Does Female Partner Preference Improve Pathogen Resistance in her Offspring?
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1. Introduction

Mating preferences, which are any biases that result in non-random mating, are widespread and can enhance an individual’s reproduction and their offspring’s survival (ANDERSSON, 1994; CLUTTON-BROCK and MCAULIFFE, 2009). By mating with certain males, females can obtain direct benefits (resources and parental care) or indirect, genetic benefits for their offspring (JENNIONS and PETRIE, 2000; ZEH and ZEH, 2001). The most direct evidence for genetic benefits from mate choice come from studies that compare the fitness of offspring from females experimentally mated with preferred (P) versus non-preferred (NP) males (HETTYEY et al., 2010; JENNIONS et al., 2012; KOTIAHO and PUURTINEN, 2007). For example, sexual selection experiments with house mice (*Mus musculus*) examined the fitness consequences of both males’ and females’ partner preferences and found that offspring from P individuals had higher viability and increased reproductive success compared to NP matings (DRICKAMER et al., 2000).

It is often suggested that mate choice functions to increase the immune resistance of offspring against infectious diseases (HAMILTON and ZUK, 1982; FOLSTAD and KARTER, 1992; PENN and POTTS, 1999). Therefore, females are expected to prefer disease-resistant males, and mating preferences should enhance offspring fitness (JOHNSON et al., 2000; PENN, 2002). In house mice (*Mus musculus*), for example, females show mating preferences for uninfected versus experimentally parasitized males (EHMAN and SCOTT, 2001). How do females recognize disease-resistant males? Studies on a wide variety of species find that males with more parasites or weak immune responses generally have reduced secondary sexual traits (e.g. colorful plumage, antlers) (HAMILTON and POULIN, 1997; MØLLER et al., 1999), which can be assessed by the female. And in mice, urinary scent marks are secondary sexual traits, and females are able to recognize and they prefer the urinary scent of healthy, uninfected versus experimentally infected males (KAVALIERS et al., 2005; PENN et al., 1998; ZALA et al., 2004). Resistance to a variety of infectious diseases is heritable in house mice (WAKELIN, 1996), and females prefer the scent of genetically modified mice that are resistant to infectious
diseases compared to susceptible controls (ZALA et al., 2008). Although numerous studies have examined how males' secondary sexual traits reveal disease resistance, there have been no studies to our knowledge that have experimentally tested whether female choice increases offspring resistance to infectious diseases.

We conducted a study with wild-derived house mice (*Mus musculus musculus*) to test whether experimental sexual selection increases the resistance of females’ offspring to an experimental challenge of *Salmonella enterica* (serovar Typhimurium). This is an enteric mouse pathogen that becomes systemic by invading the intestinal mucosa and by replicating intracellularly within host macrophages (MITTRÜCKER and KAUFMANN, 2000; SANTOS et al., 2001). We bred mice by experimentally mating females with either P or NP males in a partner preference assay and then experimentally infected the mice to assess their ability to resolve infection (by measuring bacterial loads over three weeks after infection). There are several genes known to control resistance to *Salmonella* in mice, including MHC (major histocompatibility complex) genes, e.g. toll-like receptor 4 (Tlr4) (ROY and MALO, 2002). MHC heterozygosity increases pathogen clearance and survival following *Salmonella* infection in congenic mice (ILMONEN et al., 2007; MCCLELLAND et al., 2003; PENN et al., 2002), and as well enhances reproductive success in wild-derived house mice (THOß et al., 2011). To understand the genetic basis of pathogen clearance, we examined whether MHC heterozygosity influenced the pathogen clearance of offspring to *Salmonella* infection.

We predicted that offspring sired by a P male should have better pathogen clearance compared to offspring produced by NP males, and that such differences might be explained by enhanced MHC heterozygosity of offspring from mating with P males.

