universität wien

DISSERTATION

Titel der Dissertation

„Promiscuity in female house mice (*Mus musculus musculus*)“

verfasst von

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angestrebter akademischer Grad

Doctor of Philosophy (PhD)

Wien, 2013

Studienkennzahl lt. Studienblatt:	A 094 437
Dissertationsgebiet lt. Studienblatt:	Biologie
Betreut von:	Univ.- Doz. Dr. Dustin J. Penn

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I General Introduction

Polyandry or multi-male mating (MMM) is widespread, occurring in at least 133 species in mammals (Wolff 2004) for example, but the evolutionary significance of this behaviour remains elusive and controversial (Jennions and Petrie 2000; Hosken and Stockley 2003; Gowaty 2012). Bateman's principle states that males' reproductive success will increase with the number of mates they obtain, whereas females' should be able to obtain sufficient sperm to fertilize their eggs from one male, and should not increase their reproductive success by obtaining more mates (Bateman 1948; Trivers 1972). Polyandry is especially puzzling since mating with multiple males has so many potential costs. For example, multiple mating can potentially increase females' risk of predation (Rowe 1994), disease transmission (Magnhagen 1991), injuries (Siva-Jothy 2006) and energetic loss (Daly 1978). So why do females of so many species mate multiply?

Several (non-mutually exclusive) hypotheses have been proposed to explain how females might increase their survival and lifetime reproductive success through polyandry. First, females can potentially enhance their survival (and offspring's survival) by mating with multiple males that provide food, paternal investment or other resources (*hypothesis 1: direct survival benefits*) (Hosken and Stockley 2003). Second, polyandry might function to increase females' fertility if their mates are sperm limited (*hypothesis 2: direct fertility benefits)* (Hoogland 1998). Third, MMM may enable females to enhance their fitness by increasing the quality, compatibility, or diversity of mates (Jennions and Petrie 2000; Simmons 2005) (*hypothesis 3: indirect or genetic benefits)*. Females can achieve genetic benefits either through the acquisition of (a) "good genes" in promoting sperm and other forms of male-male competition (Kempenaers et al. 1992) (*good gene hypothesis*); (b) enhanced genetic compatibility of maternal and paternal genomes (Zeh and Zeh 1997), including inbreeding avoidance (Tregenza and Wedell 2002) (*compatible gene hypothesis*); or (c) increased offspring genetic diversity though a bet-hedging and a non-bet-hedging mechanism (Yasui 1998) (g*enetic diversity hypothesis*). There have been many attempts to test these hypotheses, though most of these studies are observational rather than experimental, and we still understand very little about the potential genetic benefits of polyandry.

My aim was to conduct experiments to test hypotheses about the variation and adaptive functions of polyandry in wild-derived house mice *(Mus musculus musculus)*. House mice provide the premier model species for genetics and biomedical sciences, but, while the vast majority of this work is conducted with inbred, laboratory strains, there are still surprisingly few studies on polyandry in *wild,* outbred house mice (*Mus musculus domesticus* from Western Europe and Northern America and *Mus musculus musculus* in Central and Eastern Europe). Therefore, in Chapter 1, we conducted a field survey to investigate the frequency of multiple paternity in wild house mice (*Mus musculus musculus*) poulations. Male house mice compete for territories, and females mate with the territorial male. Studies conducted with mice in semi-natural enclosures find that females sometimes mate with multiple males (Oakeshott 1974; Carroll et al. 2004; Ehman and Scott 2004; Montero et al. 2013). The first studies to examine multiple paternity in free-ranging, wild house mice confirm that polyandry is fairly common (i.e., ca. 25% of pregnancies contain offspring from more than one sire) (Dean et al. 2006; Firman and Simmons 2008a). Interestingly, there is high variability in the rate of multiple paternity, ranging from 6 to 43% of pregnancies in different populations. The reasons for the enormous variation in MMM are unclear, and it could be the result of females changing their behaviour depending upon their condition or circumstances (conditional, phenotypic plastic mating tactic), genetic differences among populations (genetic polymorphisms), or both (Taborsky et al. 2008). Studies are needed to understand the nature of this variation in multiple paternities, especially since such work would provide an important step towards understanding the functions of MMM. Therefore, in Chapter 2, we repeatedly tested individual females and assessed whether they showed consistent individual variation in multiple paternity or not.

Male house mice provide no parental care, and so it has been difficult to understand how MMM could provide females with direct benefits. Rather than providing benefits, multiple mating may allow females to reduce costs from sexual conflict: females may mate multiply to reduce sexual harassment (*convenience polyandry*) (Thornhill and Alcock 1983; Clutton-Brock and Parker 1995) or infanticide (*infanticide avoidance*) (Hrdy 1979; Wolff and Macdonald 2004). In bank voles *(Myodes glareolus*)*,* for example, females that mated with multiple males had higher offspring survival than monogamous females, likely due to reduced infanticide (Klemme and Ylönen 2010). Infanticide is very common in house mice (Huck et al. 1982; Elwood and Ostermeyer 1984), and yet there

are no studies to my knowledge that have tested the infanticide avoidance hypothesis in house mice. In Chapter 2, we aimed to test whether females actively engage in MMM when unconstraint by male coercion and are more likely to mate with multiple males when they perceive a higher risk of infanticide.

Male mice scent mark their territory and females can assess male quality on the basis of urinary scent (Zala et al. 2004). It is often assumed that scent marking is a secondary sexual trait that enhances males' mating and reproductive success, though direct evidence for this hypothesis is lacking. In Chapter 3, we tested this hypothesis in assessing whether the amount of male scent marking is related to male reproductive success when females can freely choose among males.

Since male house mice provide no resources to females, other than territorial defence, multiple mating may also function to provide indirect, genetic benefits. For example, females that mated with three different males within one oestrus cycle had increased post natal pup than females that mated three times with the same male (Firman and Simmons 2008c). Also, it has been shown that multiply mated females could bias paternity towards unrelated males thereby facilitating inbreeding avoidance (Firman and Simmons 2008b). Finally, a recent study revealed that females from polyandrous selection lines had increased reproductive benefits as their sons were more successful in siring offspring under semi-natural conditions (Firman 2011). All these studies focused on testing good genes or compatible genes hypothesis and only little attention has been paid to the genetic diversity hypothesis. Increased genetic diversity among offspring can have positive effects on litter performance and survival, but it has never been experimentally tested whether females actively engage in MMM when they have the opportunity to enhance the genetic diversity of their offspring. Therefore in Chapter 4, we aimed to test this hypothesis. One way how increased genetic diversity among offspring can enhance female fitness is in reducing the prevalence and intensity of infections. Various studies in social insects revealed that multiply sired colonies are less vulnerable to infectious disease (Baer and Schmid-Hempel 1999; Seeley and Tarpy 2007). However, as it is not known whether such benefits also apply to other taxa than insects we finally tested in Chapter 5 whether multiple sired litters are better able to resist and clear experimental infections than single sired ones.

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Chapter 1

Multiple paternity in wild house mice (*Mus musculus musculus*): effects on litter genetic diversity and offspring body mass

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This manuscript is under review in "Journal of Evolutionary Biology".
Date of submission: May 31st, 2013

ABSTRACT

Multi-male mating is common in many species, but it is unclear whether multiple paternity enhances offspring genetic diversity or fitness. We conducted a survey on wild house mice (*Mus musculus musculus)*, and we found that in 73 pregnant females, 29% of litters had multiple sires, which is similar to the 23% and 26% found in feral populations of *Mus musculus domesticus* in the USA and Australia respectively. We found no evidence that multiple paternity resulted in larger litters, contrary to the fertility assurance, the good genes or the compatible genes hypotheses. Interestingly, multiple paternity was associated with reduced mean and variance in offspring body mass. This finding suggests that females allocate fewer resources, or that there is increased sibling rivalry in multiple versus single sired litters. We found increased allelic diversity (though not heterozygosity) in multiple versus single sired litters, as predicted by the genetic diversity hypothesis. Finally, we found that the dams' heterozygosity was correlated with the mean heterozygosity of their offspring in single and multiple sired litters, suggesting that outbred, heterozygous females were more likely to avoid inbreeding than inbred, homozygous females. The similar rates of multiple paternity in wild *Mus musculus musculus* and *Mus musculus domesticus*, suggests that multi-male mating has been maintained in these subspecies at similar levels since their evolutionary divergence ca. 2800 to 6000 years ago. Future studies are needed to examine the fitness consequences of increased genetic diversity of litters and smaller mean (and variance) of offspring body mass associated with multiple paternity.

INTRODUCTION

Polyandry or multi-male mating (MMM) is common in diverse animal taxa ranging from insects to mammals (Arnqvist and Nilsson 2000; Wolff and Macdonald 2004) and although this behaviour has been studied extensively over the past decades, there is still continuous debate over its function. Male reproductive success is usually limited by the number of mating partners acquired, whereas female reproductive success is potentially limited by the number of ova produced (Bateman 1948; Trivers 1972). Therefore, unlike males, it is not obvious how females can increase their reproductive success by MMM. There is accumulating evidence that females in many species actively engage in MMM (Berteaux et al. 1999; Rolland et al. 2003; Westneat and Stewart 2003), despite a variety of potential costs, including an elevated risk of disease transmission, predation and

injuries from potential mating partners (Daly 1978; Magnhagen 1991; Siva-Jothy 2006). These costs suggest that there are compensating benefits for MMM, and several non-mutually exclusive hypotheses have been proposed (Jennions and Petrie 2000; Hosken and Stockley 2003). The benefits are divided into three groups and they can either be direct, cryptic or indirect (see Table 1). In non-resource based mating systems in which males provide no parental care, explanations of polyandry largely rely on indirect or genetic benefits (Simmons 2005). Females can gain such benefits either through (1) good genes (Kempenaers et al. 1992), (2) increased genetic compatibility (Zeh and Zeh 1997), including inbreeding avoidance (Tregenza and Wedell 2002) or (3) enhanced genetic diversity for their offspring (Cohas et al. 2007). The good genes and compatible genes hypotheses assume that multiple mating enhances female fitness in increasing the number or quality of offspring produced (Madsen et al. 1992; Tregenza and Wedell 1998; García-González and Simmons 2005; Fisher et al. 2006), whereas the genetic diversity hypothesis assumes that females gain fitness benefits from MMM in producing genetically more diverse clutches (Yasui 1998). This strategy serves as a bet-hedging mechanism against unstable environments or fast evolving parasites (Baer and Schmid-Hempel 1999) and ensures that at least some genotypes within a clutch will fit the current environmental conditions and survive. The genetic diversity hypothesis does not necessarily predict an increase in offspring number or quality if females mate with multiple mates, but that the variation in fitness among multiple sired litters is reduced in comparison to the variation among single sired litters (Table 1).

The mating system of the house mouse (*Mus musculus)* was long thought to be harem polygyny in which females mate exclusively with the dominant territorial males, but genetic paternity analyses revealed that MP was common in enclosure populations (Potts et al. 1991; Montero et al. 2013) and feral populations of house mice (*Mus musculus domesticus)* in the USA (Dean et al. 2006) and Australia (Firman and Simmons 2008a). In addition, behavioural observations indicated that female mice actively engage in MMM (95% of females mated with both males when given a choice between a dominant and a subordinate male, Rolland et al. 2003). Also comparative analyses on testis size suggest that MMM is common in house mice (Firman and Simmons 2008a; Soulsbury 2010). On average 25% of the wild *Mus musculus domesticus* litters are multiple sired, though it is unclear why there is so much variation among different populations (6% to 43%, Dean et al. 2006; Firman and Simmons 2008a) and how this relates to *Mus*

musculus musculus populations in Europe. Population density and the number of males in female vicinity both correlate with MP (Dean et al. 2006; Klemme et al. 2007). In addition, the rate of MMM might show seasonal variation as food availability – an important determinant of population dynamics – varies strongly over seasons. However, this hypothesis has never been investigated before to our knowledge. Experiments under laboratory conditions revealed that female house mice gain indirect genetic benefits from MMM. For example, females can increase post-birth pup survival when mating with three different males in comparison to mating three times with the same male (Firman and Simmons 2008c). Also, polyandry facilitates inbreeding avoidance (Firman and Simmons 2008b) and produces sons that are superior in sperm competition (Firman 2011). However, it is unclear whether or how these findings apply to natural populations as selection is likely to be stronger in the wild and the degree of MMM might vary according to population demographics and environmental circumstances.

In this study we investigated the frequency of MP in wild house mice (*Mus musculus musculus)* and we compared two distinct populations over different seasons. This is the first such study on this subspecies and the first survey on house mice in Europe. We assessed whether single or multiple paternity differentially affected female fitness in measuring litter size, pup body mass, male sex ratio, litter genetic diversity and litter observed heterozygosity. In addition, we used these fitness measurements to assess what kind of benefits females can gain from polyandry.

Table 1: Overview of the potential fitness benefits females can gain from polyandry and the expected consequences in a natural population of house mice.

Function	Hypothesis	Description	Expected fitness consequences	References
Direct benefits	Material benefits hypothesis	Polyandry provides females with material benefits (e.g. nuptial gifts, parental care, or other resources from males)	Female house mice are unlikely to gain material benefits from polyandry as they live in a non-resource based mating system where males provide no parental care	(Arnqvist and Nilsson 2000; Hosken and Stockley 2003)
Cryptic benefits	Convenience polyandry	Polyandry functions to avoid costs arising from rejecting multiple males as mates	MP rate is not positively correlated with litter size or pup body mass MP rate is not positively correlated with litter genetic diversity or heterozygosity	(Thornhill and Alcock 1983)
	Infanticide avoidance	Polyandry serves to conceal paternity to prevent infanticide from unmated males	MP rate is positively correlated with litter size but not with pup body mass MP is not positively correlated with litter genetic diversity or heterozygosity	(Hrdy 1979; Wolff and Macdonald 2004)
	Fertility assurance	Polyandry protects against sperm depletion or genetically incompatible males	MP rate is positively correlated with litter size but not with pup body mass MP in not positively correlated with litter genetic diversity or heterozygosity	(Hoogland 1998; Stockley 2003)
Indirect benefits	Good gene hypothesis	Polyandry provides females with intrinsic male quality which increases offspring viability	MP rate is positively correlated with litter size and pup body mass MP rate is not positively correlated with litter genetic diversity or heterozygosity	(Hosken et al. 2003; García-González and Simmons 2005)
	Genetic compati-bility hypothesis	Polyandry provides females with more compatible genes (e.g. inbreeding avoidance)	MP rate is positively correlated with litter size but not pup body mass MP rate is not positively correlated with litter genetic diversity but with offspring heterozygosity	(Tregenza and Wedell 1998; Tregenza and Wedell 2002)
	Genetic diversity hypothesis	Polyandry as a bet-hedging strategy against fast evolving parasites or unpredictable environments	MP is not positively correlated with litter size or pup body mass Fitness variance is smaller in multiple than single sired litters MP rate is positively correlated with litter genetic diversity but not with offspring heterozygosity	(Yasui 1998; Cohas et al. 2007)

Footnote: Adapted from (Wolff and Macdonald 2004) and (Lane et al. 2008) to the relevance of the house mouse mating system.

MATERIALS AND METHODS

Animal trapping and housing

Trapping was conducted from January to September (winter, spring and summer season) on regular intervals in the years 2004 to 2007 and 2010. In total we trapped 73 pregnant female house mice at two different sites in and around Vienna, Austria (KLIVV: 48°12'38"N, 16°16'54"E, *N*=65; Safaripark: 48°18'22"N, 16°43'48"E, *N*=8).

For animal trapping we used Sherman live traps. Traps were provided with food (piece of apple, peanut butter and dry bread) and nesting material (wood shavings and cotton). Trapping was conducted either during dusk or dawn, and traps were checked regularly for occupancy. Pregnant females were housed in Type IIL cages (Tecniplast, 32.5 × 16 × 14 cm). 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*. Offspring were weaned at 21 ± 1 days and kept under standard colony conditions. All pups were sexed at weaning and litter size was recorded at birth. In 2010 we additionally measured pup body mass (g) at weaning (*N*=30). Ear punches were collected for individual identification, and tissues were stored at -20°C for subsequent genetic analyses.

Genotyping and paternity analysis

DNA was extracted from ear punches using a proteinase K/isopropanol protocol (Sambrook et al. 1989). A total of 73 adult females and 369 offspring were genotyped at a subset of 16 microsatellite loci (D1Mit404, D1Mit456, D2Mit252, D2Mit380, D5Mit25, D6Mit138, D7Mit227, D9Mit34, D9Mit135, D10Mit20, D11Mit150, D15Mit16, D17Saha, D17Mit28; D17Mit 21, D19Mit39; see Mouse Microsatellite Data Base of Japan) using a Multiplex-PCR MasterMix (Qiagen Multiplex PCR kit). In the years 2004 to 2007, females and offspring were typed on average at 12.1 loci; however, no individual was typed at less than 10 loci. In 2010, mice were typed on average at 13.3 loci and at a minimum of 11 loci. The markers are located on 11 different chromosomes and were previously screened to confirm that they were polymorphic. The markers include three microsatellites closely linked to major histocompatibility complex (MHC): D17Saha and D17Mit21 are located within the MHC class II E beta locus and A beta locus, respectively (Saha and Cullen 1986; Meagher and Potts 1997), D17Mit28 is adjacent to MHC class I K locus (Dietrich et al. 1996; Meagher and Potts 1997). 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 analysed 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. Estimated number of fathers per litter was obtained using the program GERUD 2.0 (Jones 2005).

Estimating genetic diversity

Mean number of alleles per locus was calculated separately for each litter using the program FSTAT developed by Jérôme Goudet (downloadable from: http://www2.unil.ch/popgen/softwares/fstat.htm). Observed multilocus heterozygosity (number of heterozygous loci divided by the total number of genotyped loci) was calculated using IRmacroN4, a macro for Microsoft Excel written by Amos (downloadable from: http://www.zoo.cam.ac.uk/zoostaff/amos/#ComputerPrograms).

Statistical analyses

We conducted the following statistical analyses. First, to test for differences in the rate of multiple paternity among populations or trapping seasons, and to assess whether dams' heterozygosity was correlated with multiple paternity we applied a generalized linear mixed effects model (GLMM) with a binomial distribution and a logit link function using paternity (single or multiple) as the dependent variable, population and trapping season as fixed factors and dam's observed heterozygosity as a covariate. We included trapping year as random factor to control for the variation and non-independence across trapping years. Second, to determine whether litter size was affected by paternity or differed over population or trapping season, we applied a linear mixed effects model (LMM) with litter size as dependent variable, paternity, population and trapping season as fixed factors and trapping year as a random factor. As the likelihood of detecting multiple paternity increases with litter size, we also used a second measure, the paternity share, which is independent of litter size. Paternity share is an estimate of the probability that a pup was sired by another male than the primary male. Paternity share was calculated using the method of Eccard and Wolf (2009). Third, to test whether litter size predicted mean pup body mass, we ran a general linear model (LM) with mean pup body mass as the dependent variable and litter size as a covariate. We could not test for population differences in mean pup body mass as offspring body mass data were only available for the KLIVV population in 2010. Homogeneity of variances was tested using Levene Test.

To determine if multiple paternity affected offspring sex ratio, we calculated a GLMM with a binomial distribution and a logit link function with the number of male offspring as dependent variable, litter size as the binomial denominator and paternity, population and trapping season as fixed factors. Again we included trapping year as a random factor. Fourth, to test for differences in offspring genetic diversity we applied a LMM with the mean number of alleles per litter as dependent variable, paternity, population and season as fixed factors and litter size as a covariate. We included trapping year as a random factor. Finally, to test which factors influence offspring observed heterozygosity, we ran a LMM with mean offspring heterozygosity within the litter as dependent variable and paternity, population and season as fixed factors. We included observed heterozygosity of the dam as a covariate into the model to test whether the dam's observed heterozygosity correlated with mean offspring heterozygosity. Again we included trapping year as a random factor. We verified that model assumptions (i.e., normally distributed residuals and homogeneity of variances) were fulfilled and transformed data if necessary. We applied a backward stepwise removal procedure (Grafen and Hails 2002) to avoid problems due to inclusion of non-significant terms (Engqvist 2005) and the removed variables were re-entered one by one to the final model to obtain relevant statistics. Statistical analyses were performed using 'R' (version 2.14.1) (R Development Core Team 2012). We implemented linear mixed effects models using the 'lme' function of the 'nlme' package, and generalized mixed effects models using the 'lmer' function in the 'lme4' package. For post-hoc analyses we used the 'glht' function of the 'multcomp' package.

RESULTS

Overall, we found that 21 of 73 litters had multiple sires, and all litters had two sires except for one litter, which was sired by three males. Thus, multiple paternity was estimated as 29% (95% confidence interval: 19.2%-38.4%). The paternity share was estimated as 6.6% (95% confidence interval: 4.2%-9.2%). We found no difference in the frequency of multiple paternities between the populations (GLMM: χ^2=0.549, N=73, P=0.459) or between seasons (GZMM: χ^2=2.658, N=73, P=0.264).

We examined how multiple paternity correlated with litter size and pup body mass and we found that MP did not increase litter size (LMM: $F_{1,64}$=2.411, P=0.125) (Figure 1a). We found no difference in litter size between populations (LMM: $F_{1,64}$=0.180, P=0.673) or trapping seasons (LMM: $F_{2,64}$=1.529, P=0.225). Unexpectedly, we found that mean

and variance of pup body mass within litters were significantly smaller in multiple versus single sired litters (Wilcox rank sum test: W=153, N=30, P=0.037; Levene test: F=4.971, P=0.034, N=30) (Figure 1b). Mean pup body mass was not affected by litter size (LM: $F_{1,28}$=2.209, P=0.148). We found no evidence that MP affected the sex ratio of litters (GLMM: χ^2=0.344, N=63, P=0.557). Male sex ratio did not differ between populations (GLMM: χ^2=0.162, N=63, P=0.687) or over trapping seasons (GLMM: χ^2=4.892, N=63, P=0.087).

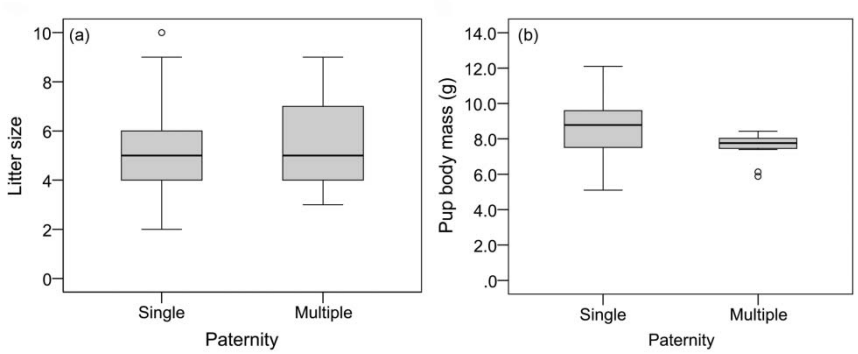

Figure 1: (a) Litter size of single and multiple sired litters and (b) mean pup body mass (g) within single and multiple sired litters.

