Im Sinne der „Heterozygotie-als-Gute-Gene"-Hypothese sollten Weibchen sich bevorzugt mit ausgezüchteten, heterozygoten Männchen verpaaren, um so die Heterozygotie des Nachwuchses zu erhöhen; jedoch fehlen eindeutige Belege.

Wir untersuchten in wilden Hausmauspopulationen (*Mus musculus musculus*), welchen Einfluß genomweite Heterozygotie auf verschiedene Fitnessparameter, im Besonderen Verpaarungs- und Reproduktionserfolg, Überlebenswahrscheinlichkeit und soziale Dominanz, hat und belegen zum ersten Mal, dass Heterozygotie am Haupthistokompatibilitätskomplex (MHC) Verpaarungs- und Reproduktionserfolg verbessern kann, sogar wenn Inzuchteffekte ausgeschlossen sind (Kapitel I und II). Des Weiteren finden wir assortative Verpaarungen in Bezug auf MHC-Heterozygotie und belegen zum ersten Mal eine Korrelation der Heterozygotie zwischen Eltern und deren Nachkommen bei einem Säugetier. Außerdem zeigen wir, dass seltene Allele die Erblichkeit von Heterozygotie im weiteren Sinne erklären können. Wie andere Studien finden auch wir einen geringen Verpaarungs- und Reproduktionserfolg bei homozygoten Männchen, jedoch können wir dieses Ergebnis nicht der reduzierten Aggressivität der homozygoten Männchen zuschreiben, da Inzucht keinen Einfluß auf die

Wahrscheinlichkeit hat, dass ein Männchen kurze Kämpfe einem Arenaaufbau gewinnt (Kapitel III). Der geringe Verpaarungs- und Reproduktionserfolg von homozygoten Männchen ist wohl eher auf Verpaarungspräferenzen der Weibchen zurückzuführen, da wir zeigen konnten, dass Weibchen den Geruch von ausgezüchteten, heterozygoten Männchen präferieren und diese Präferenz ist bei ingezüchteten, homozygoten Weibchen besonders stark ausgeprägt (Kapitel IV).

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