2. Materials and Methods

2.1. Study animals

The mice used in this experiment were F2 descendants of wild-trapped house mice (*Mus musculus*) (Fig.1) from Vienna (48° 12’ 38” N; 16°16’54” E). The parental generation were trapped at 14 different locations within a 500 m radius and then bred between locations. The F1 mice were used for a social partner preference test (DRICKAMER et al., 2000). A total of 30 females were allowed to move freely between two unrelated males without opportunity to mate. Females were then experimentally paired and mated with their partner preference: either the preferred (P) or the non-preferred (NP) male and the resulting offspring (F2 generation) were used for experimental infection. All F2 mice were weaned at the age of 21±1 days and kept individually in standard mouse cages (26.5 × 20.7 × 14 cm) with wooden bedding (Abedd: aspen wood chips), enrichment material consisting of nesting material (Abedd: aspen wood shavings), nest boxes and food (Altromin rodent diet 1324), and water ad libitum. A standard 12:12 h light cycle was maintained and temperature ranged from 22 to 25°C. During the infection experimental animals were housed in individually ventilated cages (36.5 × 20.7 × 14 cm, IVC, Fig. 2) system in special IVC racks. IVC housing provides the advantage to maintain low ammonia, CO2 concentration, and humidity, as well as to reduce spread of infective agents to other mice (mice are coprophilic). All other housing conditions remained consistent to conditions prior to infection. IVC system contains a cage top with a food hopper, a water bottle holder and a filter lid. It is supplied by a ventilation unit with HEPA (High Efficiency-Particulate Air)-filtered, conditioned room air. As a rule, 50-70 air changes per hour take place in the cage. Since the cage (including a cage bottom, a cage top with a food hopper, a water bottle holder and a filter lid) is closed with a special cover and supplied by a ventilation unit with a HEPA (High Efficiency-Particulate Air)-filter. As a rule, 50-70 air changes per hour take place in the cage. All cages were cleaned on a weekly basis to reduce the potential of infection. During the cage changing, each mouse was weighed. The cages were disinfected with Clidox (Clidox-S Base Pharmacel and Clidox Activator, Pharmacal Research Laboratories, Inc.) mixed with water. Clidox is a chlorine dioxide
based sterilant, which is sporicidal, tuberculocidal, bactericidal and fungicidal. After a reaction time of 20 minutes, the cages were washed regularly.

2.2. *Salmonella* infection

To assess pathogen clearance, 72 mice were experimentally infected, half of them on one day and the other half the next day, and then balanced for partner preference (P/NP) and sex. All mice were adults (15-21 weeks old) and received an intraperitoneal (IP) infection of 200 μl *Salmonella enterica* serovar Typhimurium (10^3 cfu mL^−1, strain LT2). The natural infection route of *Salmonella* in mice is oral e.g. infection transfer by feces. We opted for the IP method, as it allows using a lower dosage and the knowledge of the exact amount of the inoculum. As during oral inoculation the dosage is higher because the bacteria has to pass through the gut system and the mice must be restricted from food and water before to rule out variation in systemic infection due to food in the gut. The bacteria (stored as slants at 4°C originated from frozen stocks at -80°C) were cultured in 7.5 ml of heart-brain infusion at 37°C for 13 h (overnight) while shaking at 170 rpm. The overnight solution was diluted with sterile phosphate buffered saline (PBS) until the desired dilution of 10^3 was reached. To verify the concentration, serial plating of the dilution (50 μl per plate and three plates per solution) was performed. The animals (N=72) were split into three different groups to obtain a sample size of 24 individuals per week during the three-week experimental period. The groups were balanced for partner preference and sex. Individuals were euthanized and dissected over three weeks: group 1 after 7 days (N=24), group 2 after 14 days (N=24), and group 3 after 21 days post inoculation (N=24). Moreover, all animals were weighed weekly. The health and condition of the mice was examined daily by visual inspection throughout the experiment. Wild and outbred mice are resistant to *Salmonella*, and in our pilot study (same methods as described above), all animals survived the *Salmonella* infection, and therefore, we did not anticipate mortality. For comparison, we also monitored the survival of additional animals in the colony (same age class), which were not experimentally infected. All individuals were euthanized humanely using an overdose of CO₂. The pathogen load of 53 out of 72 animals was examined, since only spleens were included from animals that survived until euthanasia. To
determine pathogen loads of experimentally infected animals, spleens of euthanized animals were immediately removed and homogenized (Dispergierstation, T 8.10, IKA®-Werke) in 1ml PBS under sterile conditions. Afterwards, 50 μl of each homogenate was plated on selective agar plates (Önöz Salmonella agar Merck, Darmstadt, Germany), and incubated overnight for 18 hours at 36°C. Pathogen loads per spleen were determined by calculating the bacterial concentration (cfu/spleen) of spleen homogenates (ILMONEN et al., 2008; PENN et al., 2002) using the mean of three replicate plates. All work reported herein was in accordance with ethical standards and guidelines in the care and use of experimental animals of the Ethics Committee of the University of Veterinary Medicine of Vienna, Austria.