We tested whether multiple paternity enhanced the genetic diversity of dams' litters. We found no difference in the mean observed heterozygosity between single and multiple sired litters (LMM: $F_{1,62}$=0.006, P=0.939) (Figure 2a). Nonetheless, we found the mean observed heterozygosity to be significantly greater in the Safaripark population (LMM: $F_{1,63}$=11.469, P=0.001) (Figure 2b) and a significant effect of season (LMM: $F_{2,63}$=3.585, P=0.034): Heterozygosity was significantly lower in litters trapped in winter compared to spring and summer (winter:spring: t=2.75, P=0.020; winter:summer: t=3.389; P=0.003, spring:summer: t=0.030, P=0.999) (Figure 3). Unlike heterozygosity, we found that the mean number of alleles within litters was significantly higher in multiple compared to single sired litters (LMM: $F_{1,65}$=4.235, P=0.044) (Figure 4a). Litter size had no influence on the mean number of alleles within litters (LMM: $F_{1,64}$=0.074, P=0.786). Also, we did not detect any seasonal differences (LMM: $F_{2,63}$=0.319, P=0.728). However, litters from the Safaripark population had a significantly higher number of alleles than litters from the KLIVV population (LMM: $F_{1,65}$=15.582, P<0.001) (Figure 4b).

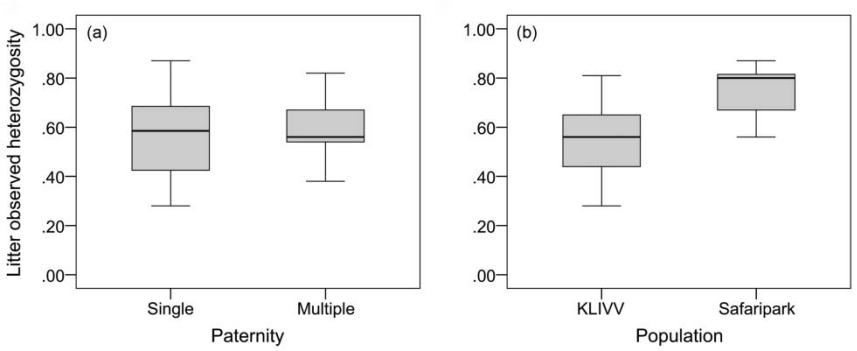

Figure 2: (a) Offspring mean observed heterozygosity of single versus multiples sired litters. (b) Offspring mean observed heterozygosity in the KLIVV and Safaripark populations.

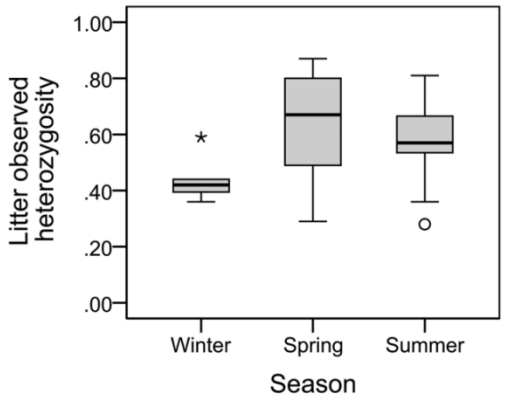

Figure 3: Offspring mean observed heterozygosity in litters born in winter, spring or summer.

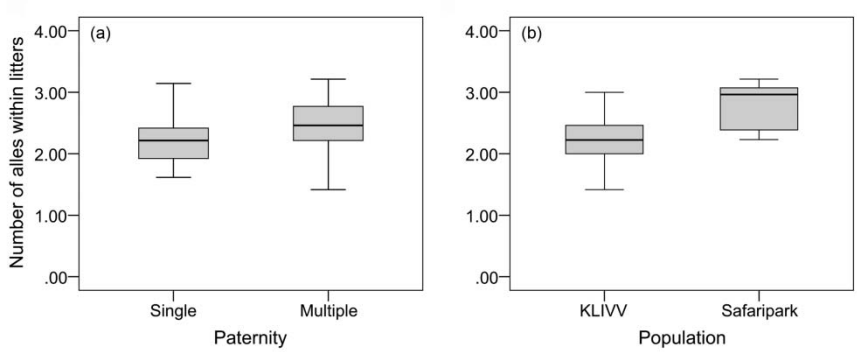

Figure 4: (a) Offspring mean number of alleles within single and multiple sired litters. (b) Offspring mean number of alleles within litters from the KLIVV or Safaripark population.

Finally, we tested whether more heterozygous mothers produce more heterozygous litters. We found that the dam's heterozygosity was significantly positively correlated with offspring mean heterozygosity (LMM: $F_{1,63}$=20.695, β=0.337, SE=0.074, P<0.001) (Figure 5). However, we found no evidence that more heterozygous females were more likely to have multiply sired litters (GLMM: χ^2=2.159, N=73, P=0.142).

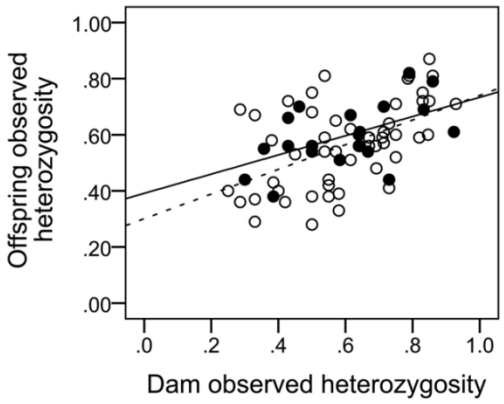

Figure 5: Correlation between dams' observed heterozygosity and offspring mean heterozygosity for single (white, dashed line R^2=0.27) and multiple (black, solid line, R^2=0.29) sired litters.

DISCUSSION

We found that MP in free ranging *Mus musculus musculus* populations is common (29% of litters were multiple sired), and though we found no differences between the two populations we surveyed (contrary to previous studies), this average rate is surprisingly comparable to feral *Mus domesticus* populations in the USA and Australia (average rate of 23% in the USA, Dean et al. 2006; and 26% in Australia, Firman and Simmons 2008a). Our findings indicate that MP is common in wild *Mus musculus musculus,* as well as feral *Mus musculus domesticus*, which suggest that MMM has been selectively maintained in these subspecies at similar levels since their evolutionary divergence (2800 to 6000 years ago, Boursot et al. 1993). Although we found that on average 29% of the litters were multiple sired, the actual rate of multiple mating might be higher depending on the competitive sire skew between males (Dean et al. 2006). A high competitive skew (one male sires the majority of offspring within a litter) requires an increased rate of multiple mating to detect multiple paternity. In house mice observational data from the field (Firman and Simmons 2008a) and laboratory experiments (Firman and Simmons 2008c) showed that paternity is strongly biased towards one male, indicating that our measurement of MP paternity is a conservative estimate of the rate of multiple male mating. The high variation in MP among populations was suggested to be due to population density (Dean et al. 2006; but see Firman and Simmons 2008a); however, we did not find any seasonal differences in the MP rate, as would be expected if MP is density dependent (Briese and Smith 1974). Also, we did not find any seasonal effects on litter size or male sex ratio.

There are a variety of potential effects from MMM on females' and offspring fitness, and we examined the effects of MP on litter size and pup body mass. We found no difference in the litter size of single versus multiple sired litters, contrary to the good genes, the

genetic compatibility or the fertility assurance hypotheses. Unlike ground squirrel species, which restrict mating to a very short time period after hibernation (Murie and Michener 1984), house mice can reproduce all year round and do not synchronize oestrus. Therefore, sperm depletion in males might be rare and unlikely to explain female multiple mating behaviour in this species. Surveys of feral *Mus musculus domesticus* populations in the USA and Australia found no effects of MP on litter size (Dean et al. 2006; Firman and Simmons 2008a). Therefore, MP does not appear to increase litter size in house mice living under natural conditions. Interestingly though, a recent study by Firman & Simmons (2012) revealed that females kept under laboratory conditions in a polyandrous mating regime significantly increased litter size in comparison to monandrously mating females over 15 generations. This result indicates that polyandry could lead to increased litter sizes – at least under laboratory conditions without nutritional limitations. However, whether this increase in litter size is beneficial for females' fitness (lifetime reproductive success) still needs to be determined.

We found that MP did not increase mean pup body mass (offspring quality indicator), as expected if MMM functions to obtain good genes, and on the contrary, pup body mass was significantly reduced in multiple compared to single sired litters. This reduction is particularly surprising considering that the litters were born in our colony (food and water were available *ad libitum* and temperature was regulated). It is unclear how MP might cause reduced offspring body mass, but it is not explained by a trade-off between offspring number and quality, as pup body mass was not related to litter size. If MP is due to sexual coercion or infanticide avoidance, then females may reduce their maternal investment when coerced into mating with non-preferred males (Drickamer et al. 2000). A non-mutually exclusive hypothesis for our result could be increased sibling rivalry (Hager and Johnstone 2006; Hudson and Trillmich 2008). Regardless of the underlying mechanisms, since pup body mass at weaning is known to increase offspring survival in the wild (Baker and Fowler 1992), our finding indicates that MP could have negative fitness effects for females and their offspring, which is consistent with sexual conflict hypotheses (convenience polyandry and infanticide avoidance). On the other hand, there may still be an adaptive benefit to multiple paternity despite the reduced offspring body mass associated, as we address below.

Although we found no evidence that MP increased observed heterozygosity of litters, we found that the number of alleles within multiple sired litters was higher compared to

single sired litters. Similar findings have been made in the alpine marmot (*Marmota marmota*) where multiple sired litters also show increased litter genetic diversity (Cohas et al. 2007). These findings contrast with the assumption that MP will not increase offspring genetic diversity, as the amount of genetic diversity during meiosis is so high that MMM is unlikely to elevate the genetic diversity among offspring (Williams 1975). Increased genetic diversity of litters can elevate female fitness, such as through bet-hedging (Yasui 1998), especially since gene-by-environment interactions on fitness are widespread (Narraway et al. 2010). Our finding that multiple sired litters show reduced variance in offspring body mass is consistent with the bet-hedging hypothesis (assuming that body mass is a good indicator of fitness, see (Baker and Fowler 1992). By reducing either the individual variance in fitness or fitness correlations between individuals from the same genetic lineage, bet-hedging could favour MP even despite a reduction in arithmetic mean fitness of offspring (Philippi and Seger 1989; Starrfelt and Kokko 2012). However, genetic bet-hedging seems unlikely in explaining the evolutionary origins of polyandry (Yasui 2001) and increased genetic diversity may be merely a by-product from other direct or indirect benefits (e.g. avoiding infanticide or harassment).

If MMM functions to facilitate inbreeding avoidance, we would expect the MP rate to be higher in populations with reduced genetic variation. We found a significant difference in the genetic diversity among populations. In the Safaripark population both the number of alleles detected and the observed heterozygosity within litters was significantly higher compared to the KLIVV population. This result shows that there is genetic variation among populations although the number of alleles within both populations was low (KLIVV: 2.25; Safaripark: 2.79; similar to Australian populations, Firman and Simmons 2008a) in comparison to populations in the USA (Dean et al. 2006). However, we did not find a difference in the rate of MP between populations and multiple sired litters did not show increased heterozygosity. Also, less heterozygous females were not more likely to give birth to multiple sired litters. Taking together, our data do not support the idea that MMM functions to gain more compatible genes through inbreeding avoidance. This result is surprising, as we found that both populations showed evidence for increased levels of inbreeding during winter (i.e., offspring observed heterozygosity was significantly lower in winter compared to spring or summer) and there are negative fitness consequences from inbreeding in house mice (Meagher et al. 2000; Ilmonen et al. 2008).

Finally, we found that dam and offspring heterozygosity was correlated, and this result was significant in single and multiple sired litters. Heterozygosity has been shown to increase disease resistance (Coltman et al. 1999; Reid et al. 2005; Charpentier et al. 2008), survival (Richardson et al. 2004) and reproductive success (Foerster et al. 2003; Kempenaers 2007), thus females can increase their fitness by producing heterozygous offspring. The idea that heterozygosity might be heritable has been questioned but there is now accumulating evidence that support this idea (Mitton et al. 1993; Bensch et al. 2006; Hoffman et al. 2007). Future studies are needed to determine the fitness consequences of increased allelic diversity of litters and smaller mean (and variance) offspring body mass, which were associated with MP. Also, future studies are needed to reveal how more heterozygous females produce more heterozygous offspring.

Acknowledgements

The work was supported by the Austrian Science Fund (FV_FP27024711).

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Chapter 2

Why do female mice mate with multiple males?

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This manuscript has been accepted at to the Journal "Behavioral Ecology and Sociobiology".
Date of acceptance: June 26th 2013

ABSTRACT

Females often show multi-male mating (MMM), but the adaptive functions are unclear. We tested whether female house mice (*Mus musculus musculus*) show MMM when they can choose their mates without male coercion. We released 32 females into separate enclosures where they could choose to mate with two neighbouring males that were restricted to their own territories. We also tested whether females increase MMM when the available males appeared unable to exclude intruders from their territories. To manipulate territorial intrusion, we introduced scent marked tiles from the neighbouring males into males' territories, or we rearranged tiles within males' own territories as a control. Each female was tested in treatment and control conditions and we conducted paternity analyses on the 57 litters produced. We found that 46% of litters were multiply sired, indicating that multiple paternity is common when females can choose their mates. Intrusion did not increase multiple paternity, though multiple paternity was significantly greater in the first trial when the males were virgins compared to the second trial. Since virgin male mice are highly infanticidal this finding is consistent with the infanticide avoidance hypothesis. We also found that multiple paternity was higher when competing males showed small differences in their amount of scent marking, suggesting that females reduce MMM when they can detect differences in males' quality. Finally, multiple paternity was associated with increased litter size but only in the intrusion treatment, which suggests that the effect of multiple paternity on offspring number is dependent on male-male interactions.

INTRODUCTION

The adaptive significance of multi-male mating (MMM) or polyandry is unclear and controversial (Jennions and Petrie 2000; Hosken and Stockley 2003; Simmons 2005; Gowaty 2012). Unlike males, females are not expected to increase their reproductive success by mating with multiple individuals (Bateman 1948; Trivers 1972). Moreover, polyandry can incur a number of costs for females, in terms of time and energy expenditure (Daly 1978), elevated risks of predation (Rowe 1994), injuries (Siva-Jothy 2006) and sexually transmitted diseases (Magnhagen 1991), suggesting that there are compensating benefits for females. Several non-mutually exclusive hypotheses have been proposed to explain how females can potentially gain fitness benefits from polyandry (Jennions and Petrie 2000; Simmons 2005). MMM could provide females with direct

benefits, such as parental care, nuptial gifts, or other resources from males (Arnqvist and Nilsson 2000; Hosken and Stockley 2003). In non-resource based mating systems, polyandry might function to increase females' fertility (fertility assurance hypothesis) (Hoogland 1998) or to obtain a variety of indirect, genetic benefits for offspring (Simmons 2005), such as eliciting sperm competition to gain "good genes" (Kempenaers et al. 1992), increasing genetic compatibility of maternal and paternal genomes (Zeh and Zeh 1997), including inbreeding avoidance (Tregenza 2002), and increasing offspring genetic diversity (Yasui 1998; Yasui 2001). Most studies on polyandry have focused on birds and insects, whereas relatively little attention has been paid to mammals (Clutton-Brock and McAuliffe 2009) or sexual conflict hypotheses (Arnqvist and Rowe 2005). Sexual conflict hypotheses suggest that polyandry may be due to sexual coercion, so that females obtain no benefits (Wolff 1985; Smuts and Smuts 1993; Clutton-Brock and Parker 1995), or it may function as an alternative mating tactic (Taborsky et al. 2008) to reduce sexual harassment (convenience polyandry) (Thornhill and Alcock 1983) or infanticide (infanticide avoidance hypothesis) (Hrdy 1979; Agrell et al. 1998). Infanticide is the main cause of offspring mortality in many mammals and polyandry has evolved more often in mammal species whose young are vulnerable to infanticide (Wolff and Macdonald 2004). However, there are surprisingly few experimental tests of whether polyandry functions to reduce infanticide or sexual coercion in any species. Here we conducted a study with wild-derived house mice (*Mus musculus musculus)* to test whether females show MMM when they can choose their mates and are not constrained by sexual coercion, and whether multiple paternity affects offspring number or size when females can select their own mates.

Male house mice are territorial and females usually mate with the dominant, territorial male, though sometimes females also mate with neighbouring territorial males (Oakeshott 1974; Bronson 1979; Potts et al. 1991; Montero et al. 2013). Surveys in wild populations of house mice (*Mus domesticus*) have found that 6 to 43% (mean 25%) of litters are multiply sired (Dean et al. 2006; Firman and Simmons 2008a). It is unclear why there is so much variation in multiple paternity among wild populations. This variation may be due to changes within females' MMM (conditional mating tactic) or differences between females (heritable or non-heritable personality trait) (McFarlane et al. 2011). It could also be due to differences in social or ecological conditions, as multiple paternity has been found to be correlated with population density (Dean et al. 2006; but see Firman and

Simmons 2008a). Females may increase MMM under high density because dominant males can no longer defend their territories from intruders (Anderson 1961), and since females have less protection, they likely face more sexual coercion and risk of infanticide (Calhoun 1962; Ebensperger 1998). Infanticide is very common in mice (Huck et al. 1982; Elwood and Ostermeyer 1984; Manning et al. 1995) and several studies suggest that males kill pups that are not likely to be their offspring. For example, virgin males are highly infanticidal (Labov 1980; Huck et al. 1982; vom Saal and Howard 1982; Elwood and Ostermeyer 1984; Elwood 1985), whereas copulation reduces infanticidal behaviour (Soroker and Terkel 1988). Territorial males kill pups outside their own territory and non-territorial males commit infanticide when they have not sired any offspring (Manning et al. 1995). Although it is often suggested, it is not known whether female house mice show more MMM when they encounter strange or infanticidal males or whether MMM reduces their risk of infanticide. A study on bank voles (*Myodes glareolus*) examined the consequences of monogamy versus polyandry and found that offspring of socially polyandrous females had higher survival than offspring from socially monandrous females (all litters were genetically polyandrous) (Klemme and Ylönen 2010). This finding supports the infanticide avoidance hypothesis; however, to explain the variation in multiple paternity, studies are also needed to test whether females are more likely to mate multiply when they perceive a higher risk of infanticide from males.

MMM has been shown to provide several indirect, genetic benefits in female house mice. First, females have increased mean pup survival when they mate with three different males within one oestrus cycle compared to females that mate three times with the same male, indicating that polyandry increases offspring viability (Firman and Simmons 2008c). Second, paternity is biased towards non-siblings when a female mates with both a sibling and a non-sibling, indicating that polyandry facilitates inbreeding avoidance and enhances the genetic compatibility (Firman and Simmons 2008b). Third, female house mice from polyandrous selection lines (16 generations) have increased reproductive benefits compared to females from monandrous selection lines, as their sons achieve higher reproductive success under natural conditions (Firman 2011). However, in all these studies, matings were arranged and it is not known whether multiple paternity provides indirect, genetic benefits when females are able to select their own mates – though the benefits may be even greater compared to when females are forced to mate with randomly selected males in terms of quality. Females show preferences for males of

high quality (Ilmonen et al. 2009) and female mate preferences can provide indirect benefits (Drickamer et al. 2000), but it is unclear how variation in male quality affects female MMM or the consequences of multiple paternity. Male mice scent mark their territories and counter-mark the marks of intruding males (Gosling 1982; Hurst 1990), and females use males' scent marks to recognize territorial males (Drickamer 1992) and to assess males' competitive ability (i.e., males' ability to exclude intruders) (Rich and Hurst 1998; Rich and Hurst 1999). Females may prefer to mate with competitive, territorial males to reduce their risk of infanticide ('pup defense hypothesis') (Ebensperger 1998), as well as obtaining indirect benefits. Female mice also use male scent marking to assess other aspects of male quality, including health (Zala et al. 2004) and genetic disease resistance (Zala et al. 2008a) and females may not show MMM when they can detect differences in the quality of the available males. Also, females may be more likely to engage in extra-pair matings when they have a poor quality mate ('trade-up hypothesis') (Kempenaers et al. 1992). Thus, previous studies indicate that female mice can obtain indirect, genetic benefits by MMM, but studies are still needed to determine whether MMM is influenced by variation in male quality (or females' perception of male quality) and how multiple paternity affects offspring fitness when females can select their mates.

In our study, we allowed female mice (wild-derived *Mus musculus musculus*) to choose to mate with either one or two neighbouring males, which both had their own territory but were restricted from leaving it, and we conduced genetic paternity analyses to determine whether females produce single or multiple sired litters (we assume that multiple sired litters were more likely to be the result of MMM than single sired litters). We also aimed to test whether females show more MMM, estimated by multiple paternity, when males are unable to defend their territories and exclude intruders, as occurs in high population densities. To test this hypothesis, we experimentally exchanged scent marks between the neighbouring males' territories to simulate intrusion and manipulated males' apparent ability to exclude intruders (territorial intrusion). For controls, we relocated males' scent marks within their own territories. The experimental manipulation may alter females' perception of males' quality (in particular their ability to defend their territory) and apparent risk of infanticide**.** We expected that if females use intruders' marks to assess males' ability to defend their territory and the risk of infanticide, then females should be more likely to have multiple sired litters when males are unable to prevent intrusion. We

also quantified male scent marking (as measure of males' quality), to test whether differences in male quality affected the rate of multiple paternity. As only few studies have investigated the consistency or repeatability of females' MMM (Dietrich et al. 2004; Whittingham et al. 2006), we tested each female under territorial intrusion and control conditions. Finally, we examined whether multiple paternity resulted in increased number of offspring when females could select their own mates, as predicted by the fertility assurance, the intrinsic male quality and the genetic compatibility hypotheses.

METHODS

Experimental animals and housing

All experimental animals were F1 from wild-derived house mice (*Mus musculus musculus),* which were trapped at 14 locations within a 500 m radius in Vienna (48°12'38"N; 16°16'54"E) and crossed between sites. The resulting F1 mice were weaned at the age of 21 ± 1 days and were thereafter housed individually in standard mouse cages (type I cages, 26.5 × 20.5 × 14 cm) under standard conditions (12:12 h light cycle) until the experiment was conducted. All cages were equipped with wooden bedding (ABEDD), wood shavings and food (Altromin rodent diet 1324) and water *ad libitum*. We conducted ear punches for individual identification, and tissues were collected and stored at -20°C for subsequent genetic analyses. Animals were between three and five months old when the experiment began. Mice were released into semi-natural enclosures where the experiment was conducted.

Experimental mate choice assay

Each female (*N*=32) could choose to mate with either one or both of two males (*N*=64), which were located in two neighbouring territories. Each territory (1 × 1.7 × 0.8 m) contained one nest box, one shelter, one mouse cage, one water dispenser, food (Altromin rodent diet 1324) and nesting material (Figure 1). The males' enclosures were separated from each other by an opaque plastic divider to prevent them from entering and marking each other's territories. The divider had four holes (4 cm in diameter) at the base, which were mesh-sealed to allow visual and olfactory contact between males. Females could move freely between the males' territories through a small passage (plastic tube installed at the bottom of the divider, 3 cm diameter), whereas the males were prevented from entering the passage by collars (2.5 mm-wide cable ties with two attached wires that provided a mechanical barrier at the opening of the tube). Males were collared two days

prior to their introduction to provide them with sufficient time to become habituated to the collar. A separate shelter cage was placed in each male's territory, which was only accessible to females through a narrow tube entrance that allowed females to escape sexual harassment.