2.3. Genotyping

DNA from ear punches of a total of 72 animals was extracted previously using a proteinase K-isopropanol protocol (SAMBROOK et al., 1989). All individuals were genotyped at three MHC loci in one multiplex run (D17SAHA; D17Mit21; D17Mit28; see Mouse Microsatellite Data Base of Japan) using a Multiplex-PCR Master Mix (Qiagen Multiplex PCR kit). Heterozygosity at D17SAHA was previously confirmed to be strongly correlated with heterozygosity at the MHC class II Eb locus using SSCP (single-strand conformation polymorphism (THOß et al., 2011). Marker D17Saha is located within the MHC class II Eb locus (SAHA and CULLEN, 1986), marker D17Mit21 is adjacent to MHC class II Ab locus, and marker D17Mit28 is adjacent to MHC class I K locus (DIETRICH et al., 1996; MEAGHER and POTTS, 1997). All three loci are closely linked (i.e., within 1 cM and suggesting a 1% probability of recombination) and should be inherited as a single haplotype. PCR conditions and cycling parameters were identical to those used in another study. After that, the amplification products were analysed using an automated sequencer (Beckman Coulter CEQ 800). Allele scoring was conducted using Beckman Coulter CEQ 8000 System software, and allele sizes were determined with SLS+400 as size standard.
2.4. Statistical analyses

To increase normality and homogeneity of variances, we transformed individual mass and count data (pathogen load) using log-transformation. We performed a linear mixed-effects model (LMM) with offspring sex and partner preference of sires (P, NP) as fixed effects and week (time to euthanasia, see methods) as a covariate. We also entered the starting date of the experiments as a random effect to control for statistical non-independence of trials started on one or the other date. The distribution of body mass was random across offspring of preferred and non-preferred sires ($F_{1,68}=2.66; P=0.11$) and across weeks ($F_{1,68}=0.22; P=0.64$), while males were heavier than females (males: $21.29\pm0.54g$ (mean±SE); females: $18.95\pm0.34g; F_{1,69}=15.36; P<0.001$). All two-way interactions were non-significant (all $P>0.15$). To avoid entering strongly correlated effects as independent variables into subsequent models, we calculated residuals of body mass on sex and used these as deviations from the within-sex mean in further analyses.

We analysed pathogen loads using two separate LMMs, entering partner preference (P/NP), observed MHC-heterozygosity, and sex as fixed effects, week as a covariate and starting date of the experiments and family (N=23) as random effects. There were very few individuals that were not either completely heterozygous or homozygous at all three loci (N=4: 0.33; N=2: 0.66), and for simplicity, we excluded these from our analyses. We included all two-way interactions into initial models and applied a backward stepwise removal procedure (GRAFEN et al., 2008) to avoid problems due to the 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 LMMs using the ‘lme’ function of the ‘nlme’ package, and GZLMM using the ‘glmmPQL’ function of the ‘MASS’ package. Furthermore, the coefficient of variation (CV) was calculated for the three plates of the $10^2$ dilution of individual spleen homogenate. CV is defined as the ratio of the standard deviation SD to the mean $\bar{X}$. We analysed whether the plate-to-plate variation CV value was different during the experimental duration (1-3 weeks) using a Kruskal Wallis test in SPSS Version 17.
3. Results
Pathogen load of the offspring was unrelated to partner preference of sires (P/NP), duration of infection (week), observed heterozygosity or sex (LMM, sex: $F_{1,14}=0.05; P=0.83$; partner preference: $F_{1,35}=0.82; P=0.37$; week: $F_{1,14}=2.42; P=0.14$; observed heterozygosity: $F_{1,13}=0.12; P=0.73$) (Fig.3). Female preference (P or NP) had no effect on offspring observed MHC heterozygosity (Mann-Whitney U test: $W=97, Z=-1.23, P=0.291$). MHC-heterozygous offspring had similar pathogen loads irrespective of partner preference, whereas homozygous offspring of P sires tended to have lower pathogen loads compared to those of NP sires (the interaction between partner preference and MHC heterozygosity was marginally significant ($F_{1,12}=4.63; P=0.053$; see Fig. 4). All other two-way interactions remained non-significant (all $P>0.1$). The coefficient of variances for pathogen load were not different between weeks: median week 1: 57.13% (interquartile range (IQR): 9.5 - 87.9); week 2: 47.28 % (IQR=9.0 - 91.6) and week 3: 33.33 % (IQR: 0 - 58.7) (Kruskal-Wallis, time: $\chi^2=2.4; df=2; P=0.3$).
4. Discussion