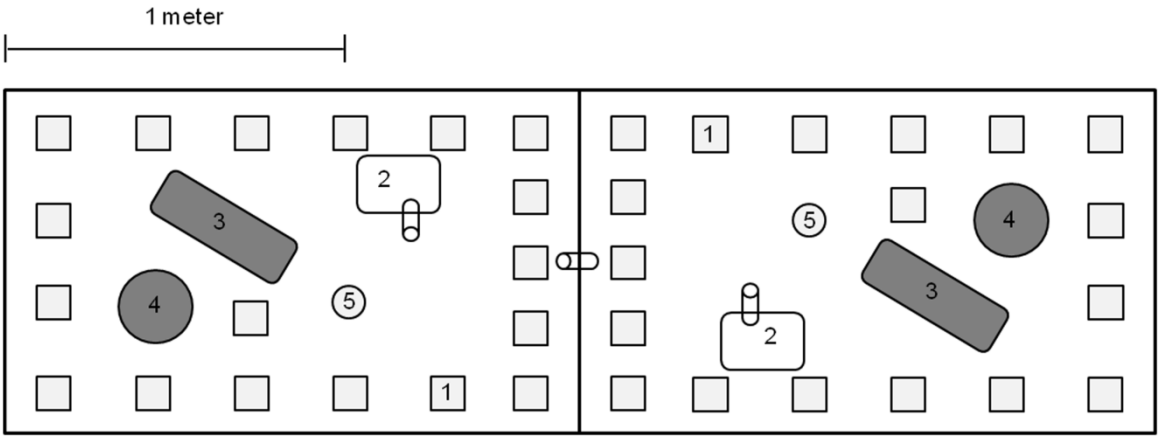

Figure 1: Neighbouring males' compartments with a connection tube that allowed females to move between the male territories. Both compartments contained 18 tiles (1), a cage accessible only to females (2), a shelter box (3), a nest box (4), and a water dispenser (5). In the intrusion treatment, all the tiles in the two males' compartment were exchanged with each other, whereas in the controls, the tiles were rearranged within the males' own compartments.

Scent marks and simulated intrusion

To collect scent marks, we placed 18 PVC tiles (10 × 10 cm) on the floor of each male's enclosures along the borders and next to nesting sites, covering approximately 11% of the enclosure's surface area (Figure 1). Each tile was individually labelled underneath and was assigned to an exact position within the enclosure. Males were introduced into the enclosures two days before the females to enable them to scent mark their compartment and to establish a territory. Simultaneously to male introduction, 20 µl of female urine (pool of seven females collected on five consecutive days) were deposited between the nest box and the shelter as female urine has been shown to stimulate males to increase scent marking (Zala et al. 2008b). In the experimental intrusion treatment (N=32 females), the males' tiles were exchanged with those of their neighbour's compartment (to simulate intrusion), while in the controls (N=32 females), the males' tiles were collected and replaced within their own compartment. Tile shifting began at day three of the experiment, shortly before females were released into the experiment, and was conducted on a daily bases around noon, when animals were inactive. To prevent

spreading scent among the tiles and enclosures, observers wore one-way plastic shoe covers and tiles were handled with one-way latex gloves. We took photographs of all tiles before relocating them to assess the amount of males' scent marking for six days starting at the day of female introduction.

Experimental design

Each of the 32 females was used in two trials, once in the intrusion treatment and once with a different pair of males under control conditions (within subject design). The time between the first and the second trial was two month. The order of treatments versus control was determined by applying stratified randomization to avoid sequential effects. Due to space limitations we could not test all 32 females simultaneously, so we ran two groups per trial, where we tested 16 females and 32 males each. The number of treatment and controls was balanced within groups. The 64 males were also tested twice; however, males were assigned to new pairs for the second trial. We ensured that none of the experimental animals were familiar with or related to one another and male pairs were body mass matched within 0.5 g. Male body mass (g) was measured shortly before they received their collar and female body mass (g) was measured at the day we released them into the experiment. We determined the differences between female and male body mass by calculating the mean body mass of male pairs and subtracting female body mass. All females had given birth to one litter before this experiment to control for potential order effects due to comparing virgin versus non-virgin females. The males were all virgins on the first trial and at least 61% were sexually experienced in the second trial (61% of males sired offspring, but the number of males that mated could be higher). The mice in the experiment were allowed to interact for 18 days and then all animals were returned to the colony. Males' collars were removed immediately and females were placed individually in type IIL mouse cages (32 × 20.5 × 14 cm) to give birth under controlled conditions. Reproductive success (litter size at birth and mean pup body mass at weaning [litter mass at weaning/ litter size at weaning]) was measured and genetic paternity analyses were conducted.

Scent mark analysis

Photographs of tiles were recorded in a black box (60 × 60 × 80 cm) under UV light, emitted by two 18 W strip lights (90 cm, OMNILUX) fixed on the ceiling of the box. The 18 tiles within each territory were photographed in two sets of nine tiles each. Tiles were placed centrally on the bottom of the box in the same order and the same position. Digital

photographs were recorded with a camera (Canon EOS 400 D Digital camera, 0.8" exposure time and 4.5 aperture value) from a fixed position on top of the box. We recorded the photographs of the first group in trial one (see experimental design) in JPG format, but we excluded these data from our analyses as the image quality was inadequate and only analysed subsequent photographs which were recorded in CR2 format. The box was cleaned with 70% Ethanol after each photo to prevent odour contamination. Photographs were imported into Adobe Photoshop CS5.1 for image analyses and interpolated (10 cm ≙ 1000 pixel) before a threshold was assigned. To assess the amount of individual male's scent marking we determined the proportion of freshly marked tile area each day and calculated the sum of the freshly marked area over time starting after female introduction (sum of five days). For further analyses we calculated the *difference* in the sum of marked tile area within male pairs (hereafter '*difference* in the two males' scent marking'). In addition we determined the measurement error in taking three photographs of the same set of tiles, and analysed the photographs with the afore mentioned method. The marked tile area differed in <0.05% between the three photographs.

Genetic paternity analyses

We conducted paternity analyses to define single versus multiple sired litters. DNA was extracted from ear punch samples using a proteinase K/isopropanol protocol (Sambrook et al. 1989). Individuals were genotyped at a minimum of 6 polymorphic microsatellite loci. If paternity could not be assigned by complete exclusion, we genotyped additional loci. A maximum of 16 microsatellite loci was used for paternity analyses (D11Mit150, D9Mit34, D9Mit135, D17Saha, D17Mit28, D10Mit20, D2Mit252, D6Mit138, D15Mit16, D5Mit25, D19Mit39, D7Mit227, D1Mit456, D2Mit380, D17Mit21, D1Mit404, see Mouse Microsatellite Data Base of Japan) using a Multiplex-PCR MasterMix (Qiagen Multiplex PCR kit). 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 analysed using an automated sequencer (Beckman Coulter CEQ 800). Allele scoring was performed using Beckman Coulter CEQ 8000 System software, and allele sizes were determined with SLS+400 as size standard. Paternity assignment was assessed using complete exclusion. In addition, paternity results were confirmed with a 95 to 99% trio

confidence (dam-sire-offspring relationship) using the program CERVUS 3.0.3 (Kalinowski et al. 2007).

Statistical analyses

To test the effect of intrusion treatment and male scent marking on the rate of multiple sired litters we ran a generalized linear mixed effects model (GLMM) with a binomial error distribution and a logit link function. We entered paternity (single or multiple) as the dependent variable, trial and treatment as fixed effects and female body mass, the body mass difference between males and females and the *difference* in the two males' scent marking as covariates. As females were repeatedly tested we included female ID as a random factor to control for non-independence. Fitness effects of multiple mating were analysed using a general linear mixed effects model (LMM) with either litter size or mean pup body mass as dependent variables, trial, treatment and paternity as fixed effects and female body mass as a covariate. Female ID was again included as a random factor. To test for the relationship between mean pup body mass and litter size under intrusion, we ran a linear model (LM) with mean pup body mass as the dependent variable and litter size as a covariate. Female ID was not included as a random factor as each female was tested only once under intrusion. We tested whether model assumptions (i.e., normally distributed residuals and homogeneity of variances) were fulfilled and transformed data if necessary. We only included biologically meaningful two-way interactions into all initial models and applied a backward stepwise removal procedure (Grafen and Hails 2002) to avoid problems due to inclusion of non-significant terms (Engqvist 2005). Removed variables were re-entered one by one to the final model to obtain relevant statistics. Statistical analyses were performed using 'R' (version 2.14.1). We implemented linear mixed effects models using the 'lme' function of the 'nlme' package, and generalized mixed effects models using the 'lmer' function in the 'lme4' package (R Development Core Team 2011).

RESULTS

We found that 26 of the 57 litters (46%) had multiple sires, however, the intrusion treatment did not significantly affect multiple paternity (GLMM: z=-0.283, N=44, P=0.777, Figure 2). Yet, the rate of multiple paternity was significantly greater in the first trial when the males were all virgins (15/26 or 58% of litters) compared to the second trial (11/31 or 35% of litters) (GLMM: z=-2.306, N=44, P=0.021, Figure 2). Also, paternity was significantly predicted by the *difference* in the two males' scent marking: we found

that multiple paternity was higher when males showed smaller differences in their marking whereas single paternity was higher when the differences in males marking increased (GLMM: z=-2.472, β=-0.373, SE=0.151, N=44, P=0.013, Figure 3). We found no evidence that female body mass (GLMM: z=0.911, N=44, P=0.362) or the differences in body mass between females and males (GLMM: z=-0.198, N=44, P=0.843) affected multiple paternity.

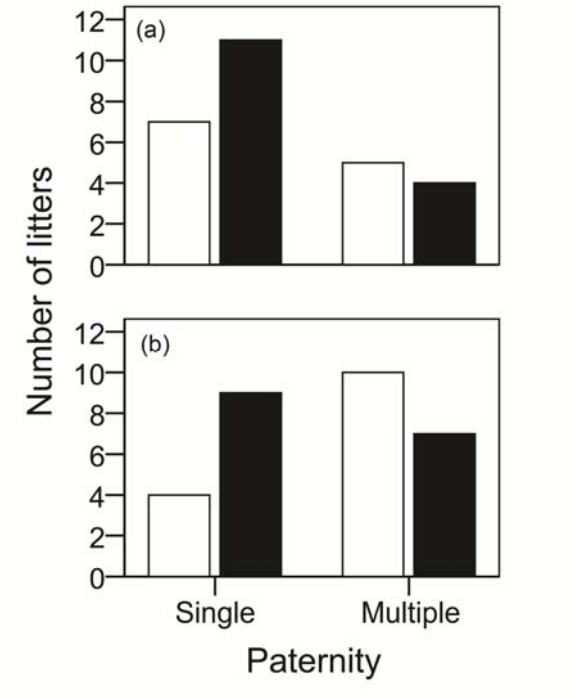

Figure 2: Frequency of single and multiple sired litters in the first (white bars) and second (black bars) trial of the experiment under (a) intrusion treatment and (b) control conditions.

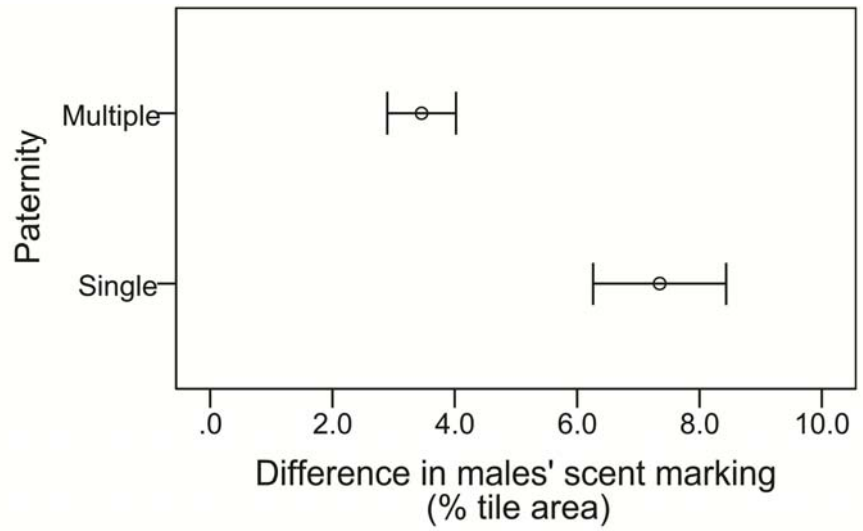

Figure 3: Difference in the two males' scent marking in single and multiple sired litters. Means ± SE are indicated.

We found no overall effect of multiple paternity on litter size (LMM: $F_{1,20}$=2.861, N=57, P=0.106,), but we found a significant interaction such that the effect of paternity on females' litter size was dependent on the experimental treatment (LMM: $F_{1,20}$=4.671, N=57, P=0.043, Figure 4).

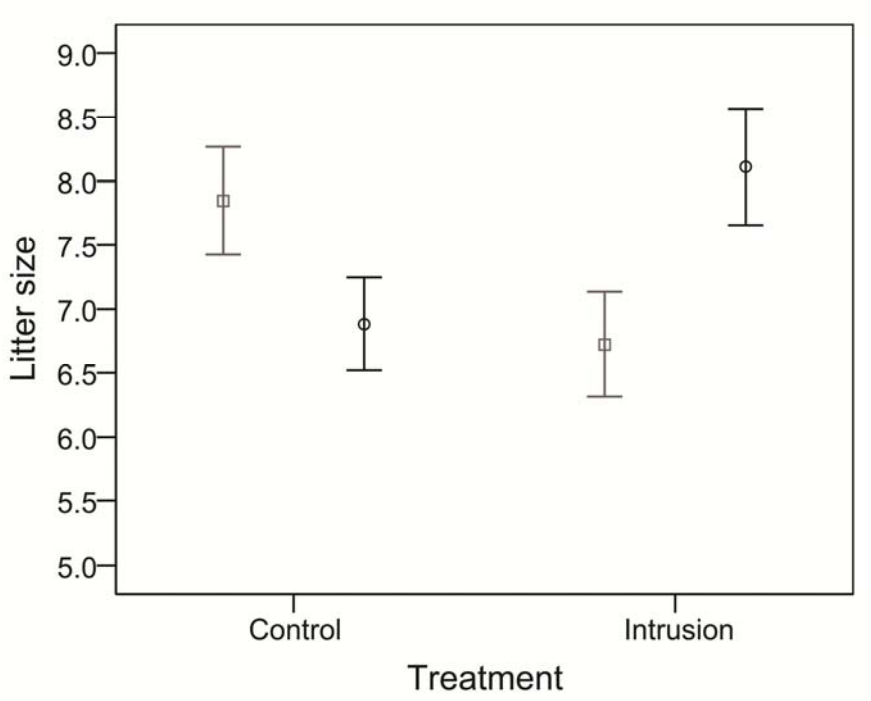

Figure 4: Litter size of single (grey, square) and multiple (black, circle) sired litter under intrusion treatment (single paternity: 6.72 ± 0.4, N=18; multiple paternity: 8.11 ± 0.5, N=9) and control conditions (single paternity: 7.85 ± 0.4, N=13; multiple paternity: 6.94 ± 0.4, N=17). Means ± SE are indicated.

We therefore examined the effect of paternity independent for the intrusion treatment and the controls. In the intrusion treatment, litter size was significantly larger in multiple than single sired litters (T test: t=-2.267, N=27, P=0.034) whereas under control conditions paternity had no significant effect on litter size (T test: t=1.677, N=30, P=0.106). There was no change in litter size over trials (mean ± SD: 7.26 ± 1.60) (LMM: $F_{1,19}$=0.537, N=57, P=0.473) but heavier females had larger litters (LMM: $F_{1,20}$=6.925, β=0.258, SE=0.098, N=57, P=0.016). Female body mass did not predict mean pup body mass (LMM: $F_{1,18}$=0.822, N=56, P=0.376), though mean pup body mass significantly increased from the first to the second trial (from 8.57 g to 9.32 g) (LMM: $F_{1,19}$=12.686, N=56, P=0.002). The interaction between treatment and paternity also had a significant effect on mean pup body mass (LMM: $F_{1,19}$=7.739, N=56, P=0.012). We therefore, again,

examined the effect of paternity independent for the intrusion treatment and the controls. In the intrusion treatment, mean pup body mass was significantly smaller in multiple sired litters (T test: t=3.391, N=27, P=0.002) whereas in the control treatment we did not find an effect of paternity on mean pup body mass (T test: t=-0.529, N=29, P=0.601). Thus, under intrusion, litter size increased whereas mean pup body mass decreased with multiple paternity. This result could be explained by the marginally non-significant negative relationship of litter size and mean pup body mass (LM: $F_{1,25}$=3.634, β=-0.264, SE=0.131, N=27, P=0.068).

DISCUSSION

In total, 46% (26 out of 57) of the litters were multiply sired, which is in the high end of the range of multiple paternity found in wild populations of house mice (Dean et al. 2006; Firman and Simmons 2008a). Thus, our findings show that females actively mate multiply when they have the opportunity to freely choose their mates and that they even increase MMM when they are unconstrained by males or other factors in the wild. A previous study on house mice (using offspring from crosses of female laboratory mice (ICR) with wild male mice (*Mus musculus domesticus*)) observed that 95% of females actively mated multiply when they could choose to mate with a dominant versus a subordinate male (Rolland et al. 2003). Taken together, these findings support the hypothesis that MMM is due to female choice in house mice, whereas they are inconsistent with the hypothesis that females are forced to mate multiply (sexual coercion) (Wolff 1985; Smuts and Smuts 1993; Clutton-Brock and Parker 1995). We did not explicitly test consistency in MMM (for review see Nakagawa and Schielzeth 2010) but since multiple paternity was inconsistent across trials, our findings do not provide evidence for consistent individual variation (personality trait or true alternative mating strategy) in multiple paternity. Although multiple paternity was not consistent between treatment versus controls, we found no evidence that multiple paternity was increased in the intrusion treatment, as predicted by the infanticide avoidance hypothesis. Females may have failed to detect any differences between intrusion versus control conditions, though this seems unlikely as the males scent marked significantly more under intrusion compared to control conditions (Chapter 3). Alternatively, females may not assess males' competitive ability based on the frequency of intruders' scent marks, as previously suggested (Rich and Hurst 1998), or males' competitive ability is unrelated to the risk of infanticide, contrary to the 'pup defense' hypothesis (Ebensperger 1998). Thus, we cannot

exclude the idea that multiple paternity depends on the risk of infanticide or males' perceived quality, especially since we found evidence for both hypotheses, as we explain below.

We found that the rate of multiple paternity was significantly higher in the first compared to the second trial, and females likely faced a higher risk of infanticide during this time. In the first trial, the available males were all still virgins – which are highly infanticidal (Labov 1980; Huck et al. 1982; vom Saal and Howard 1982; Elwood and Ostermeyer 1984; Elwood 1985). After copulation and cohabitation with a female, males reduce infanticidal behaviour towards their mates' offspring and even other females' pups (Soroker and Terkel 1988). Thus, our finding suggests that female mice mate with multiple males when they encounter virgin males, as predicted by the infanticide avoidance hypothesis. It is not known whether female mice can recognize virgin or other infanticidal males, but females might discriminate differences in their scent, ultrasonic vocalizations, aggression, or other behaviours. Alternatively, the reduction in multiple paternity we observed may be due to other changes in males' behaviour over time, unrelated to infanticidal behaviour, or changes in females' behaviour (despite that experimental females were non-virgins in both trials). Experience might allow females to avoid male harassment and coercion, though this explanation seems unlikely, as females had a refuge and the differences in body mass between females and males had no influence on the multiple paternity rates. Alternatively, experience may allow females to more effectively defend their offspring against infanticidal males.

We measured males' scent marking since this behaviour is expected to influence females' mating preferences, and indeed we found that the *difference* in the two males' scent marking explained the variation in single versus multiple paternity: in single sired litters the males displayed significantly larger differences in their scent marking compared to multiple sired litters. Males' scent marking is a quality indicator display, and female mice can assess several aspects of quality on the basis of males' scent marking, including social status (Drickamer 1992), competitive ability (Rich and Hurst 1998; Rich and Hurst 1999), and health (Zala et al. 2004). Therefore, this finding suggests that females mate singly when they can detect differences in the males' quality and otherwise they mate multiply. There are several (non-exclusive) hypotheses to explain why females might use such a strategy. First, when females cannot detect differences in males' quality, they may mate multiply to incite sperm competition to increase the genetic quality of their

offspring. This idea assumes that males' sperm competitiveness and offspring fitness are genetically correlated and males of high genetic quality sire more viable offspring (intrinsic male quality hypothesis) (Yasui 1997; García-González and Simmons 2005). Second, if male sperm competitiveness is heritable, multiply mated females would have a selective advantage over single mated females as the former are fertilized by the most competitive sperm and will have sons which have superior sperm competitive abilities. A study on house mice showed that polyandrous females can gain fitness benefits by producing sons that achieve high reproductive success in a competitive environment (Firman 2011). Third, if females cannot detect differences in males' quality, MMM could provide females with good genes as females avoid sampling errors caused by inadequate mate discrimination (bet-hedging) (Yasui 1998). Fourth, this result may not be due to female choice, but rather to male sperm competitiveness and male-male interactions. Females may have generally mated multiply, but males' scent marking might have honestly reflected male sperm competitiveness and males that marked at similar rates were equally good in sperm competition, or alternatively, higher marking males could have been better in intimidating rivals, which then in turn transferred less sperm. Future studies are needed that include direct observations of female and male behaviour to determine whether our results can be explained by female choice, male-male competition or an interaction of both.

Finally, we aimed to determine whether multiple paternity enhanced females' reproductive success (litter size) when they are able to select their mates. We found no overall effect of multiple paternity on litter size, but we found an unexpected interaction that masked the effect of paternity on litter size: multiple paternity increased litter size in the intrusion treatment, whereas there was no significant effect in the controls. Thus, although intrusion treatment had no effect on multiple paternity, the effects of multiple paternity on offspring number crucially depended on intrusion where male-male interactions were intensified (and the within-subject design controls for other potential confounds). This finding suggests that multiple paternity provided reproductive (fitness) benefits for females (e.g. as predicted by fertility assurance, the genetic compatibility and intrinsic male quality hypotheses), but why did this effect only occur under intrusion treatment? One possible explanation is that males perceived higher competition with intrusion and they increased the number of sperm transferred during mating when they perceive a high risk of sperm competition (Parker 1990; Parker 1998; Wedell et al. 2002).

One study on house mice supports this hypothesis (Ramm and Stockley 2009a), but a second study found no support (Ramm and Stockley 2009b) and a third study found that males reduced the number of sperm transferred when mating in the presence of a rival male (Ramm and Stockley 2007). Therefore, it is unclear whether males transfer more sperm when they perceive an increased risk of sperm competition. Moreover, it is unlikely that the females were sperm limited under intrusion but not under control conditions. Interpreting the positive correlation is not straightforward because increasing offspring number does not necessarily enhance females' fitness, contrary to what is often assumed, as there are sexual conflicts over the optimal number of offspring (Penn and Smith 2007), as well as over parental investment. If females engage in multiple mating to reduce the costs from sexual harassment or to reduce infanticide, then increases in litter size from MMM may be costly rather than beneficial for females' fitness. The situation becomes even more complicated when we consider that multiple paternity exacerbates sibling rivalry, as well as sex conflicts, and offspring can potentially influence maternal investment and litter size (Royle et al. 2004; Drake et al. 2008). Males and their offspring may influence the number of eggs that females produce or the number of embryos reabsorbed (Hager and Johnstone 2003). An increasing number of studies find that male-male competition influences female mate choice (Wong and Candolin 2005), but this is the first study to our knowledge that shows that the effects of multiple paternity on offspring number depend on females' exposure to male-male interactions. Thus, future studies are needed to determine why the effects of multiple paternity on offspring number depend on male-male interactions and to disentangle the underlying proximate causes and the evolutionary fitness consequences.

Litter size was also influenced by maternal body mass with heavier females producing larger litters. Given that reproduction incurs fitness costs (Reznick 1985), and especially in mammals (Speakman 2008) where gestation is followed by lactation, females in better condition and larger body mass could probably better afford the costs of producing larger litters. Yet, mean pup body mass did not depend on female body mass. Instead pup body mass significantly increased over trials, indicating that older or more experienced females invested more resources into their average offspring or that they reduce investment when exposed to infanticidal virgin males. Females were repeatedly tested and could gain experience in mate choice and the raising of pups. Although not all females became pregnant during the first trial, all of the females in our study were sexually experienced

and gave birth to one litter before used in this experiment. This way we ensured that any differences in the litter sizes between the trials were not due to comparing virgin and non-virgin females. Pup body mass at weaning is known to correlate with offspring survival in the wild (Baker and Fowler 1992). Our results thus suggest that offspring number depends on female body mass whereas offspring quality (e.g. mean pup body mass) depends on females' age or experience. We also found a negative relationship between litter size and mean pup body mass under intrusion, indicating a negative trade-off between offspring number and quality (Smith and Fretwell 1974).

In summary, we found high rates of multiple paternity even when females can select their mates, indicating that MMM is due to female choice rather than sexual coercion. We found no evidence that females were more likely to give birth to multiple sired litters when males' territories were intruded by neighbouring males, as expected if MMM functions to reduce infanticide. We found that multiple paternity was significantly increased in the first trial when the available males were virgins, which are known to be highly infanticidal. However, experimental tests are needed to determine whether MMM is increased when females are exposed to virgin or otherwise infanticidal males. Also, multiple paternity was influenced by the difference in the two males' scent marking, which is a condition-dependent secondary sexual trait (quality-indicator). This finding suggests that females mate singly when they are able to detect significant differences in male quality and otherwise they mate multiply (e.g., bet-hedging hypothesis). Finally, we found that multiple sired litters were larger than single sired litters under intrusion, though studies are needed to determine why this effect only occurred under intrusion when male-male interactions were intensified. Future studies should be aware that the effects of multiple paternity on female reproduction (offspring number) can be masked and even reversed by male-male interactions.

Ethical Standards

This study has been discussed and approved by the Institutional Ethics Committee in accordance with Good Scientific Practice guidelines and national legislation.

Acknowledgements

We thank S. M. Zala and three anonymous reviewers for their valuable comments on earlier versions of the manuscript. We also want to thank T. Klaus and E. K. Straßer for assistance in data collection. Research was supported by the DARPA program (ARO

Contract DAAD19-03-1-0215), the FWF (FV_FP27024711), Attila Hettyey was supported by the "Lendület" programme of the Hungarian Academy of Sciences (MTA, LP2012-24/2012; www.mta.hu) and S. Raveh was supported by Swiss National Science Foundation fellowships (PBNEP3-132801 and PBNEP3-140190).

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Chapter 3

Scent marking enhances male reproductive success

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This manuscript has been accepted at the Journal "Animal Behaviour".
Date of acceptance: June 19th, 2013

ABSTRACT

Scent marking is often assumed to be a secondary sexual trait that enhances males' mating and reproductive success, though direct evidence for this hypothesis is lacking. We conducted a study with wild-derived house mice (*Mus musculus musculus*) to test whether scent marking increases males' reproductive success when females can freely choose between two territorial males. We also experimentally manipulated males' competitive scent marking by exchanging scent marked tiles between the neighbouring males' territories (intrusion treatment) or we relocated males' tiles within their own territory (control). Experimental animals were tested twice and we examined whether individual males were consistent in their marking. We found that males marked more in the intrusion treatment than controls and more at shared territorial borders than elsewhere. We found high day-to-day variation in most individual's scent marking, and yet the sum of individuals' scent marking was consistent over time and across different social conditions. Genetic paternity analyses revealed that males' scent marking significantly increased their reproductive success in both intrusion treatment and the controls. Surprisingly, however, female social preference was not positively correlated with male scent marking. These results provide direct evidence that scent marking enhances males' reproductive success when females can choose their mates, even though it did not increase females' social preferences.

INTRODUCTION

Scent marking is common among male mammals and other terrestrial vertebrates, and this behaviour plays an important role in communication within and between the sexes (Ralls 1971). Scent marking has a number of costs, including an increased risk of predation and reduced growth rates (Viitala et al. 1995; Gosling et al. 2000; Hughes et al. 2009; Hughes and Banks 2010). Darwin (1874) originally suggested that the evolution of odour glands in male mammals is "intelligible through sexual selection, if the most odouriferous males are the most successful in winning the females and in leaving offspring to inherit their gradually perfected glands and odours" (p. 809). Subsequently, many studies have found evidence that scent marking functions to intimidate rivals and attract females (Ralls 1971; Gosling 1982; Hurst 1990a; Gosling and Roberts 2001). For example, studies on house mice (*Mus musculus*) have found that males produce many small scent marks and that they increase the quantity of scent mark deposition in the

presence of sexually mature females (Ralls 1971; Reynolds 1971; Maruniak et al. 1974; Arakawa et al. 2007) or females' scent (Wolff and Powell 1984; Hurst 1989; Zala et al. 2004). Moreover, female mice are capable of assessing males' quality (Lenington 1983; Kavaliers and Colwell 1995; Penn et al. 1998) and compatibility (Yamazaki et al. 1976) from their scent. Therefore, males' scent appears to be a secondary sexual trait, analogous to colourful visual displays of birds and fish (Penn and Potts 1998), though unlike conventional displays, scent marks provide an example of an extended phenotype (Penn 2006) or extra-bodily ornament (Schaedelin and Taborsky 2009).

Although many studies provide evidence that females can assess potential mates by the *quality* of their urinary scent or bedding odour, it is unclear why males produce so many scent marks, as there are fewer studies on the benefits of scent marking *per se*. In house mice, infection reduces the amount of males' scent marking and the attractiveness of their scent marks to females (Zala et al. 2004). Moreover, females prefer the scent marks of males genetically engineered (transgenic 'knock-in') to be resistant to infection compared to susceptible controls (Zala et al. 2008), but it is unclear whether females prefer to mate with high versus low markers. Only two studies to our knowledge have tested whether female mate choice is influenced by the quantity of males' marking, and they found no evidence that males' scent marking enhances female social preferences or mate choice in prairie voles (*Microtus ochrogaster*) (Thomas 2002; Mech et al. 2003). However, these studies measured males' scent marking for only 30 minutes, which may have been insufficient to accurately assess quantitative differences in males' marking, and furthermore, they did not test whether scent marking enhanced males' actual reproductive success (paternity).

Most studies on scent marking have examined their function in male-male interactions, and these studies show that male marking provides an honest indicator of males' territorial ownership and social status (Ralls 1971; Gosling 1982; Hurst 1990a; Gosling and Roberts 2001). In house mice, for example, dominant males mark more than subordinates (Desjardins et al. 1973; Drickamer 2001) and territory owners counter-mark the scent marks of intruders (Hurst 1990a; Rich and Hurst 1999). Scent marking is a cheat-proof indicator of a males' social status because it is physically impossible for subordinates to fake territory ownership and occupation (Hurst and Rich 1999; Gosling and Roberts 2001). Scent marking mediates competitor assessment, and high marking is suspected to intimidate rivals and detour unnecessary fights (Gosling 1982; Hurst 1990a;

Gosling and Roberts 2001). Dominant male mice aggressively defend their territories (Crowcroft 1955; Crowcroft and Rowe 1963; Hurst 1990a), and subordinate males avoid areas marked by dominant individuals (Jones and Nowell 1973), most likely to evade agonistic encounters. Competition in many species induces males to increase their signalling effort and to produce more conspicuous and costly displays. For example, in Thomson's gazelles (*Gazella thomsoni*), scent marks are more dense in territories under the threat of intrusion than elsewhere (Walther 1978) and male orbi (*Ourebia ourebi)* mark more at common boundaries where other males were located (Brashares and Arcese 1999). However, these findings are observational, and we know of no study that has experimentally tested whether male-male conflict increases males' scent marking, and if so, whether increased scent marking subsequently improves males' reproductive success.

Scent marking behaviour likely evolves through both inter- and intra-sexual selection, and female choice may be influenced by male-male competition (Wong and Candolin 2005). For example, females may be able to assess male quality by eavesdropping on male's competitive scent marking, and females may change their preferences after observing male-male interactions. Studies investigating female preferences for male scent usually control for male-male effects, though several studies indicate that female preferences for male odour are influenced by male-male interactions. Female mice preferentially mate with territorial, socially dominant males (Rolland et al. 2003) or winners in agonistic encounters (DeFries and McClearn 1970; Parmigiani et al. 1982b). Dominant males produce more scent marks than subordinates (Desjardins et al. 1973) and females prefer the odour of dominant males (Drickamer 1992). Female mice also prefer the odour of males that counter-mark the scent marks of competitors (Rich and Hurst 1999); however, it is unclear whether females use males' scent marks (quality or quantity) to choose *among dominant* territorial males. Thus, studies are needed to test whether male-male interactions among territorial males affect female preferences and males' actual reproductive success.

In this study, we investigated wild-derived house mice (*Mus musculus musculus*) in large enclosures to test whether scent marking increases males' reproductive success when females can freely choose their mates, which has never been tested before to our knowledge. We also measured individual variation in scent marking over time (daily amount of marking over five days) and analysed individual consistency in marking (in another five days with a different female and male competitor), which has not been

studied before to our knowledge. In addition, we experimentally introduced scent marks from neighbouring males into males' territories ('territorial intrusion'), which increases male-male interactions, and we tested whether territorial intrusion affected males' marking, females' social preferences and males' reproductive success. In addition to eliciting male counter-marking, territorial intrusion likely has additional effects on males' behaviour and females' perception of males' competitive ability. We expected that increased scent marking would improve males' reproductive success when females can select their mates, and we tested whether this effect is eliminated or enhanced by male-male interactions in our intrusion treatment.

METHODS

Animals and housing

Experimental animals were F1 offspring of wild-caught house mice (*Mus musculus musculus*), which were trapped at 14 locations within a 500 m radius in Vienna (48°12'38"N; 16°16'54"E) and crossed between sites to avoid inbreeding. The F1 mice were weaned at the age of 21 ± 1 days and then housed individually in standard mouse cages (type II, 26.5 × 20.5 × 14 cm) containing wooden bedding (ABEDD), wood shavings and a nest box. Food (Altromin rodent diet 1324) and water was provided *ad libitum* and a 12:12 h light: dark cycle was maintained. Keeping temperature was 22 ± 2°C. At weaning all animals received an ear punch which was necessary for individual identification. Animals were three to five months old when the experiment began.

Mate choice assay

Each female (N=32) was released into a large (3.4 m^2) enclosure where she could choose to interact and mate with either one or both of two unrelated males (N=64). The enclosure was divided in half by an opaque plastic wall (divider), which separated the males on either side of the neighbouring compartments (1.7 m^2 each). Females could move freely between the males' compartments through a small passage tube at the base of the divider. Males were prevented from entering the passage by small collars to ensure that both establish their own territory and to avoid injuries due to fighting. At the base of the divider, four mesh-sealed holes (4 cm diam.) allowed visual and olfactory contact between the neighbouring males to stimulate their signalling effort towards competitors and females. Each male compartment contained one nest box and one shelter both

equipped with bedding and nesting material, one water dispenser and randomly distributed food. We provided females with a cage within each male's compartment (including separate water and food), which was accessible only to females through another passage tube, and thus allowed them to escape male harassment.

To assess females' 'social preferences', we recorded females' presence in the males' compartments once per day on six days a week to determine how often a female was located in each male's compartment over the course of the experiment. Localization of females was performed always at the same time (10:30 am ± 30 min). To assess females' actual mating preferences, we conducted paternity analyses on offspring.

Male scent marks and experimental treatment

To manipulate and quantify males' scent marking, we placed 18 PVC tiles (10 × 10 cm) on the floor of each of the males' compartments before males were introduced. Tiles were arranged along the periphery of their compartments and next to nesting sites, covering approximately 11% of the compartments' total area. Each tile was assigned to a pre-defined position within the compartment. Males were released into the experiment two days before females were introduced to establish a territory and to assess the amount of males' initial scent marking. To simulate territorial intrusion, all of the tiles in a males' compartment were collected and exchanged with his neighbour's tiles ('intrusion treatment'), whereas for the control group, the males' tiles were collected and relocated within their own territory. A study by Desjardins et al. (1973) revealed that males which are located in neighbouring compartments and are separated from each other by a mesh both became dominant markers. The tile shifting started at day three of the experiment, shortly before females were introduced and was conducted on a daily basis bases at the same time (11:00 am ± 30 min to minimize disturbance) until the end of the experiment. To quantify males' scent marking, we took photographs of all tiles before relocating them for six consecutive days starting on the day of female introduction. To prevent spreading scent between the tiles or the enclosures, observers wore one-way plastic shoe covers and tiles were handled with one-way latex gloves.

Experimental design

Each of the 32 females was used for two trials, once in the intrusion treatment and once with a different pair of males under control conditions (within subject design). The 64 males were also tested twice; however, males were assigned to new pairs for the

second trial. Male pairs were always size-matched within 0.5 g body mass and we ensured that none of the experimental animals were familiar with or related to one another. Due to space limitations we could not test all 32 females and 64 males at the same time, so we ran two groups per trial, where we tested 16 females and 32 males each. The number of treatment and controls was balanced within each group. The time between the first and the second trial was two months.

All females had given birth to one litter before the experiment began to control for potential order effects due to comparing virgin versus non-virgin females. Males were sexually inexperienced in their first trial, which allowed us to test for differences in scent marking behaviour between naïve and sexually experienced males. The mice in each group were allowed to interact in the experiment for 18 days and then all animals were returned to the colony. We measured male body mass prior to the experiment shortly before they received the collar and once again immediately after the experiment when we removed the collars to calculate male body mass change during the experiment. Females were placed individually in type IIL mouse cages (32 × 20.5 × 14 cm) to give birth under controlled conditions. Reproductive success was measured in applying genetic paternity analyses on the 57 litters produced.

Scent mark analysis

To quantify males' scent marking, we took photographs of scent marked tiles (Figure 1) each day for six consecutive days. The first photograph was taken shortly before females were introduced to the experiment to quantify the amount of male 'initial scent marking' marking (sum of male scent marking within the first two days of the experiment). The other photographs were used to assess the daily amount of newly marked tile area and to calculate the sum of marked tile area for each male after female introduction ('sum of male scent marking'). We did not analyse number of marks to quantify male scent marking because we found that when males added new marks, previous marks often merged into a single mark, erroneously reducing the number of marks. This merging likely explains why we found no significant increase and in fact a negative trend between the number of marks and the total area marked (Pearson correlation: r=-0.175, N=96, P=0.088). Photographs of tiles were recorded inside of a dark box (60 × 60 × 80 cm) under UV light, emitted by two 18 W strip lights (90 cm, OMNILUX) attached to the ceiling of the box. The 18 tiles within each territory were photographed in two sets of nine tiles each (Figure 1). Set 1 included 5 tiles which were located at the males' shared

territorial border with their neighbouring competitor and 4 tiles along a non-shared territorial border ('shared border'), whereas the second set included 9 tiles from non-shared territorial borders ('non-shared border'). To photograph tiles, each set was placed centrally on the bottom of the box in the same order and exact the same position. Digital photographs were recorded (Canon EOS 400 D, 0.8" exposure time and 4.5 aperture value) from a fixed position on top of the box. The box was cleaned with 70% ethanol between photographs to prevent odour contamination. We recorded the photographs of the first group in trial one (see experimental design) in JPG format, but we excluded these data from our analyses as the image quality was inadequate and only analysed subsequent photographs which were recorded in CR2 format. Photographs were imported into Adobe Photoshop CS 5.1 for image analyses and interpolated (10 cm ≙ 1000 pixel) before a fixed threshold was assigned to determine the proportion of marked tile area. We assessed our measurement error by photographing the same set of tiles three times and found that the sum of marked area differed in ≤ 0.05% between the three photographs.

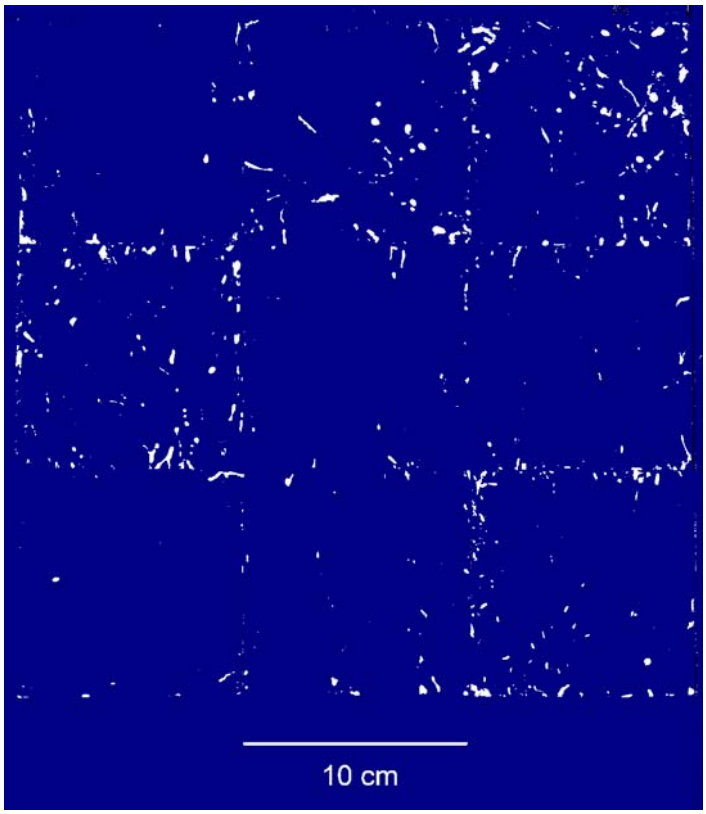

Figure 1: Photograph of scent marked tiles. Photograph of male scent marks on one set of nine tiles. For photographs the nine tiles of each set were collected from the male compartment and placed centrally in the same order and the same position on the bottom of a dark box under UV box.

We could not control for female scent marking; however, it has been shown that female house mice show very low levels of scent marking (Kimura and Hagiwara 1985), independent of oestrus cycle or sexual experience (Maruniak et al. 1975). Even in response to males, females only mark very little (Hurst 1990b), and therefore, they are expected to have a relatively small effect on the overall marking. Our study confirms this assumption since marking did not increase after female introduction and the amount of scent marking was not higher in territories where females were found more often.

Genetic paternity analyses

For genetic paternity analyses DNA was extracted from ear punch samples using a proteinase K/isopropanol protocol (Sambrook et al. 1989) and individuals were genotyped at a minimum of six and a maximum of 16 microsatellite loci (D11Mit150, D9Mit34, D9Mit135, D17Saha, D17Mit28, D10Mit20, D2Mit252, D6Mit138, D15Mit16, D5Mit25, D19Mit39, D7Mit227, D1Mit456, D2Mit380, D17Mit21, D1Mit404, see Mouse Microsatellite Data Base of Japan).Whenever paternity could not be assigned by complete exclusion, we genotyped additional loci. 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. PCR products were analysed using an automated sequencer (Beckman Coulter CEQ 800) and allele scoring was performed using Beckman Coulter CEQ 8000 System software. Allele sizes were determined with SLS+400 as size standard. Paternity results were confirmed with a 95 to 99% trio confidence (dam-sire-offspring relationship) using the program CERVUS 3.0.3 (Marshall et al. 1998).

Statistical analyses

We applied a linear mixed effects model (LMM) to test whether the sum of male scent marking was affected by the intrusion treatment. We included the sum of male scent marking as the dependent variable, trial and treatment as fixed factors and male body mass and body mass change during the experiment as covariates. As males were tested in pairs we included male pair as a random factor to control for non-independence. Male scent marking data referred to proportions, thus, we performed arcsine-square root transformation on this variable.

To test whether males' scent marking predicted their reproductive success we used a generalized mixed effects model (GLMM) with a binomial distribution and a logit link

function. We calculated two models, where we assessed the influence of male marking under intrusion treatment and controls separately as we found a significant interaction between treatment and female social preference (number of observations in a male territory). For both models, the number of offspring sired was included as the dependent variable and litter size as the binomial denominator. Trial was included as fixed factor and the sum of male marking, male body mass change and female social preference was included as a covariate. We included male pair as a random factor to control for non-independence. To test whether female social preference was predicted by the sum of male marking, we applied a generalized mixed effects models (GLMM) with a binomial distribution and a logit link function. We separately assessed female social preference for intrusion treatment and controls as we found a significant interaction between the sum of male marking and treatment. For both models, female social preference was included as the dependent variable and the total number of observations (15) as the binomial denominator. We included trial as fixed factor and the sum of male marking and body mass change as a covariate. Again we included male pair as random factor to control for non-independence. For all models we tested for co-linearity within predictor variables and ensured that model assumptions were fulfilled. We only included biological meaningful two-way interactions into initial models and applied a backward stepwise removal procedure (Grafen and Hails 2002) to avoid problems due to inclusion of non-significant terms (Engqvist 2005). Removed variables were re-entered one by one to the final model to obtain relevant statistics. Statistical analyses were performed using 'R' (version 2.14.1). We implemented linear mixed effects models using the 'lme' function of the 'nlme' package, and generalized mixed effects models using the 'lmer' function in the 'lme4' package (R Development Core Team 2011).

Ethical note

This study had been discussed and approved by the institutional ethics committee in accordance with Good Scientific Practice guidelines and national legislation. We worked with F1 from wild trapped house mice (*Mus musculus musculus*) and trapping of the founder individuals was conducted overnight with Sherman life traps. Each trap was equipped with a piece of apple and bred with peanut butter and nesting material (wood shavings and cotton). Traps were checked twice during the night for occupancy and trapped individuals were immediately removed and placed individually into standard mouse cages (type II, 26.5 × 20.5 × 14 cm) for three weeks of quarantine before joining

the colony. Quarantine conditions were identical to standard keeping conditions in the colony. Trapping was in accordance with national legislation and has been approved by the MA 22 (Municipality for Environment and Conservation of Vienna, Austria). At weaning all animals received an ear punch for individual identification. Ear punches were conducted with a small (2 mm diam.) hole punch device and we collected and stored ear punch tissues at -20°C for genetic analyses, thereby avoiding any additional pain or distress to the animals by any other tissue sampling. We did not administer analgesics to the animals before the ear punch as this procedure would have caused additional handling and stress to the animals. All animals were inspected on a regular basis after the ear punch to monitor animal behaviour and health. No infection ever occurred after ear punching. Males received collars, which consisted of cable ties (2.5 mm wide) with two wires (2.5 cm, 1 mm diam) to provide a mechanical barrier at the shared territory border. Male collaring was necessary to prevent males from fighting, as males aggressively defend their home range against intruders and to protect females from male harassment and coercion by blocking males' entrance into females' cages (see mate choice assay). Males received collars 2 days prior to the experiment to allow them to habituate to the collar in their home cage, while they were inspected on regular intervals. In order to put the collar on, we gently held the mouse in one hand and moved the collar over its head with the other hand to adjust it behind the ears. Collars were loose enough to be moved but tight enough to prevent its removal by the fore-paws of the animal. Collars did not cause any tissue irritation or injury and collared males did not show altered behaviour.