Our results did not support the hypothesis that females’ preferences can increase offspring pathogen clearance, though pathogen clearance does not necessarily indicate how well individuals survive or cope with an infection. We found that the coefficient of variation did not differ between weeks, therefore, our results were not biased due to measurement error in assessing pathogen load during the period of the experiment. Females’ preferences did not increase offspring MHC-heterozygosity, contrary to the MHC-disassortative mating hypothesis (APANIUS et al., 1997; PENN and POTTS, 1999), and we found no difference in the pathogen clearance of MHC-heterozygotes versus homozygotes. Interestingly though, we found an interaction (p=0.053) between female’s partner preference (P/NP) and MHC-heterozygosity on offspring pathogen clearance: offspring sired by P-males had lower pathogen loads than those sired by NP-males, but only among the MHC homozygotes, whereas there was no such difference in loads among the MHC heterozygotes (Fig. 4). This result suggests that females’ preferences improved offspring pathogen clearance, but these benefits were only detectable after controlling for variation in MHC-heterozygosity. Another possible interpretation is that MHC-heterozygotes had better pathogen clearance than homozygotes, but only among the offspring from NP males, whereas there were no differences among offspring sired by P males. Thus, females’ preferences appeared to improve pathogen clearance among MHC-homozygous offspring.
5. Summary and Conclusions

Mate choice may allow females to obtain indirect, genetic benefits for their offspring, including enhanced immune resistance to infectious diseases (FOLSTAD and KARTER, 1992; HAMILTON and ZUK, 1982). To test this hypothesis, we conducted an experimental sexual selection study with wild-derived house mice (*Mus musculus musculus*). Females were experimentally mated with their preferred (P) or non-preferred (NP) male after a partner preference test, and in this study the resulting offspring were experimentally infected with *Salmonella enterica* (serovar Typhimurium). Every week (total duration three weeks), the bacterial load (pathogen clearance) per individual spleen was quantified. No difference was found between the pathogen loads of the offspring from P versus NP males. Immune resistance to *Salmonella* infection is controlled by several genetic loci, including the genes of the major histocompatibility complex (MHC). However, we detected no effect of females’ preferences on offspring MHC-heterozygosity, and there was no difference in the pathogen clearance of MHC-heterozygotes and homozygotes. Yet, the effect of females’ preferences on offspring pathogen clearance depended on their MHC-heterozygosity: among MHC-homozygous offspring, those sired by P males had lower pathogen loads compared to those from NP males, whereas females’ preferences had no effect on offspring pathogen clearance among heterozygous offspring. Future studies are needed to investigate whether mate choice improves that ability of offspring to cope with or survive infection.
6. Zusammenfassung und Schlussfolgerungen

7. References


8. Appendix

**Table 1:** Effects of sex, partner preference of sires (P/NP), week (1-3), observed MHC heterozygosity, and body mass on pathogen load as calculated using generalized linear mixed effects modelling procedures.

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*: P<0.1
Figure 1

Hausmaus *Mus musculus musculus*
Generously provided by © Dr. K. Musolf
Figure 2

Individually Ventilated Cage (IVC)

Accessed: 2012-10-07
Figure 3

Pathogen loads of infected mice comparing offspring from preferred (white triangles) versus non-preferred males (black circles) over the three weeks.
Figure 4

Pathogen loads of infected mice comparing offspring from preferred (white triangles) versus non-preferred males (black circles) and their relative MHC heterozygosity (P=0.053).