RESULTS

Variation in male scent marking

We tested whether intrusion treatment elicited increased scent marking, and we confirmed that the sum of male scent marking was significantly increased in the intrusion treatment compared to controls (LMM: $F_{1,46}$=5.132, P=0.028, Figure 2). We found no evidence that male body mass (LMM: $F_{1,47}$=0.050, P=0.824), body mass change (LMM: $F_{1,46}$=0.464, P=0.499) or trial (LMM: $F_{1,47}$=1.027, P=0.316) influenced the sum of male scent marking. We found that males scent marked around the entire periphery, but they marked significantly more at the shared versus the non-shared border in both controls (Paired T test: t_{49}=18.46, P<0.001, Figure 2a) and intrusion treatment (Paired T test: t_{45}=16.55, P<0.001, Figure 2a). In addition to this spatial pattern, we also found high within and between individual variation in the daily amount of male scent marking after female

introduction (Figure 2b). Despite this day-to-day variation, individual males' sum of scent marking was correlated between the two trials (Pearson correlation: r=0.584, N=30, P=0.0007, Figure 3a).

Females' introduction into the enclosures did not change males' scent marking rank: The amount of males' initial marking was positively correlated with the sum of scent marking after female introduction (Pearson correlation: r=0.311, N=96, P=0.002, Figure 3b).

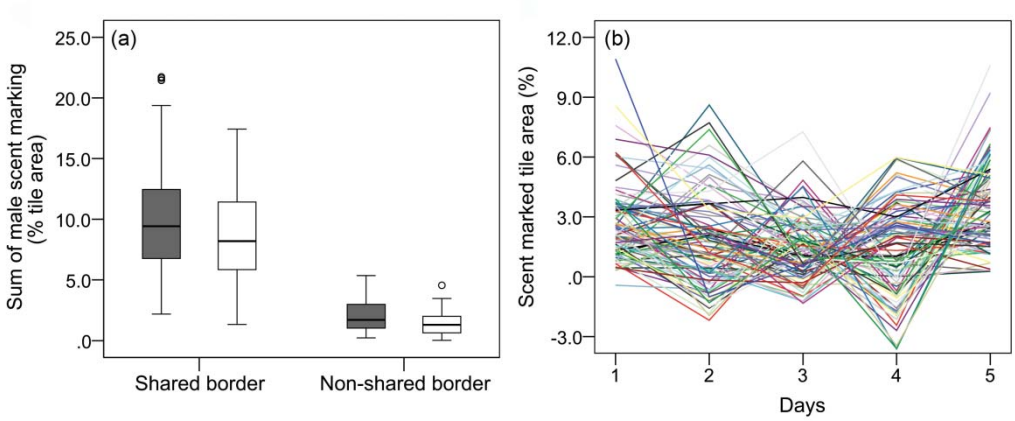

Figure 2: Variation in scent marking over time and space. (a) Amount of male scent marking at the shared territorial borders versus the non-shared territorial borders under the intrusion treatment (grey) and control conditions (white). (b) Within and between individual variation in the daily amount of newly marked tile area after female introduction.

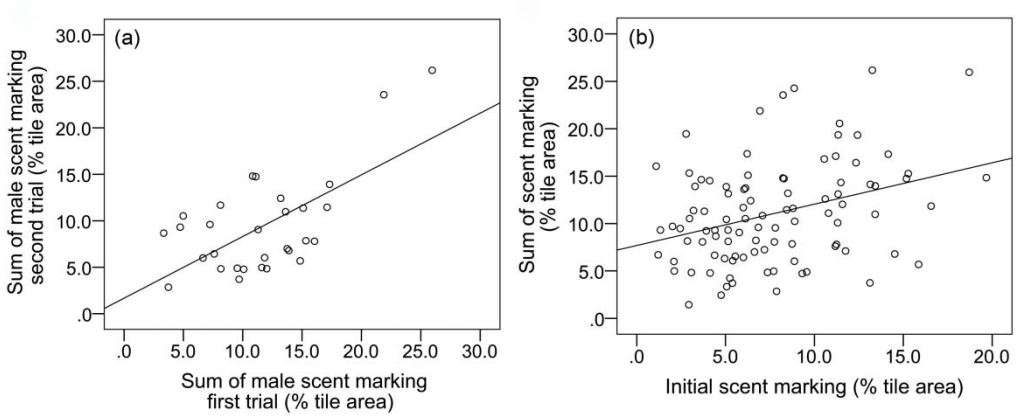

Figure 3: Consistency of male scent marking. (a) Relationship of the sum of male scent marking in their first and the second trial of the experiment. (b) Relationship of males' initial scent marking and their sum of scent marking.

Male scent marking and reproductive success

To determine whether scent marking enhanced males' reproductive success, we conducted paternity analyses on the offspring. We found that the sum of male scent marking was positively and significantly associated with the number of offspring sired, and this effect was stronger in the intrusion treatment compared to the control (GLMM: intrusion treatment: z=4.358, β=0.095, SE=0.021, N=44, P<0.001; control: z=1.972, β=0.055, SE=0.028, N=44, P=0.049, Figure 4). Females' social preferences influenced male reproductive success, but this effect depended on the intrusion treatment: under intrusion female social preference was positively correlated with male reproductive success (GLMM: z=3.299, β=0.109, SE=0.033, N=44, P<0.001), whereas in the controls, female social preference was negatively correlated with male reproductive success (GLMM: z=-2.242, β=-0.064, SE=0.029, N=44, P=0.025). We found no evidence that trial had any influence on male reproductive success in either the intrusion treatment (GLMM: z=0.567, N=44, P=0.571) or the controls (GLMM: z=-0.954, N=44, P=0.340). Male reproductive success was not related to male body mass change in the intrusion treatment (GLMM: z=0.118, N=44, P=0.906), whereas in the control, there was a positive correlation between male body mass gain and male reproductive success (GLMM: z=2.683, β=0.220, SE=0.082, N=44, P=0.007). Individual male reproductive success was correlated across experimental trials (Spearman rank correlation: r_s=0.425, N=62, P=0.003).

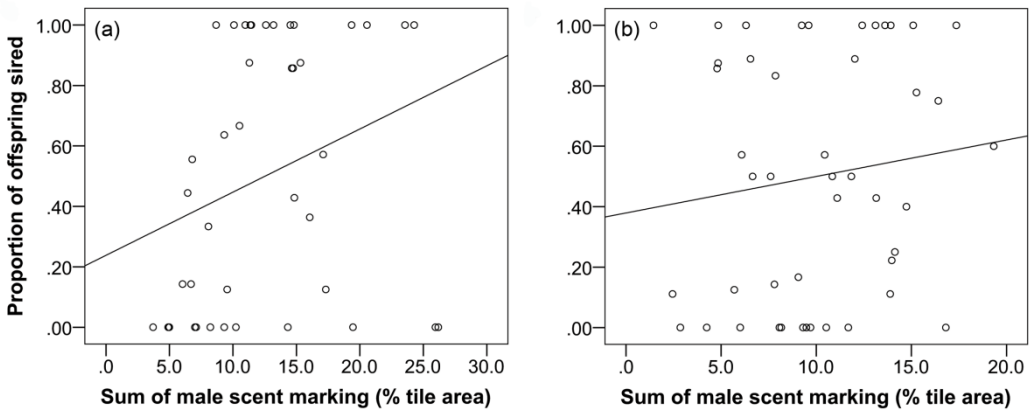

Figure 4: Male scent marking and reproductive success. The amount of male scent marking in relation to the proportion of offspring sired within a litter under (a) intrusion treatment and (b) control conditions. Depicted is the proportion of offspring sired although male reproductive success was calculated as a binomial response with one male's reproductive success being dependent on the second male's reproductive success.

Male scent marking and female social preference

We conducted further analyses to assess whether male marking may have increased their reproductive success by increasing female social preference. In the intrusion treatment, we found no evidence that the sum of male scent marking (GLMM: z=-0.926, N=46, P=0.354, Figure 5a), male body mass change (GLMM: z=0.764, N=64, P=0.445) or trial (GLMM: z=0.275, N=64, P=0.784) influenced female social preference. In the controls, female social preference was negatively associated with the sum of males' scent marking (GLMM: z=-4.030, β=-0.071, SE=0.018, N=50, P<0.001, Figure 5b). Male body mass change (GLMM: z=1.404, N=48, P=0.160) and trial (GLMM: z=-0.432, N=50, P=0.665) had no influence on female social preference.

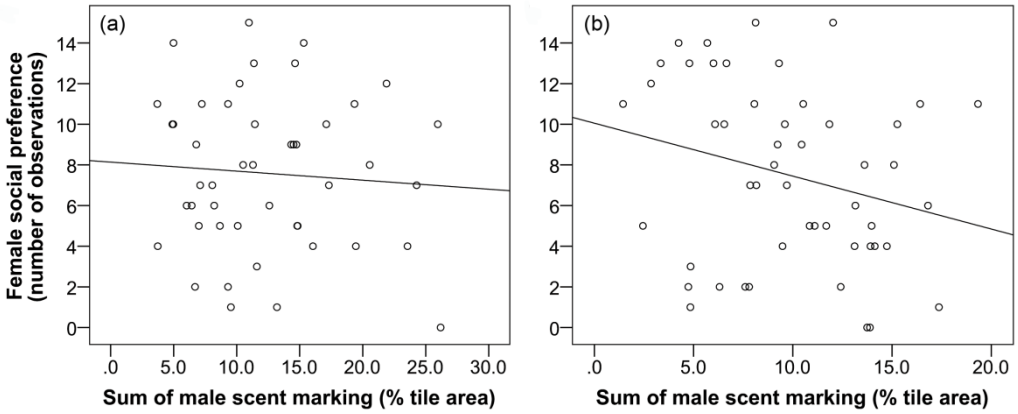

Figure 5: Male scent marking and female social preference. Female social preference (number of female observations in male territories) in relation to male scent making under (a) intrusion treatment and (b) control conditions.

DISCUSSION

Our results indicate that males that deposited more scent marks had higher reproductive success than other males, which provides the first direct evidence to our knowledge that scent marking is maintained by sexual selection. Our finding contrasts with previous studies on prairie voles (*Microtus ochrogaster*) that found no evidence that scent marking increased males' mating success when females could freely choose between two males (Thomas 2002; Mech et al. 2003). However, as we explain below, our results suggest that the 30 min time scale used in these studies was probably insufficient to assess quantitative differences in males' scent marking. Also, these studies did not measure paternity to directly assess male reproductive success. Our results may have been due to female mate choice (inter-sexual selection), as females could choose their mates without sexual coercion, but our observations of females' social preferences provide only mixed

evidence for this hypothesis. We address these results and our other main findings below in more detail.

Variation in male scent marking

We analysed the quantitative variation in males' scent marking, and we found several interesting results. First, we found that males significantly increased their marking in the intrusion treatment compared to controls. This result shows that the introduction of a competitors' scent marks into males' territories elicits increased scent marking from territorial males. Second, we found that males' marking was especially pronounced at the shared territory border in both the intrusion treatments and the controls. If scent marking functions to advertise the ownership of the resident male to potential intruders, then males are expected to mark mainly at their territorial borders where competition for ownership is most likely (Ralls 1971; Gosling 1982). Our study is the first to our knowledge that experimentally manipulated male scent marks to assess the influence of male-male competition on the amount of male marking, and the first to quantify the distribution of scent marks within a males' territory. Our results provide additional support for the hypothesis that male marking and counter-marking advertises a males' territory ownership and mediates intra-sexual competition (Gosling 1982; Hurst 1990a; Gosling and Roberts 2001). Third, we found that males' scent marking showed surprisingly large differences between and within individuals over time. The high day-to-day variation in scent marking suggests that studies measuring scent marking for only a short time period could erroneously classify a low marker as a high marking individual and vice versa. The only other study to our knowledge that repeatedly measured male scent marking over time found that male mice (strain C57BL/J6) showed a rapid decline in marking when repeatedly exposed to the same individuals or the same environment (Arakawa et al. 2008), though we did not find evidence for such habituation effects. For some days, our estimates of marked area yielded negative values, indicating that more scent marks were lost during this period than were added, presumably due to the volatility of pheromones. For the intrusion treatment, negative values could also be due to males removing the scent marks of competitors, although this hypothesis has been questioned for two reasons: (1) Male scent marks are so broadly scattered that it is doubtful that residents can remove them (Hurst and Rich 1999); and (2) males may benefit by the presence of counter-marks if they provide a record of the owner's success in repelling intruders (Hurst and Rich 1999). Nonetheless, it might be beneficial for males to remove competing scent marks to

advertise their sole scent marks to others (Gosling 1982; Rich and Hurst 1998). Regardless of the causes for the negative values in marking, our finding help to explain why males must constantly scent mark their territory to honestly advertise their social status and competitive ability. Finally, although we found large fluctuations in most males' daily scent marking, the sum of each male's scent marking was correlated between the two experimental trials. This finding indicates that males show individual consistency in their overall scent marking, despite large day-to-day fluctuations and interactions with different individuals. Also, since males' initial scent marking was correlated with their sum of marking, female introduction did not change the individual males' rank order of marking. Low markers remained low markers regardless of the availability of females, which suggests that males' low marking during females' absence was not due to a strategic reduction of reproductive investment for the future, and that low marking males may be poor quality and unable to afford higher marking rates.

Male scent marking and reproductive success

We analysed how the sum of males' scent marking influenced their reproductive success, and we found a small but significant correlation between males' marking and their reproductive success, as expected if scent marking is a sexually selected trait. The correlation between male marking and reproductive success was stronger under intrusion treatment compared to controls, though the increase was not significantly different. In this analysis, we also examined female social preferences, and surprisingly, we found that female social preferences had different effects on male reproductive success depending on the experimental treatment: under intrusion, males' reproductive success was positively correlated with female social preference, as expected, whereas in the controls, males' reproductive success was negatively correlated with how often females were observed on their territories. In other words, under the threat of male intrusion, females were more likely to mate with their social partner than his rival, whereas without this threat, females spent more time with their social mates' competitor. This result is difficult to understand, but perhaps females in control conditions – with reduced male-male interactions – were attempting to incite male-male competition, as observed in other mammals (Cox and Le Boeuf 1977). Regardless of the cause, this interaction shows that the effect of females' social preferences on males' reproductive success depended on male intrusion (male-male interactions) (Wong and Candolin 2005). Thus, our findings emphasise that female social preferences are not so straightforward as often assumed, and

that they do not necessarily correspond to actual mating preferences, which is why it is important to measure paternity and not only females' social preferences (Gubernick and Addington 1994).

We also found that male reproductive success was positively correlated with increased body mass during the experiment, though only under control conditions. This finding indicates that increasing body mass enhanced males' reproductive success, which may have been due to females preferring to mate with males in better condition. In this experiment male pairs were closely matched for initial body mass, but females may use changes in body mass as an indicator of male quality. We would expect such a preference in both intrusion treatment and controls, but perhaps females pay more attention to males' condition under low levels of male-male competition. We cannot rule out effects due to male-male competition, and a non-exclusive alternative explanation for this result could be that males that gained more body mass had more competitive sperm or were more effective in intimidating their rivals.

Male scent marking and female social preference

We conducted further analyses to test whether males' scent marking increased their reproductive success by making themselves or their territories more attractive to females (female social preferences). Although male scent marking and female social preference both correlated with male reproductive success under intrusion, male marking had no effect on females' social preferences under intrusion and surprisingly male marking was associated with reduced social preferences in control conditions. This result suggests that even though males' scent marking enhanced their reproductive success, it was not explained by enhancing females' social preferences. A previous study on wild-derived *Mus musculus domesticus* found that females are attracted to the scent of competitive marking males (males in controls versus intrusion), but not to their territories (Rich and Hurst 1998). A possible explanation why females prefer to mate with high marking males, but do not prefer their territories might be that high markers are more aggressive (Drickamer 2001) and females are more likely to get attacked by these males. Female mice prefer dominant over subordinate male odours (Jones and Nowell 1974; Drickamer 1992), however, females prefer to dwell with subordinates when observed overnight as subordinates are less aggressive towards them (Mainardi and Pasquali 1973; Parmigiani et al. 1982a).

Finally, although we controlled for male sexual coercion and allowed female to select among territorial males, we cannot rule out the possibility that the reproductive benefits of scent marking were due to cryptic male-male interactions (intrasexual selection). For example, high markers may have produced more competitive ejaculates in sperm competition and thus sired more offspring. Dominant male mice have increased sperm motility, sperm density and preputial gland weight compared to subordinates (Koyama and Kamimura 1999; Koyama and Kamimura 2000) but it is not known whether differences in sperm production among dominant, territorial males is correlated with their scent marking. We also found that male reproductive success was correlated between the first and the second trial of the experiment. This result indicates that male reproductive success is robust as it is repeatable over time and across different social conditions. High quality males might be constantly superior in male- male interactions including sperm competition or better in attracting females. Further studies are needed to determine whether male scent marking affects male reproductive success through female choice, male-male interactions, or both, and how male-male interactions affect female choice for male scent marking (and vice versa).

Acknowledgements

We thank S. M. Zala for her valuable comments on the study and the manuscript. We also thank T. Klaus and E. K. Straßer for assistance in data collection. Research was supported by the FWF (FV_FP27024711) and Attila Hettyey was supported by the "Lendület" programme of the Hungarian Academy of Sciences (MTA, LP2012-24/2012; www.mta.hu) and S. Raveh was supported by Swiss National Science Foundation fellowships (PBNEP3-132801 and PBNEP3-140190).

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Chapter 4
Does female multiple mating depend on the genetic diversity of potential mates?

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This manuscript has been submitted to the Journal "Behavioral Ecology".
Date of submission: July 3rd 2013

ABSTRACT

Multiple paternity is associated with increased genetic diversity of litters, suggesting multi-male mating (MMM) may function to enhance offspring diversity. We tested whether female house mice (*Mus musculus musculus*) are more likely to mate with multiple males when they have the opportunity to increase the genetic diversity of their litters. In our experiment, females could choose to mate with either one or two males, which were genetically similar (siblings with identical MHC genotypes) or dissimilar (unrelated with different MHC genotypes) to each other. Furthermore, we compared inbred and outbred females since MMM may also depend on females' condition or inbreeding status. Overall, we found no evidence that females increased MMM when they have the opportunity to enhance litter genetic diversity – regardless of whether females were genetically inbred or outbred. Moreover, multiple paternity had no effect on offspring size or number, though mean offspring body mass significantly decreased as litter size increased, and this trade-off between offspring quantity versus quality was more pronounced in inbred than outbred females. Finally, multiple paternity had no effect on offspring sex ratio, yet females produced significantly male-biased litters when they were inbred and when their potential mates were siblings. Our findings do not support the idea that female mice engage in MMM when they have the opportunity to enhance the genetic diversity of offspring, as expected from the genetic diversity hypothesis; however, our findings suggest that females bias offspring sex ratio towards the dispersing sex (males) when they are inbred or perceive a higher risk of inbreeding.

INTRODUCTION

Females in many species show polyandry or multi-male mating (MMM) despite various costs (Daly 1978; Arnqvist and Rowe 2005), and though several potential benefits have been suggested, the functions of this behaviour remain unclear and controversial (Jennions and Petrie 2000; Hosken and Stockley 2003; Simmons 2005). Various studies show that females can increase the number or quality of offspring produced when mating with multiple males (e.g. Madsen et al. 1992; Hoogland 1998; Tregenza and Wedell 1998; García-González and Simmons 2005; Fisher et al. 2006; Firman and Simmons 2008b) and three types of hypotheses have been proposed to explain the evolutionary functions of polyandry through indirect, genetic benefits (Simmons 2005): (1) increased offspring fitness ('good genes') (Kempenaers et al. 1992); (2) increased genetic

compatibility (Zeh and Zeh 1996; Penn and Potts 1999; Tregenza 2002); and (3) enhanced offspring genetic diversity (Yasui 1998; Cohas et al. 2007). Most studies have focused on the good genes and compatible genes hypotheses whereas the genetic diversity hypothesis has received relatively little attention (but see Cohas et al. 2007; Schmoll et al. 2007). Increased offspring genetic diversity can have positive effects on litter performance and survival, as it may serve as a hedge against unstable environments or other unpredictable selective factors. Alternatively, increased genetic diversity can provide benefits through a non-bet-hedging mechanism, as genetic differences in offspring parasite resistance could prevent infections from spreading efficiently within a clutch. For example, in tree swallows (*Tachycineta bicolor*) the immune responses of nestlings from multiple sired clutches were stronger compared to single sired clutches (Dunn et al. 2009). Similarly, a study in the bumble bee (*Bombus terrestris*) showed that high-diversity colonies had fewer parasites and increased reproductive success compared to low diversity colonies (Baer and Schmid-Hempel 1999). However, to our knowledge, it has never been experimentally tested whether females actively engage in MMM when they have the opportunity to enhance the genetic diversity of their offspring. In fact, no studies on polyandry to our knowledge have manipulated the genetic differences of potential fathers.

To test whether females show MMM depending upon the genetic similarity of their potential mates, we conducted a mate choice experiment with wild-derived house mice (*Mus musculus musculus*). Female house mice actively engage in MMM (Rolland et al. 2003) and 46% of litters have two sires when females can choose to mate with one or two sires (Chapter 2). The rate of multiple sired litters is 25% on average in wild populations, though rates of multiple paternity show much variation among populations (6 to 43%, Dean et al. 2006; Firman and Simmons 2008a). It is unclear why females show MMM only sometimes but not other times, or why the rate of multiple paternity varies among populations. One study found that the rate of multiple sired litters increases with population density, suggesting that MMM correlates with the number of potential mating partners (Dean et al. 2006), but another study found no evidence for this hypothesis (Firman and Simmons 2008a). Another possibility is that MMM varies depending upon the degree of genetic diversity among populations. Females may be more likely to seek extra-pair matings when there is more genetic diversity among potential mating partners, and hence more possibilities for genetic benefits. This hypothesis was supported by a

comparative study in birds that found that the degree of extra-pair paternity in populations and species increases with the genetic diversity (Petrie et al. 1998). This hypothesis suggests that females may engage in MMM when their potential mates are more genetically dissimilar to each other, and they have the opportunity to increase the diversity among offspring.

In a previous study we found that multiple paternity is associated with increased litter genetic diversity (Chapter 1). Here, we aimed to test whether females show more MMM when they have the opportunity to increase the genetic diversity of their offspring. Females could choose to mate between two males, which were either genetically unrelated to each other ('genetically dissimilar') or siblings ('genetically similar'). Our hypothesis predicted that females would be more likely to show MMM when the available males were genetically unrelated compared to when they were siblings, and we assessed MMM using genetic paternity analyses. MMM may be influenced by the genes from the major histocompatibility complex (MHC) as a mechanism to enhance offspring MHC diversity (Evans et al. 2012 but see Bollmer et al., 2012). For example, in the Seychelles warbler (*Acrocephalus sechellensis*), females were more likely to seek extra pair copulations when their social mate had low MHC diversity (Richardson et al. 2005). Similarly, a study with house mice suggested that females seek extra-pair matings with males that are more disparate at MHC than their social mate (Potts et al. 1991). Therefore, we also tested whether females are more likely to engage in MMM when they have the opportunity to enhance the MHC diversity of their offspring. MHC genes are good candidates to assess the genetic benefits of mate choice, as they are highly polymorphic, they control immune resistance to infectious diseases, and they influence disassortative mating preferences in mice (Penn and Potts 1999; Penn 2002). As MHC genes control resistance to pathogens and parasites (Apanius et al. 1997), increasing MHC diversity might be advantageous for the survival of litters, as it might allow a broader range of pathogens to be detected and combated. However, it has never been experimentally tested whether females actively engage in MMM when they can enhance the MHC diversity of their offspring.

Finally, we considered the possibility that inbred females (from first-sib matings) are more promiscuous than outbred females. Female mate sampling and mate preferences can depend on females' condition (Hunt et al. 2005; Burley and Foster 2006; Cotton et al. 2006), including their infection (Buchholz 2004) and inbreeding status (Mazzi et al.

2004). For example, in house mice females prefer the odour of outbred versus inbred males and this preference was more pronounced in inbred versus outbred females (Ilmonen et al. 2009). Moreover, a study in the red flour beetle (*Tribolium castaneum*) showed that females with an inbreeding history had higher rates of polyandry compared to outbred controls and that polyandry effectively doubled previously inbred females' reproductive success (Michalczyk et al. 2011). Therefore, we tested whether inbred females are more promiscuous than outbred ones, and whether they are more likely to diversify their offspring when they have the opportunity.

METHODS

Experimental animals

All experimental animals were second generation descendants of wild trapped house mice *(Mus musculus musculus)* in Vienna (48°12'38"N; 16°16'54"E). Progenitor mice were trapped at 14 different locations within a 500 m radius and crossed between trapping sites. Before we assigned the breeding pairs, we genotyped mice to exclude individuals carrying *t* alleles, since these alleles cause meiotic drive and may influence females' mating preferences (Lenington 1991). F1 mice were arranged in two breeding lines to generate both, inbred and outbred mice. Inbred mice resulted from one generation of brother-sister matings and outbred mice resulted from matings of unrelated individuals. Experimental mice were weaned at the age of 21 ± 1 days and kept in standard mouse cages (type I cages, 26.5 × 20.5 × 14 cm). All cages were equipped with wooden bedding (ABEDD), wood shavings and food (Altromin rodent diet 1324) and water *ad libitum*. Ear punches were made for individual identification and tissues were stored at -20°C for genetic paternity analyses. A standard 12:12 h light cycle was maintained. All animals were sexually naive and between 10 and 22 weeks of age when the experiment started.

Mate choice assay

Each female could choose to mate with one or both of two males and these males were either siblings (genetically similar) or unrelated (genetically dissimilar) to each other. Males were located in two neighbouring enclosures (each measuring 1 × 1.7 × 0.8 m) separated by an opaque plastic divider. Males were introduced into their enclosures one day before females to enable them to establish a territory. Simultaneously to male introduction, 20 µl of female urine (pool of seven females collected on five consecutive days) were deposited between the nest box and the shelter to sexually stimulate males.

Females could move freely between the males' enclosures through a small passage tube at the base of the divider (3 cm diameter). Males were prevented from entering the passage by collars (2.5 mm-wide cable ties with two attached wires that provided a mechanical barrier at the opening of the tube). Males were collared two days prior to their introduction to provide them with sufficient time to become habituated to the collar. At the base of the divider, four mesh-sealed holes (4 cm diameter) allowed visual and olfactory contact between males. Each enclosure contained one nest box and one shelter both equipped with bedding and nesting material, one water dispenser and randomly distributed food (Altromin rodent diet 1324). To prevent male harassment, we provided a cage within each male's enclosure (including separate water and food), which was accessible only to females through another passage tube.

To assess females' 'social preferences', we recorded females' presence in the males' compartments once per day over the course of the experiment to determine how often a female was located in each male's compartment. Localization of females was determined around noon when animals were inactive. To assess females' actual mating preferences, we conducted paternity analyses on offspring.

Genetic similarity

Experimental males were selected in matched pairs according to their degree of relatedness and MHC genotypes (see MHC genotyping). 'Genetically similar' males were full siblings that shared identical MHC genotypes, whereas genetically dissimilar pairs were unrelated males that only shared one allele at each MHC locus (see Table 1). In total we had 24 pairs of males, 12 genetically similar and 12 genetically dissimilar. We tested a total of 48 females, thus males pairs were used twice, and 24 of the females were inbred; all other experimental animals were outbred. The genetic background of male pairs (similar or dissimilar) was balanced for female inbreeding status (inbred or outbred). Inbred and outbred females always shared the same number of alleles with both males they were tested with independent of whether the males were genetically similar or dissimilar. However, the number of new alleles females could potentially obtain for their litter by mating with two genetically dissimilar males is twice to what they gain from mating with two genetically similar males (see Table 1).

Table 1: Example of MHC genotypes (class II Aα and Eβ locus) in genetically similar versus dissimilar males in relation to the tested females. Regardless of female inbreeding status, females always shared the same number of alleles with potential mating partners, both when males were genetically similar or dissimilar to each other. However, in the genetically dissimilar treatment, the number of potential new alleles females could gain for their litters from multiple mating was doubled.

Treatment	♂ relatedness	♂ MHC genotype		OB* ♀ MHC genotype		IB* ♀ MHC genotype		# shared alleles	# new alleles
Genetically similar	Brothers	ab ab	cd cd	ag	ch	aa	cc	2	2
Genetically dissimilar	Unrelated	ab ae	cd cf	ag	ch	aa	cc	2	4

*OB = outbred; IB = inbred

Experimental procedure

Females were sexually naïve and always unrelated and unfamiliar with the males with which they were tested. Males were also sexually inexperienced in their first trial. We measured individual body mass (g) the day we introduced the animals into the enclosures to assess whether female mate choice is related to their own or male body mass. The mice were allowed to interact in the experiment for 14 days before all animals were returned to the colony. Male collars were removed immediately and females were placed individually in type IIL mouse cages (36.5 × 20.5 × 14 cm) to give birth under controlled conditions. Reproductive success (litter size and mean pup weight at weaning [litter weight at weaning/litter size]) was measured and genetic paternity analyses were conducted.

MHC Genotyping

For MHC genotyping two class II MHC loci Aα and Eβ on mouse chromosome 17 were screened with single strand conformation polymorphism (SSCP). Therefore, genomic DNA was extracted from frozen ear punch samples using a proteinase K/isopropanol protocol (Sambrook et al. 1989). A two-step PCR (Biometra-T1 thermocycler) was used to amplify the products. The first denaturation step started at 94°C for 2 min followed by 10 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s, and extension at 72°C for 1 min. The second step was followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, extension at 72°C for 1 min and a final extension at 72°C

for 10 min. The PCR for Eβ differed in the two annealing temperatures which were of 53°C and 48°C. The PCR reaction contained 1 µl DNA (100ng/ µl), 1 µl 10x B-buffer, 1 µl dNTPs (2 mM), 1.5 µl $MgCl_2$ (25mM), 0.2 µl Taq-Polymerase (1U/ µl), 0.3 µl of both Aα forward and reverse primer and 0.5 µl of both Eβ forward and reverse primer (modified after Schad et al. 2004). Nucleotide sequence of primers for both loci were: Aα-Forward: 5'-ACCATTGGTAGCTGGGGTG-3'; Aα-Reverse: 5'- CTAAATCC ATCAGCCGACC-3'); Eβ-Forward: 5'- GAGTGTCATTTCTACAACGGGACG-3'; Eβ-Reverse: 5'- GATCTCATAGTTGTGTCTGCA-3'. Reaction volume was 10 µl and ddH_2O was added to reach the desired volume.

For the CE-SSCP analyses 1 µl of the diluted (1:60) PCR product was added to 9 µL loading dye mix (8,5 µL Hi-DiTM formamide,0.5 µL GeneScan ROX 350 standard [Applied Biosystems]). The reaction was denatured at 95°C for 5 min and immediately chilled on ice before analysed by capillary electrophoresis on an ABI PRISM 3130xl automated DNA Sequencer (Applied Biosystems). The CE-SSCP polymer consisted of 5% Conformational Analysis Polymer (CAP) which is made of 9% CAP, 10x Genetic Analyze Buffer, 100% glycerol and HPLC-water and a 1x ABI running buffer was used. The separation of the allelic variants was achieved by using the following running conditions: injection voltage at 1.2 kV, injection time of 18 s, run voltage at 12 kV for 40 min, run temperature at 22°C. The retention times of the allelic variants were identified relative to the ROX 350 standard. GeneMapper software packages 4.05 from Applied Biosystems was used to analyse the SSCP data.

Genetic paternity analyses

Paternity analyses were used to assess differences in female MMM, which is a conservative approach (multiple paternity indicates MMM, whereas single paternity is not necessarily due to single male mating, as one males' sperm can be eliminated by cryptic female choice or sperm competition). Although genetic paternity analyses may underestimate the rate of MMM, this approach does not bias our assessment. Moreover, the genetic benefits from increased litter genetic diversity only apply to multiple sired litters, and not to multiply mating females per se. Thus to understand how selection acts on polyandry through increased genetic diversity, it is more informative to assess paternity of litters rather than to observe female mating behaviour.

DNA was extracted from frozen ear punch samples using a proteinase K/isopropanol protocol (Sambrook et al. 1989). Individuals were genotyped at a minimum of 6 and at maximum of 24 microsatellite loci (D9Mit34, D9Mit135, D10Mit20, D11Mit150, D17Saha, D17Mit28, D17Mit21, D1Mit404, D1Mit456, D2Mit252, D2Mit380, D5Mit25, D6Mit138, D7Mit227, D15Mit16, D19Mit39, D4Mit 17, D4Mit 164, D4Mit 139, D4Mit 243, D4Mit 288, D4Mit 217, D4Mit 241, D4Nds6, see Mouse Microsatellite Data Base of Japan) which were arranged in multiplex PCRs using a Multiplex-PCR MasterMix (Qiagen Multiplex PCR kit). Whenever paternity could not be assigned by complete exclusion we genotyped additional loci. 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 analysed 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. Paternity assignment was made by complete exclusion. Paternity results were additionally confirmed with a 95 to 99% trio (dam-sire-offspring) confidence using the program CERVUS 3.0.3 (Kalinowski et al. 2007).

Statistical analyses

To test the effect of male genetic similarity and female inbreeding status on multiple paternity, we applied a generalized linear mixed effects model (GLMM) with a binomial distribution and a logit link function. Paternity (single or multiple) was included as the dependent variable, male genetic similarity (similar or dissimilar) and female inbreeding status (inbred or outbred) as fixed factors and female body mass, the two males' body mass difference and female social preference (difference in number of observations within the male compartments) as a covariate. As males were used twice in the experiment, we included male pair as a random factor to control for non-independence. To test the effect of paternity on litter size we applied a linear mixed effects model (LMM) with litter size as dependent variable, paternity, male genetic similarity and female inbreeding status as fixed factors and female body mass as a covariate. Male pair was included as a random factor. To test which factors affected mean pup body mass we applied a LMM with mean pup body mass as dependent variable and paternity, male genetic similarity and female inbreeding status as fixed factors and female body mass and litter size as a covariate. Again, male pair was included as a random factor. To test for

differences in male sex ratio we applied a GLMM with a binomial distribution and a logit link function. The number of male offspring was included as dependent variable and litter size as the binomial denominator. Paternity was added as fixed factor and female body mass as a covariate. Again, male pair was included as a random factor. To test for the deviation from the expected mean sex ratio (0.5), we applied a one sample T test. We also applied a T test to determine differences in female body mass in relation to inbreeding status. To test which factors influenced male reproductive success, we applied a GLMM with a binomial distribution and a logit link function. The numbers of offspring sired by each male were included as the dependent variable and litter size as the binomial denominator. Male genetic similarity and female inbreeding status were added as fixed factors, male body mass and female social preference (number of observations within a male's territory) as a covariate. Male ID nested in male pair was included as a random factor to control for non-independence.

We verified that model assumptions were fulfilled in all models and transformed data if necessary. We applied a backward stepwise removal procedure (Grafen and Hails 2002) to avoid problems due to inclusion of non-significant terms (Engqvist 2005) and the removed variables were re-entered one by one to the final model to obtain relevant statistics. Statistical analyses were performed using 'R' (version 2.14.1) (R Development Core Team 2011). We implemented linear mixed effects models using the 'lme' function of the 'nlme' package and generalized mixed effects models using the 'lmer' function in the 'lme4' package.

RESULTS

We analysed the rate of multiple paternity to test whether multiple paternity depended on the genetic similarity of females' potential mating partners or females' inbreeding status. Of the 48 females tested, 78% (38/48) gave birth and 29% (11/38) of these litters were multiple sired. However, the rate of multiple versus single sired litters was not influenced by male genetic similarity (GLMM: z=-0.127, N=38, P=0.899) (Figure 1a) or female inbreeding status (GLMM: z=0.146, N=38, P=0.884) (Figure 1b). Also, none of the covariates in our model explained multiple paternity (female social preference GLMM: z=1.625, β=0.286, SE=0.176, N=38, P=0.104; female body mass GLMM: z=-1.435, β=-5.912, SE=4.121, N=38, P=0.151; body mass differences between males GLMM: z=0.887, β=0.204, SE=0.229, N=38, P=0.375).

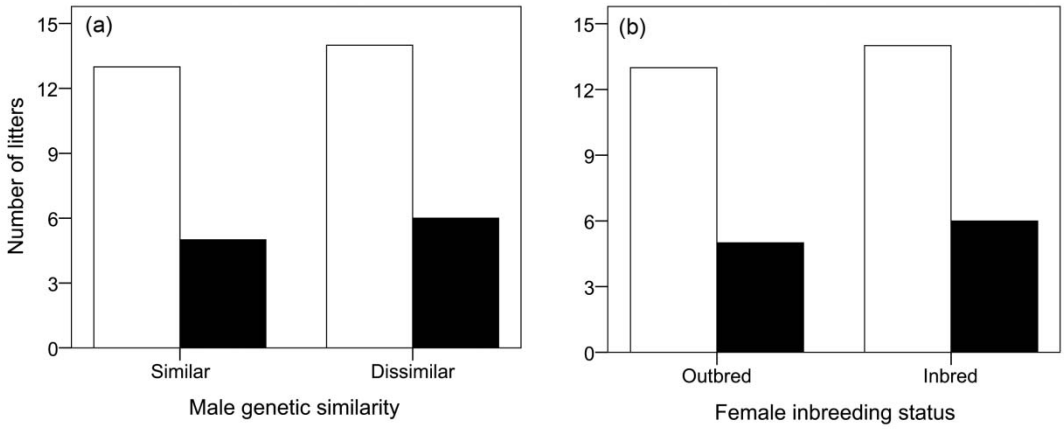

Figure 1: Number of single (white) and multiple (black) sired litters depending on (a) potential mating partners' genetic similarity or (b) female breeding status.

Although we found no support for our main hypotheses, we examined whether multiple paternity or female inbreeding status influenced offspring number, size or sex ratio. We did not find any evidence that multiple paternity affected litter size (LMM: $F_{1,14}$=0.067, N=38, P=0.799). Also, the genetic similarity of potential mates (LMM: $F_{1,14}$=0.697, N=38, P=0.414) and female inbreeding status (LMM: $F_{1,14}$=0.115, N=38, P=0.739) did not influence litter size. Only female body mass was a good predictor of litter size (LMM: $F_{1,15}$=5.135, β=0.644, SE=0.284, N=38, P=0.039), with heavier females producing larger litters. Female body mass had no influence on mean pup body mass (LMM: $F_{1,11}$=2.774, β=0.143, SE=0.086, N=35, P=0.124), but mean pup body mass was negatively correlated with litter size (LMM: $F_{1,12}$=8.921, β=-0.271, SE=0.091, N=35, P=0.011) and this effect was stronger in inbred than outbred females (Figure 2). Female body mass did not differ between inbred and outbred females (T test: t=-0.708, df=44, P=0.482). Multiple paternity (LMM: $F_{1,11}$=1.303, N=35, P=0.278), the genetic similarity of potential mates (LMM: $F_{1,20}$=0.620, N=35, P=0.440) and female inbreeding status (LMM: $F_{1,11}$=0.959, N=35, P=0.349) had no influence on mean pup body mass. Male sex ratio did not differ for single versus multiple sired litters (GLMM: z=0.469, N=35, P=0.639), nor was it correlated with female body mass (GLMM: z=-0.140, β=-0.010, SE=0.066, N=35, P=0.889). However, we found that male sex ratio significantly deviated from the expected 0.5 ratio when potential mating partners were genetically similar (genetically similar: t=3.000, df=16, P=0.008; genetically dissimilar: t=1.675, df=17, P=0.112) (Figure 3a) and when females were inbred (inbred: t=3.017, df=18, P=0.007; outbred: t=1.406, df=15, P=0.180) (Figure 3b).

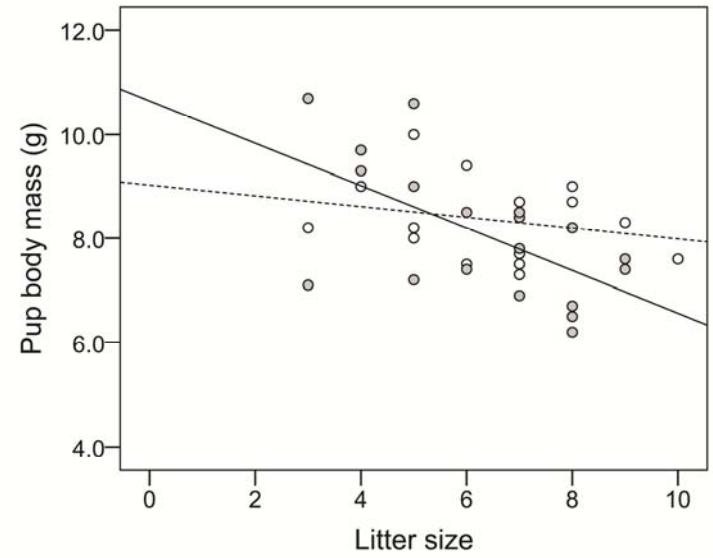

Figure 2: Relationship of litter size and mean pup body mass. Litters of inbred females are depicted as grey dots (solid line) versus outbred females are in white dots (dashed line).

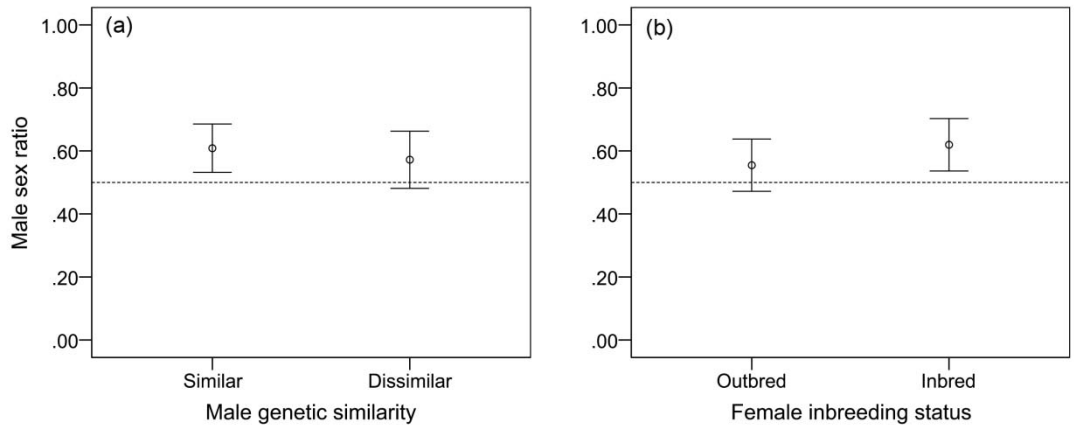

Figure 3: Offspring sex ratio in relation to (a) the genetic similarity of potential mating partners and (b) female inbreeding status. Depicted is the mean ± 95% confidence interval. Dashed lines indicate the expected sex ratio.

Finally, we tested which factors influenced male reproductive success. We found that females' social preferences correlated with males' reproductive success, though interestingly, this relationship depended upon females inbreeding status (GLMM interaction: z=-4.369, β=-0.649, SE=0.149, N=70, P=1x10^{-5}) (Figure 4): females social preference predicted male reproductive success in outbred (GLMM: z=3.900; N=34, P=9x10^{-5}), but not in inbred females (GLMM: z=-0.288, N=36, P=0.773). Male reproductive success was not affected by their body mass (GLMM: z=0.833, N=70, P=0.405) or their genetic similarity (GLMM: z=0.039, N=70, P=0.969). Males were used

twice during the experiment and male reproductive success was correlated between the two trials (Spearman rank correlation: σ=0.659, P=9.6x10^{-5}).

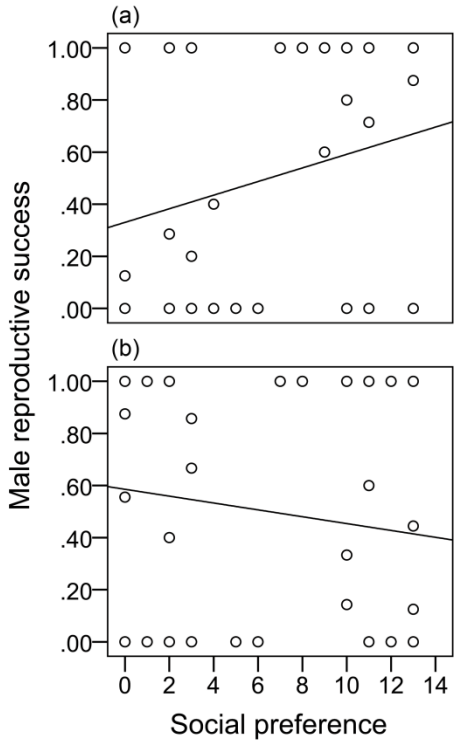

Figure 4: Relationship of female social preference (number of observations within male territories) and male reproductive success (proportion of offspring sired within the litter) in (a) outbred and (b) inbred females.

DISCUSSION

The aim of this study was to test whether females are more likely to produce multiple sired litters when they have the opportunity to increase the genetic diversity of their litters. We did not detect any difference in the rate of multiple paternity between females that could choose to mate with two males that were either genetically similar or dissimilar to each other, and thus we did not find any evidence to support this hypothesis. Our negative results could be due to an inability of females to discriminate male genetic similarity; however, this explanation seems unlikely as several studies indicate that female house mice are able to discriminate males' scent when they differ genetically (see appendix in Thom and Hurst 2004). House mice are even capable of discriminating the scent of males that differ genetically at a single MHC locus (Yamazaki et al. 1979; Penn and Potts 1998). Another possible explanation is that our experiment generated artefacts, which somehow abolished females' preferences for mating with multiple males when males are genetically unrelated. Our genetic paternity analysis indicated that 29% of the litters were multiple sired, which is identical to the average rate found in wild populations of *Mus musculus musculus* (Chapter 1) and feral *Mus musculus domesticus* populations in the USA and Australia (Dean et al. 2006; Firman and Simmons 2008a). This finding

indicates that our experiment did not artificially alter the rate of multiple paternity, and moreover, it provides additional evidence that females show MMM when they can select their males rather than being due to sexual coercion. We can also exclude possible confounds due to differences in male body mass, as the rate of multiple sired litters was not related to the difference in body mass between potential mates, and male body mass did not correlate with their reproductive success. Thus, although female mice potentially gain fitness benefits by producing genetically diverse litters (as has been shown in social insects and birds: Liersch and Schmid-Hempel 1998; Seeley and Tarpy 2007; Dunn et al. 2009), they did not increase the diversity of their litters when given the opportunity to mate with genetically dissimilar versus similar males, as expected by the genetic diversity hypothesis (Yasui 1998). Therefore, there must be some other explanation for why female mice only sometimes show MMM besides the genetic similarity of the available males.

We considered the possibility that inbred females are more likely to show MMM and are more likely to diversify their progeny than outbred ones, but we found no evidence to support these hypotheses either. A previous study with flour beetles found that inbred females (generated from experimentally bottlenecked populations) were more likely to engage in MMM than outbred controls, which enabled them to reduce the negative fitness consequences of inbreeding (Michalczyk et al. 2011). Since the inbred females in the study with flour beetles were generated by eight generations of first-sib matings, it is possible that one generation of inbreeding is not sufficient to increase female promiscuity. However, if eight generations of close inbreeding are necessary to increase female promiscuity, then the relevance of this study is rather limited. Another study on grey mouse lemurs (*Microcebus murinus*) found that polyandry was condition dependent: females with larger body mass were more likely to mate with multiple males than females with lower body mass, suggesting that females in poor condition cannot afford the costs associated with MMM (Huchard et al. 2012). However, we did not find that the rate of multiple sired litters was related to female body mass.

We examined whether females' inbreeding status affected their condition, and whether either inbreeding or condition affected offspring number or size. Inbreeding did not affect female body mass, as in previous studies (Meagher et al. 2000). We found that heavier females produced larger litters, which is commonly observed in mice. Given that reproduction incurs costs (Reznick 1985), especially in mammals (Speakman 2008) where gestation is followed by lactation, females in better condition can afford the higher

costs of producing more offspring. Yet, larger females did not produce larger offspring than smaller females (mean pup body mass was not correlated with female body mass). Inbreeding did not affect offspring size or number either. Yet, mean pup body mass was negatively correlated with litter size, and interestingly this trade-off between offspring number versus quality was more pronounced for inbred than outbred females. Previous studies find that low levels of inbreeding (one generation of sib-sib mating) are not particularly detrimental for the reproductive success of female mice (Lynch 1977; Connor and Belucci 1979; Meagher et al. 2000), however, these studies only examined offspring number or body mass but not their relationship.

We investigated whether multiple paternity enhanced female reproductive success, but we found no differences in offspring number, size or sex ratio between single versus multiple sired litters. Nevertheless, this finding does not rule out the possibility that enhanced genetic diversity improves fitness of litters in the long-term (Mattila and Seeley 2007; Seeley and Tarpy 2007). Unexpectedly, we found that females biased the sex ratio of their litters depending upon their inbreeding status and the genetic diversity of available males: sex ratios were significantly male-biased when females were inbred and when females could only choose among males that were siblings. It has been suggested that females should increase the production of male offspring when they are in good condition (Trivers and Willard 1973), which is supported in some studies on house mice (Drickamer 1990; Rosenfeld et al. 2003 but see Krackow, 1997), but our findings are inconsistent with this hypothesis. It is unclear why inbred females produced male-biased litters, but we propose that this sex ratio bias may allow inbred females to increase the dispersal of offspring (males are the dispersing sex) as a mechanism to avoid inbreeding. This hypothesis would also explain why females in our study also produced male-biased litters when their potential mating partners were siblings, similar to inbred populations. House mice populations are more inbred during winter (when dispersal is almost impossible) than other times (Chapter 1), but in spring and summer they produce more male-biased litters (Drickamer 1990), which is consistent with the idea that females bias the sex ratio of their offspring towards the dispersing sex to reduce inbreeding.

Finally, we also investigated how female social preferences (number of observations within a male territory) predicted actual reproductive outcomes. We expected that when females showed a clear social preference for one male over the other, their litters would be more likely to be single sired. However, whether females had a clear bias for one male

or not did not affect the number of fathers within a litter. We found that male reproductive success was correlated with female social preferences – but only in outbred females. We cannot explain this result, but it implies that inbred females did not spend more time with their preferred male (or the more successful male), unlike outbred females. Thus, taken together, female social preferences in house mice are not a straightforward, unambiguous indicator of mating preferences, as often assumed.

In summary, we did not find any support for the hypothesis that females are more likely to mate with multiple males when they have the opportunity to enhance the genetic diversity of their progeny. Although previous work shows higher levels of genetic diversity within multiple versus single sired litters (Chapter 1), female mice did not show increased rates of multiple sired litters when they have the opportunity to diversity their progeny. Moreover, we found no evidence that inbred females were more likely to give birth to multiple sired litters compared to outbred females, contrary to experimental findings with beetles (Michalczyk et al. 2011), regardless of the genetic diversity of the available males. Nevertheless, we found that inbreeding affected females' social preferences and it increased the trade-off between investing into offspring quantity versus quality. We also found that when females are inbred or can only choose among males that are siblings, they produce male-biased litters, which potentially functions as a mechanism to increase offspring dispersal to reduce additional inbreeding. Future studies are required, however, to experimentally test these observational findings.

Ethical note

This study has been discussed and approved by the Institutional Ethics Committee in accordance with Good Scientific Practice guidelines and national legislation.

Acknowledgments

We thank Dr. Yukio Yasui for his valuable comments on the manuscript.

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Chapter 5

Multiple paternity does not enhance litter resistance to infection

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This manuscript has been submitted to the Journal "Frontiers in Zoology".
Date of submission: July 3rd 2013

ABSTRACT

Polyandry is common in various animal taxa and increased genetic diversity has been suggested to enhance offspring resistance to parasites. We tested this hypothesis in wild derived house mice *(Mus musculus musculus)* by experimentally infecting entire litters of mice with two different strains of *Salmonella typhimurium,* and then measuring whether mice from single and multiple sired litters differed in their ability to control or resolve the infection. We found high variation in pathogen loads within litters and no significant differences between litters overall, and we found no difference in the mean or variance of pathogen loads for single versus multiple sired litters. Therefore, our results do not support the hypothesis that multiple paternity enhances resistance of litters against pathogenic infections. Nonetheless, we found a significant difference in *Salmonella* loads between the sexes, and surprisingly, males showed better ability to control infection than females. This results does not support the hypothesis that testosterone suppresses immune defences against infectious diseases.

INTRODUCTION

The evolutionary function of polyandry or multi-male mating (MMM) is unclear and controversial (Jennions and Petrie 2000; Hosken and Stockley 2003; Gowaty 2012). Multiple mating is clearly adaptive for males, as male reproductive success is limited by the number of females they mate with, whereas female reproductive success is limited by the number of ova produced (Bateman 1948; Trivers 1972). As sperm from a single male is sufficient to fertilize all of a female's eggs, MMM seems superfluous. Moreover, polyandry incurs various costs, such as increased risk of disease transmission, predation or injuries from mating partners (Daly 1978; Magnhagen 1991; Siva-Jothy 2006), and yet, females in many species actively solicit multiple matings (Birkhead and Møller 1998). Several hypotheses have been proposed to explain how females can benefit from MMM (Jennions and Petrie 2000; Hosken and Stockley 2003; Simmons 2005). In non-resource based mating systems where males provide nothing but sperm, explanations of polyandry largely rely on indirect or genetic benefits. The good genes and compatible genes hypotheses predict that MMM can enhance females reproductive success by producing more viable or higher quality offspring, as paternity could be biased towards a better or a more compatible mate (Madsen et al. 1992; Tregenza 2002; Hosken et al. 2003; García-González and Simmons 2005; Fisher et al. 2006; Firman and Simmons 2008c; Firman

and Simmons 2008b). However, even without such a paternity bias, polyandry could provide females with genetic benefits by enhancing offspring genetic diversity. Increased offspring genetic diversity can have positive effects on offspring performance and survival (Yasui 1998).

According to bet-hedging theory, sire genetic diversity would reduce the between-generation fitness variation and thereby enhance the geometric mean fitness of females (Yasui 1998). Such bet-hedging can be selectively favoured (even if the arithmetic mean fitness of females is reduced) when future environmental conditions are unpredictable as increased genetic diversity ensures that at least some genotypes would fit the prevailing circumstances. The non-bet-hedging mechanism on the other hand, predicts that increased genetic diversity within litters enhances the arithmetic mean fitness of females. This effect could be due to genetically based differences in disease resistance as increased genetic diversity could prevent infections from spreading (Hamilton 1987; Jennions and Petrie 2000). For example, increased genetic diversity within bee colonies (either generated by increased MMM of queens or mixing broods from diverse backgrounds) reduced the intensity and prevalence of parasitic infection of colonies (bumble bees, Bombus terrestris: Shykoff and Schmid-Hempel 1991; Liersch and Schmid-Hempel 1998; Baer and Schmid-Hempel 1999; honeybees, Apis mellifera: Tarpy 2003; Tarpy and Seeley 2006; Seeley and Tarpy 2007). Surprisingly though, this hypothesis has never been experimentally tested in a vertebrate species to our knowledge.

In house mice (*Mus musculus musculus*), polyandry is common and on average 29% of the litters are multiple sired (Chapter 1). The number of fathers within multiple sired litters is low and rarely more than two (Dean et al. 2006; Firman and Simmons 2008a). Females actively engage in MMM when unconstrained and allowed to choose between two males (Rolland et al. 2003). These findings suggest that females potentially obtain benefits from polyandry other than the reduction of male harassment and coercion (convenience polyandry, Thornhill and Alcock 1983). It has been shown that females can gain genetic benefits from MMM in enhancing post natal pup survival (Firman and Simmons 2008c) and in facilitating inbreeding avoidance (Firman and Simmons 2008b), though these benefits do not explain the high variation in multiple paternity among feral populations (range between 6 to 43%, Dean et al. 2006; Firman and Simmons 2008a). Given that increased genetic diversity reduces the prevalence and intensity of infections, the rate of multiple paternity within populations could be related to the exposure and the

risk of infection. In line with this assumption, it has been shown that the rate of multiple paternity is higher in high-density populations (Dean et al. 2006; but see Firman and Simmons 2008a) where there is an increased chance of transmission. As we recently found that multiple paternity enhances offspring genetic diversity in wild house mice (Chapter 1), we aimed to test in this study whether multiple paternity enhances offspring disease resistance. Here we tested whether multiple compared to single sired litters show increased immune resistance when challenged with *Salmonella enterica*, a mouse pathogen (gram-negative bacteria). Resistance to this pathogen is genetically controlled by several loci (Roy and Malo 2002), including the highly polymorphic genes of the major histocompatibility complex (MHC) (Penn et al. 2002). We infected mice from single and multiple sired litters with two strains of *Salmonella enterica* and measured the pathogen load in the spleen 17 days post-infection to assess their ability to control and clear infection. If genetic diversity enhances female fitness through bet-hedging, we expected reduced *variance* in pathogen load over multiple versus single sired litters, regardless of any differences in the mean load between the litters. However, if genetic diversity enhances female fitness through a non bet-hedging mechanism we expected the *mean Salmonella* load of multiple sired litters to be smaller from single sired litters.

METHODS

Experimental animals and housing

Experimental mice were second generation descendants of wild trapped house mice *(Mus musculus musculus)* bred for a previous experiment, in which females could choose to mate with one or two territorial males (see Chapter 2). We selected a total of 213 mice from 30 litters (15 single sired; 15 multiple sired). Litter size varied from three to eleven and did not differ significantly between single and multiple sired litters (T test: t=0.562, df=28, P=0.579). Also, there was no significant sex difference between single and multiple sired litters (Chi square test: χ^2=0.004; P=0.949). Weaning occurred at the age of 21 ± 1 days and individuals were further kept in type II standard mouse cages (26.5 × 20.5 × 14 cm) under standard conditions (12:12 h light cycle). Two days prior to infection all experimental animals were moved into a separate room and placed individually into type IIL standard mouse cages (36.5 × 20.5 × 14 cm) with filter hoods on top. All cages were equipped with wooden bedding (ABEDD), wood shavings, a nest box and food (Altromin rodent diet 1324) and water *ad libitum*. Filter hoods were used to prevent mice

from being exposed to other potential pathogens and to avoid the spread of the infection. At the start of the experiment, animals were between six and nine months old.

***Salmonella* infection**

We measured resistance to infection by assessing individuals' ability to control or resolve an experimental infection of a mouse pathogen, *Salmonella enterica* serovar Typhimurium. All experimental animals were infected with two different strains of *Salmonella:* a primary infection with strain AroA (avirulent) and a secondary infection with strain LT2 (more virulent). The bacteria from both strains were stored as slants at 4°C (originated from frozen stocks at -80°C) and cultured in 7.5 ml of heart-brain infusion at 37°C (for 13 hours while shaking at 170 rpm). We diluted the cultures with sterile PBS (phosphate buffered saline) until the desired concentration of 10^4 cfu/ml for AroA and 10^3 cfu/ml for LT2. To verify inocula concentrations we used quantitative plate counts (three plates per dilution). All animals received intraperitoneal (IP) injections of 200µl AroA inoculum and after ten days 200µl of LT2 inoculum. Due to space and time limitations we could not treat all individuals at the same day, thus we divided the experimental animals into 15 groups with each containing one single and one multiple sired litter. Fresh inocula were prepared for both infections in each group. AroA inocula concentrations varied from $4.8x10^4$ to $1.0x10^5$ and LT2 inocula from $8.2x10^2$ to $2.1x10^3$ between groups. All experimental animals were euthanized seven days after the LT2 infection using CO_2. The health condition of all mice was checked daily by visual inspection and animals were weighted before infections and after euthanasia.

Pathogen load

To assess the ability to control and clear *Salmonella* infection ('pathogen resistance') we measured the individual number of bacteria in the spleen (hereafter 'pathogen load') following previous methods (Ilmonen et al. 2007; Ilmonen et al. 2008b). Spleens from euthanized animals were immediately removed, weighed and homogenized (Dispergierstation, T 8.10, IKA®-Werke) in 1 ml PBS under sterile conditions. We plated 50µl of spleen homogenates and their 10^{-1} and 10^{-2} dilutions on selective agar plates (Salmonella-Shigella Agar, Roth) and incubated the plates for 18 hours overnight at 36°C. Pathogen loads per spleen were determined by quantitative plate counts using the mean of two replicate plates for each dilution. We confirmed that pathogen load was not affected by the variation of cfu in inocula (LMM: $F_{1,28}$=0.010, β=0.0006, SE=0.0006, P=0.921).

Statistical analyses

To test whether the pathogen load differed among litters we applied an ANOVA with individual pathogen load as dependant variable and litter as a fixed factor. To compare the mean and variance in pathogen load of single versus multiple sired litters we applied a T test and an F Test. To measure the influence of multiple paternity on individual pathogen load, we applied a general linear mixed effects model (LMM) with individual pathogen load as the dependent variable, paternity (single or multiple) and sex as fixed factors and inocula concentration, individual body mass, body mass change during the experiment and age at the beginning of the experiment as a covariate. Family ID was included into the model as a random factor to control for non-independence of individuals within families. The data were highly skewed and unlike previous studies on lab mice, they could not be normalized with a log-transformation. As model residuals were not normally distributed we used a box- cox transformation to fulfil model assumptions. We tested for sex differences between single and multiple sired litters with a Chi-square test. All statistical analyses were performed using 'R' (Version 2.14.1) (R Development Core Team 2011). We implemented linear mixed effects models using the 'lme' function of the 'nlme' package. We applied a backward stepwise removal procedure (Grafen and Hails 2002) to avoid problems due to inclusion of non-significant terms (Engqvist 2005) and removed variables were re-entered one by one to the final model to obtain relevant statistics.

Ethical Permissions

This study has been discussed by the Institutional Ethics Committee in accordance with good scientific practice guidelines and has been approved by the Austrian Federal Ministry for Science and Research (Zl.22/01/97/2012).

RESULTS

In general, we found no significant difference in pathogen load among litters (ANOVA: $F_{1,29}$=1.305, P=0.150) (Figure 1) and no significant difference in the mean (T test: $t_{1,28}$=-1.033, P=0.310) or variance (F Test: $F_{1,14}$=0.460, P=0.159) of single versus multiple sired litters (Figure 2).

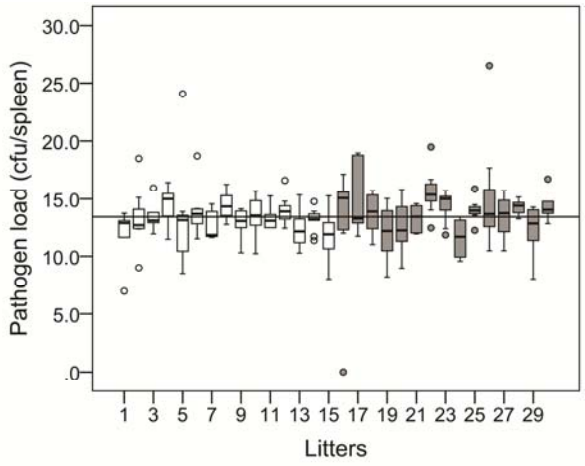

Figure 1: Differences in pathogen loads between litters. Single sired litters are depicted in white and multiple sired litters are in grey. The solid line shows the mean pathogen load of all litters. Pathogen load is box cox transformed.

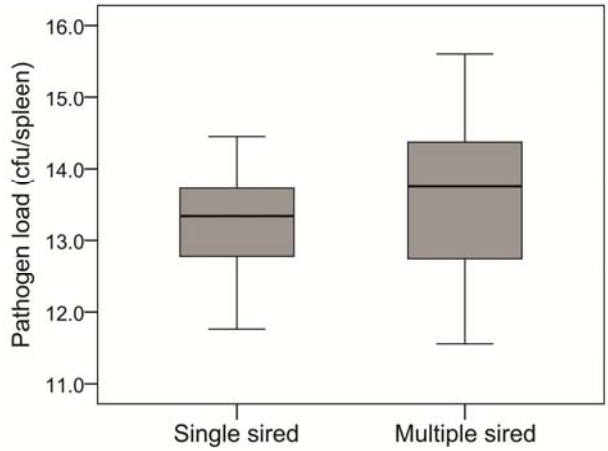

Figure 2: Pathogen load in single versus multiple sired litters. Differences in pathogen load of single (*N*=15) versus multiple (*N*=15) sired litters. Pathogen load is box cox transformed.

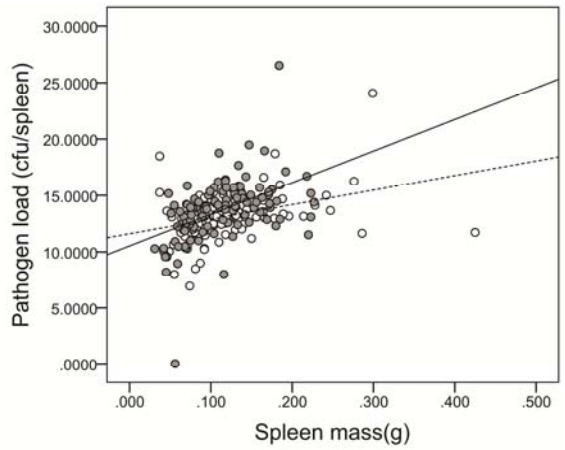

Figure 3: Relationship of spleen mass and pathogen load. Individual offspring spleen mass and pathogen load in single sired (white and dashed line, R^2=0.152) and multiple sired (grey and solid line, R^2=0.223) litters. Pathogen load is box cox transformed.

There was no correlation between individual spleen mass and body mass (Spearman rank correlation: σ=0.022, N=213, P=0.748); however, as expected, spleen mass was highly correlated with pathogen load (Spearman rank correlation: σ=0.453, N=213, P=3.4x10^{-12}) (Figure 3). Also, individual pathogen load did not differ between offspring from single versus multiple sired litters (LMM: $F_{1,28}$=2.765, P=0.108) and was not influenced by individual age (LMM: $F_{1,28}$=0.611, β=-0.006, SE=0.008, P=0.441), body mass (LMM: $F_{1,181}$=0.1446, β=0.065, SE=0.054, P=0.231) or body mass change over the course of the experiment (LMM: $F_{1,181}$=1.782, β=0.061, SE=0.045, P=0.184). However, we found that pathogen load was significantly higher in females than males (LMM: $F_{1,182}$=4.033, P=0.046) (Figure 4).

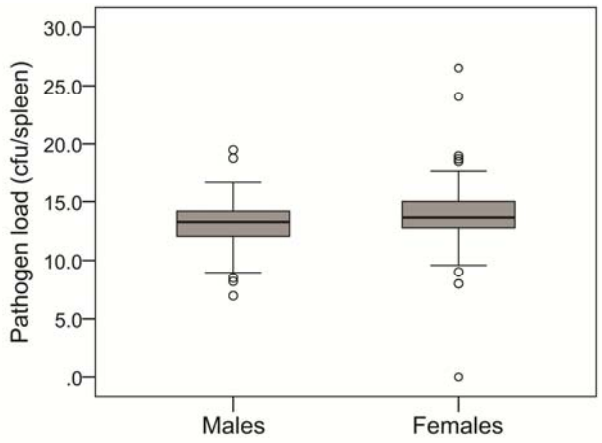

Figure 4: Sex difference in pathogen load. Individual pathogen loads for males (N=108) and females (N=105). Pathogen load is box cox transformed.

DISCUSSION

We aimed to test the hypothesis that females can increase the overall pathogen resistance of their offspring by multiple paternity. By increasing genetic diversity of litters, females might reduce the average pathogen load within litters (non- bet-hedging) or the variation in pathogen load among litters (bet-hedging). We did not find any evidence to support these hypotheses, as we detected no difference in the mean or variation in *Salmonella* load between single and multiple sired litters. Below we discuss potential explanations for our negative result.

There are several potential reasons why we may have missed to detect a difference in pathogen resistance between single versus multiple sired litters. First, our study may have lacked sufficient power, though our sample size was larger than previous studies (Liersch and Schmid-Hempel 1998; Baer and Schmid-Hempel 1999; Tarpy and Seeley 2006). On the other hand, all of these previous studies applied one-tailed tests and therefore had an increased likelihood of finding a significant result. However, even if we would apply one-

tailed tests, our results would not become significant. Second, sire genetic diversity in our study may have been too small to find an actual difference. Multiple sired litters are significantly more genetically diverse compared to single sired litters in wild populations of house mice (Chapter 1), but we did not confirm this finding in the present study. Also, multiple sired litters in our study had only two sires, whereas previous studies with bees inseminated queens with sperm from 10 different drones (Tarpy and Seeley 2006; Seeley and Tarpy 2007). In addition, because social insects produce larger broods than mice (honeybee queens produce up to 250 000 eggs per year), they have greater potential to increase the genetic diversity of their broods. Nonetheless, we aimed to test the genetic diversity hypothesis under ecologically relevant conditions and in wild populations of house mice, multiple paternity very rarely involves more than two sires (Dean et al. 2006; Firman and Simmons 2008a). Third, we measured offspring pathogen resistance against two strains of *Salmonella*, whereas genetic diversity may protect litters against challenges of multiple pathogens, either simultaneously or over time. Fourth, we assessed how individuals were able to control or clear an experimental *Salmonella* infection (pathogen load 7 d after the second infection) in wild mice, and though many studies have found that this measure predicts disease resistance and survival in laboratory strains of mice (Roy and Malo 2002), studies are needed to evaluate the fitness effects in wild mice as they might differ from lab strains. Also, pathogen resistance can be a result of either preventing an infection (decreased prevalence) or reducing the growth (decreased intensity) of the pathogen, but as we experimentally infected the animals we could not test whether increased genetic diversity provides any benefits in preventing the infection or reducing its the spread within litters.

Unexpectedly, we found that males had significantly lower pathogen loads than females, indicating that they are better able to control *Salmonella* infection. There have been numerous experimental *Salmonella* infection studies on laboratory mice (Mittrücker and Kaufmann 2000), as this is the most important host-pathogen model for typhoid fever, however, only few studies have examined sex differences to our knowledge. Early *Salmonella* infection studies on laboratory mice detected no sex differences in pathogen loads (Plant and Glynn 1976), but studies since then, usually focus on one sex or the sexes are not compared or reported. The few studies on *Salmonella* resistance on wild-derived mice also suggest sex differences in disease resistance (Ilmonen et al. 2008a) or interactions between sex and genetic effects on pathogen loads (Ilmonen et al. 2008b).

These findings suggest that sex differences in resistance to *Salmonella* may have been lost in most inbred lab strains. An X-linked locus (*btk)* controls resistance to *Salmonella* in mice through B cell functions (O'Brien et al. 1979; O'Brien et al. 1981), but it is unclear whether this locus explains sex differences in resistance. Resistance to infection is sex-dependent in many species, but our finding is surprising because males are generally the susceptible rather than the resistant sex (Zuk and McKean 1996). There are exceptions (Klein 2004; Morales-Montor et al. 2004), as male house mice are more resistant than females to several parasites, including *Toxoplasma gondii, Babesia microti, Schistosoma mansoni*, and *Taenia crassips* (Klein 2004). Sex differences in immune resistance are generally thought to be due to immunosuppressive effects of testosterone or other steroid hormones (Klein 2000; Klein 2004) – but how can we explain why females are more resistant than males? The harmful effects of *T. gondii* infection depend on estradiol as ovarectomy reduced and administration of estradiol enhanced the development of tissue cysts caused by *T. gondii* (Pung and Luster 1986). Estradiol reduces CD8+ T-cells (Boll and Reimann 1996), and CD8+ T cells control protection against virulent *S. typhimurium* (at least in secondary infections) (Mastroeni et al. 1992; Mittrücker and Kaufmann 2000). Also, mice deficient in CD8+ T cells that survived an attenuated *S. typhimurium* infection were more susceptible to a virulent *S. typhimurium* strain compared to control animals (Lo et al. 1999). Given that testosterone enhances CD4+ and CD8+ T cells (CD4+ T cells are also highly important in protection against Salmonella infection, Mittrücker and Kaufmann 2000), the sex difference in *Salmonella* load in our study may be explained by differences in steroid hormone concentration.

In summary, our findings do not support the hypothesis that multiple paternity enhances parasite resistance in litters. Future studies should investigate the relationship of pathogen clearance and resistance in wild mice and test the effects of multiple parasites on the pathogen resistance of multiple versus single sired litters. As the prevalence and intensity of infections might be sex dependent, future studies should explicitly state in which sex immune response is measured or assess differences between males and females when both sexes are tested.

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II Concluding Discussion

My general aims were to determine the frequency of multiple paternity in wild populations of house mice (*Mus musculus musculus*) and to conduct studies to determine the adaptive functions of multi-male mating and multiple paternity. In Chapter 1, I report the results of the first survey of multiple paternity in wild populations of house mice in Europe (and the first studies on *Mus musculus musculus*), and in the subsequent chapters, I provide results from studies designed to test the adaptive functions of multi-male mating, outlined in the Introduction. Below I provide a general summary of the main findings.

In Chapter 1, we conducted a survey on multiple paternity in wild *Mus musculus musculus* populations, which is the first study to investigate multiple paternity in this subspecies in Europe. We found that MMM was common since on average 29% of the litters were multiple sired. This rate is surprisingly similar to the average rate (25%) found in feral *Mus musculus domesticus* populations in the USA and Australia (Dean et al. 2006; Firman and Simmons 2008a), suggesting that MMM has been selectively maintained in both subspecies at similar rates since their evolutionary divergence approximately 2800 to 6000 years ago (Boursot et al. 1993). The actual rate of MMM might be much higher than 29%, depending on the sire skew of mates (Dean et al. 2006). In house mice, observations in the field suggested (Firman and Simmons 2008a), and laboratory experiments confirmed (Firman and Simmons 2008b), that paternity in multiple sired litters is strongly biased towards one male, indicating that multiple paternity is a conservative estimate of MMM. Although previous studies found high variation in MP among populations, we found none. However, our comparison was based on only two populations. Finally, we also investigated whether multiple paternity resulted in larger offspring or litter sizes, but we found no such evidence, contrary to genetic benefits hypotheses. Nonetheless, we found that the allelic diversity of multiple sired litters was significantly higher compared to single sired litters, supporting the hypothesis that females can gain genetic benefits from polyandry by increasing the genetic diversity of litters. Since we found that multiple paternity was associated with increased genetic diversity of litters, we conducted further studies to test whether females are more likely to engage in MMM when they have the opportunity to enhance the genetic diversity of their offspring (see Chapter 4) and whether multiple sired litters are more resistant against

infectious diseases, as predicted by the genetic diversity hypothesis (see Chapter 5). Before testing these hypotheses, however, we first aimed to test whether females show MMM when they can chose to mate singly or multiply and whether MMM is a conditional mating tactic depending upon territorial intrusion (Chapter 2).

In Chapter 2, we conducted a study to investigate whether females show MMM (estimated by the rate of multiple paternity) when they can select their mates, and whether they show increased MMM when their potential mating partners are unable to exclude intruders from their territories – as predicted by the infanticide avoidance hypothesis. Overall, we found that 46% of litters were multiple sired – which is in the high end of the range found in feral populations (Chapter 1; Dean et al. 2006; Firman and Simmons 2008a). Our findings indicate that females actively engage in MMM when unconstrained by male sexual coercion or other factors in the wild. A study on hybrids of lab and wild mice found that 95% of females actively engaged in MMM when they could freely choose to mate between two males (Rolland et al. 2003), though paternity analyses were not conducted. We found no evidence that the rate of multiple sired litters was higher under intrusion, as we predicted. Yet, we found that multiple paternity was enhanced when potential mating partners were sexually inexperienced, which previous studies found are highly infanticidal (e.g. Labov 1980). In addition, we found that multiple paternity was significantly higher when a females' potential mates showed little or no differences in their amount of scent marking, whereas we found single paternity when males show pronounced differences in their marking. As male scent marking is a condition-dependent quality indicator (Zala et al. 2004; Zala et al. 2008), this finding suggests that females may mate multiply when they cannot assess differences in males quality, as proposed from bet-hedging theory. Alternatively, this result could be explained by either the 'good sperm' hypothesis (male scent marking behavior is correlated with male sperm competitiveness and similar marking males are equally likely to sire the litter) or cryptic female choice. However, future studies are necessary to experimentally test these observational findings.

In Chapter 3, we report additional analyses on the study reported in Chapter 2 to assess how males' scent marking is related to their fitness. Scent marking is often assumed to be a secondary sexual trait that enhances males' mating and reproductive success, though surprisingly there is no direct evidence for this hypothesis. We show that male reproductive success was positively correlated with the amount of their scent marking

even when females can select their mates. This is the first direct evidence that male scent marking is maintained by sexual selection in house mice or any other species to our knowledge. Interestingly, we could not find any evidence that male scent marking enhanced female social preferences, and female social preferences was only correlated with male reproductive success when there were male-male interactions (intrusion treatment). Nonetheless, studies are needed to experimentally test whether male scent marking enhanced male reproductive success through female choice, male-male competition or both.

In Chapter 4, we aimed to test whether females are more likely to show MMM (assessed by the rate of multiple paternity) when their potential mates are genetically more diverse from each other, as a mechanism to enhance the diversity of their offspring (Petrie et al. 1998). In addition, we tested whether inbred females were more likely to engage in MMM than outbred females, as increased genetic diversity could even be more beneficial for inbred than outbred females (Michalczyk et al. 2011). We found a high rate of multiple paternity (29 %) when females can select their mates, but MP was unaffected by whether males were siblings (and MHC identical) or unrelated (and MHC dissimilar) to each other. Therefore, the increased allelic diversity found in multiple sired litters (Chapter 1) is not likely due to females increasing MMM when they encounter males that are more genetically diverse. Also, we found no difference in the rate of multiple paternity between inbred and outbred females, suggesting that female inbreeding status does not predict MMM or alternatively that the moderate level of inbreeding in our study and the associated negative fitness consequences were not sufficient to elicit female MMM. However, as we aimed to test this hypothesis under ecologically relevant conditions we did not carry female inbreeding to extreme stages. Even though there was no evidence that the genetic diversity of potential mates or female inbreeding status influenced the rate of multiple sired litters we still found that both variables directly influenced female fitness: Females produced significantly male biased litters when they were inbred or could only choose among males that are genetically similar. As males are the dispersing sex in house mice, male-biased litters might function as a mechanism to increase offspring dispersal to reduce additional inbreeding. Thus, although we found no evidence to support the hypothesis that polyandry works as a mechanism to enhance the diversity of offspring, we found that females potentially bias the sex ratio in offspring to

avoid inbreeding. However, future studies are needed to test these observational findings experimentally.

In Chapter 5, we aimed to test the hypothesis that MMM functions to increase offspring resistance to infectious diseases (Hamilton 1987; Jennions and Petrie 2000). We tested this hypothesis for the first time in a vertebrate species by experimentally infecting litters with two different strains of *Salmonella typhimurium,* and then measuring whether multiple versus single sired litters differed in their ability to control or resolve the infections. We could not detect any difference in the mean or variation in pathogen load of single versus multiple sired litters. Therefore, our finding does not support the hypothesis that multiple paternity enhances parasite resistance in litters. Interestingly though, we detected a sex bias in individual pathogen load, in males being more resistant than females. This result is surprising as males are usually the more susceptible sex, most likely due to the immunosuppressive effects of testosterone (Zuk and McKean 1996). However, also other steroid hormones can suppress the immune system (Klein 2000; Klein 2004): For example, in mice, estradiol has been shown to reduce CD8+T cells (Boll and Reimann 1996). As these cells are important to combat virulent Salmonella infections we suggest that the higher estradiol concentration in females explains their increased susceptibility to *Salmonella* infections. Thus, although we found no evidence for the hypothesis that MMM functions to enhance offspring disease resistance in house mice we could show that this species is a promising model for investigating sex differences in immune response.

In summary, this work provides important insights into the mating system of wild house mice and contributes to better understand polyandry in general and the ecology of the species in particular. Even though we found no support for any of the tested hypotheses, we could make some novel contributions in the fields of behavioral ecology and evolutionary biology and suggested promising directions for future research.

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III Summary

Polyandry or multi-male mating (MMM) is suspected to provide direct, cryptic and indirect, genetic benefits to females, and our goal was to test whether and how female house mice (*Mus musculus musculus*) can gain such benefits. We assessed the rate of multiple paternity (MP) in wild populations and we found that 29% of litters on average were multiple sired, which is similar to feral populations of *Mus musculus domesticus*, and these litters had greater allelic diversity than single sired ones. We tested whether females are more likely to engage in MMM when they have the opportunity to enhance offspring genetic diversity, but we found no evidence for this hypothesis. It has been shown that higher genetic diversity among nest mates improves collective immune defenses but we found no evidence that multiple sired litters are better protected against infection than single sired ones. MMM may be due to sexual coercion, but when we controlled sexual coercion and allowed females to select their mates, rates of MP were still high (29-46%) and comparable to the wild. We tested females repeatedly and there was no evidence for individual consistency in MP, suggesting that MMM is a conditional tactic. We found two potential explanations for the variation in MP: MP was high when females were presented with virgin males, which are highly infanticidal, and low when females' potential mates produced disparate amounts of scent marking (social status and quality indicator trait). In summary, our results indicate that MMM is common in house mice and it is due to female choice rather than sexual coercion. We found little evidence for genetic benefits, but MMM in mice may be an adaptive response triggered by exposure to infanticidal males or males with similar competitive ability, but experiments are needed to test whether MMM reduces infanticide risk or provides other direct or indirect benefits.

IV Zusammenfassung

Polyandrie ist eine sehr häufig vorkommende Paarungsstrategie im Tierreich, deren evolutionärer Ursprung sehr umstritten ist. Weibchen sind in ihrer Fitness durch die Anzahl ihrer Eizellen und nicht durch die Anzahl der Geschlechtspartner beschränkt und da multiple Verpaarungen erhebliche Kosten mit sich bringen, wie zum Beispiel ein erhöhtes Risiko an sexuell übertragbaren Krankheiten oder ein erhöhter Raubdruck, stellt sich die Frage, warum sich Weibchen mit mehreren Männchen verpaaren.

Ziel meiner Dissertation war es, Polyandrie bei Hausmäusen (*Mus musculus musculus*) zu untersuchen und zu testen welche Fitnessvorteile Weibchen durch eine solche Paarungsstrategie erhalten können. Ich habe die Häufigkeit von multiplen Vaterschaften in wilden Würfen bestimmt und festgestellt, dass 29% dieser Würfe von mehreren Vätern gezeugt wurden. Diese Rate ist mit jener von wild lebenden *Mus musculus domesticus* vergleichbar. Unabhängig von der Wurfgröße zeigten genetische Untersuchungen, dass die Nachkommen von multipel gezeugten Würfen eine signifikant höhere Allelediversität aufwiesen, als Nachkommen von Würfen mit nur einem Vater. In diesem Zusammenhang habe ich getestet, ob sich Weibchen wahrscheinlicher mit mehreren Männchen verpaaren wenn die Möglichkeit besteht die genetische Diversität der Nachkommen zu erhöhen. Meine Ergebnisse unterstützen diese Hypothese nicht. In anderen Studien wurde gezeigt, dass erhöhte genetische Diversität innerhalb von Familien die kollektive Immunantwort der Familie steigert. Ich habe diese Theorie getestet, allerdings ließ sich auch hier kein Beweis dafür erbringen, dass multipel gezeugte Würfe besser gegen eine Infektion geschützt wären als Würfe mit nur einem Vater. Polyandrie kann das Ergebnis von weiblicher Partnerwahl oder sexueller Nötigung sein. Wenn Weibchen die Möglichkeit hatten, frei von sexueller Nötigung zwischen Männchen zu wählen, war die Rate der multipel gezeugten Würfe vergleichbar mit der in freier Wildbahn. Dieses Ergebnis unterstützt die Theorie, dass Polyandrie nicht von Männchen initiiert ist. Wiederholt getestete Weibchen zeigten keine individuelle Beständigkeit in ihrem Paarungsverhalten, was darauf hinweist, dass Polyandrie eine konditionelle Paarungsstrategie ist. Zwei Faktoren standen im direkten Zusammenhang mit der Rate an multipel gezeugten Würfen: Erstens, die Häufigkeit von multipel gezeugten Würfen war höher, wenn potentielle Paarungspartner sexuell unerfahren waren. Da sexuell unerfahrene Männchen hoch infantizitär sind und Kopulation dieses Verhalten nachweislich verringert, deutet

dieses Ergebnis darauf hin, dass Weibchen sich multipel verpaaren, um die Gefahr des Infantizids zu reduzieren. Zweitens, die Häufigkeit von multipel gezeugten Würfen war niedrig, wenn potentielle Geschlechtspartner nur geringe Unterschiede in ihrem Markierungsverhalten zeigten. Duftmarkierungen sind Qualitätsmerkmale die Auskunft über den sozialen Rang und die Konkurrenzfähigkeit der Männchen geben. Weitere Analysen zeigten, dass jene Männchen, die mehr markierten auch einen höheren Reproduktionserfolg hatten. Zusammengefasst weisen diese Ergebnisse darauf hin, dass Weibchen sich multipel verpaaren, wenn sie keinen klaren Qualitätsunterschied zwischen den Männchen feststellen können, andernfalls verpaaren sie sich mit dem qualitativ hochwertigeren Männchen. Weitere Studien sind jedoch nötig, um diese Annahmen experimentell zu testen.

V Contributions

	Chapter 1	Chapter2	Chapter 3	Chapter 4	Chapter 5
Study conception and design	KT, DP	KT, DP	KT, DP	KT, DP	KT, DP
Run the experiment	KT, KM, MT	KT	KT	KT	KT, SR, MT
Data analyses	KT, KM, MT, TK	KT, AT, HB	KT, AT, HB	KT	KT
Writing the manuscript	KT, DP	KT, DP	KT, DP	KT, DP	KT, DP
Revising the manuscript	KT, DP, KM, MT	KT, DP, AT, SR	KT, DP, AT, SR	KT, DP, SR	KT, DP, SR, MT

AT Attila Hettyey

DP Dustin J. Penn

HB Helmut Beissmann

KM Kerstin Musolf

KT Kerstin E. Thonhauser

MT Michaela Thoß

TK Teresa Klaus

VI Acknowledgments

First, and most importantly I want to thank Dustin J. Penn for providing me with the opportunity to become a scientist. You always challenged and supported me and there are no words to express how much I have learned from you. You are an incredible source of inspiration.

I am grateful to Attila Hettyey, Michaela Thoß, Shirley Raveh and Sarah Zala for insightful discussions and motivation.

Many thanks to Helmut Beissmann and Renate Hengsberger for their time and their gift to solve problems.

I want to thank Michael Kuba for good advice, patience and motivation whenever it was needed.

I want to thank my family and especially my parents for paving the way and constantly supporting my decisions.

I am grateful to my friends for joining me in this endeavour and their patience and generous understanding.

Last, but surely not least, I want to thank Chris, who was always part in this journey. I am looking forward to new journeys with you.

VII Curriculum Vitae

Personal Data

Name	Kerstin Elisabeth Thonhauser
Date of birth	03.01.1984
Citizenship	Austria
Family Status	unmarried, no children

School and Professional Education

01/2010–09/2013	PhD programme Biology at the Vienna University
01/2006–04/2008	Master programme Zoology at the Karl-Franzens University Graz. Graduation: passed with honours
10/2007–12/2008	Master thesis at the Vienna Zoo: Aspects of social and discrimination learning in freshwater stingrays (*Potamotrygon falkneri*)
10/2002–01/2006	Bachelor programme Biology at the Karl-Franzens University Graz
03/2004	Bachelor Specialization: Animal Behaviour 1. Bachelor thesis: The phylogenetic development of elephants, their life in nature to the problematic of elephant husbandry 2. Bachelor thesis: The systematic of bees – their defence mechanisms with special emphasis on their poison
09/1994–06/2002	BG/BRG Köflach (Grammar School with special emphasis on languages) 22.06.2002 Matura (school leaving examination)
09/1990–06/1994	Primary School Mooskirchen

Foreign Experience

07/2013–08/2013	Guest researcher at the Max Planck Institute for Brain Research, Frankfurt am Main, Germany Research topic: Learning and cognition in cuttlefish (*Sepia officinalis*)
07/2011	Guest researcher at the Alexander Silberman Institute of Life Sciences, Hebrew University of Jerusalem, Israel Research topic: Learning and cognition in Octopus (*Octopus vulgaris*)
09/2007	Teaching assistant at the Marine Science Institute „Meeresschule Valsaline“, Pula, Croatia Topic: Marine life forms and habitats in the Mediterranean Sea

07/2007–08/2007	Research associate at the Alexander Silberman Institute of Life Sciences, Hebrew University of Jerusalem, Israel Research topic: Learning and cognition in Octopus (*Octopus vulgaris*)
08/2006–12/2006	Study abroad: Eckerd College, Florida, USA Major: Marine Science and Animal Behaviour All courses passed with honours
08/2005	Voluntary internship at Archelon (Sea Turtle Protection Society of Greece), Lakonikos, Greece Topic: Conservation of loggerhead turtles (*Caretta caretta*)

Work Experience

01/2010–09/2013	Research associate at the Konrad Lorenz Institute of Ethology, University of Veterinary Medicine, Vienna, Austria Research topic: Polyandry in house mice *(Mus musculus musculus)*
05/2009–09/2009	Research associate at the Vienna Zoo, Vienna, Austria Research topic: Thermoregulation in Morelet's crocodiles (*Crocodylus moreletii*)
03/2009–05/2009	Technical Assistant at the Department of Applied Plant Sciences and Plant Biotechnology, University of Natural Resources and Applied Life Sciences, Vienna, Austria. Topic: Host parasite interaction of *Phylloxera* on *Vitis sp.*
02/2008–02/2009	Part time employee at the Vienna Zoo, Vienna, Austria Responsibility: Zoological guide and seminars
07/2007	Project collaborator at the Vienna Zoo, Vienna, Austria Responsibility: Investigation of the foraging behaviour of freshwater stingrays (Potamotrygonidae)
01/2007–06/2007	Part time assistant at the ACC (Animal Care Center), Styria, Austria Responsibility: Assistance in health care and medical treatment
07/2006	Internship at the Federal Department of Veterinary Medicine in Styria, Austria Responsibility: Laboratory and administrative work
07/2005	Internship at the Animal and Nature Park Schloss Herberstein, Styria, Austria Responsibility: Investigation of social behaviour in Siamangs (*Hylobates syndactylus)*

Seminars, Conferences and Awards

08/2013	IEC (Animal Behaviour) conference, Newcastle, England Oral Presentation: Scent marking behaviour and reproductive success in male house mice
08/2012	ISBE (International Society for Behavioural Ecology) conference, Lund, Sweden Poster presentation: Polyandry in female house mice
08/2012	ISBE Travel grant: € 1.000
09/2008	DZG (Deutsche Zoologische Gesellschaft) Conference, Jena, Germany Poster presentation: Social learning in freshwater stingrays Poster award
05/2008	Seminar: Social development and body language of dogs by Marlie-Helene Scheib
06/2006	Seminar: Leadership and team experience with horses Intensive training in nonverbal communication by Bruno Sperl, Styria, Austria
2005	Student award of the University of Graz. € 730
2007	Student grant of the University of Graz. € 2.646

Publication list

Hettyey, A., Griggio, M., Mann, M., Raveh, S., Schaedelin, F.C., Thonhauser, K.E., Thoß, M., van Dongen, W.F.D., White, J., Zala, S.M., Penn, D.J. (2012) Peerage of Science: will it work? *Trends in Ecology & Evolution* **27**: 189-190. doi: 10.1016/j.tree.2012.01.005

Thonhauser, K.E., Gutnick, T., Byrne, R.A., Kral, K., Burghardt, G.M., Kuba, M.J. (2013a) Social learning in Cartilaginous fish (stingrays *Potamotrygon falkneri*). *Animal Cognition*: 1-6. doi: 10.1007/s10071-013-0625-z

Thonhauser, K.E., Raveh, S., Hettyey, A., Beissmann, H., Penn, D.J. (2013b) Scent marking enhances male reproductive success. *Animal Behaviour* **submitted**

Thonhauser, K.E., Raveh, S., Hettyey, A., Beissmann, H., Penn, D.J. (2013c) Why do female mice mate with multiple males? *Behavioral Ecology and Sociobiology* **submitted**

Thonhauser, K.E., Raveh, S., Penn, D.J. (2013d) Does female multiple mating depend on the genetic diversity of potential mates? *Behavioral Ecology* **submitted**

Thonhauser, K.E., Raveh, S., Thoß, M., Penn, D.J. (2013e) Multiple paternity does not enhance litter resistance to infection. *Frontiers in Zoology* **submitted**

Thonhauser, K.E., Thoß, M., Musolf, K., Klaus, T., Penn, D.J. (2013f) Multiple paternity in wild house mice (*Mus musculus musculus*): effects on litter genetic diversity and offspring body mass. *Evolutionary Biology* **submitted**