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DIPLOMARBEIT

MHC-dependent mate choice in wild house mice (*Mus musculus musculus*)

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TABLE OF CONTENTS

ZUSAMMENFASSUNG

Partnerwahl kann die Fitness des Nachwuchses verbessern indem ein Sexualpartner gewählt wird, der ein gutes Territorium kann und/oder von guter genetischer Qualität ist. Bei Hausmäusen (*Mus musculus musculus*) bevorzugen Weibchen dominante Männchen, die sie anhand von Gerüchen erkennen können. Die Gene des Major histocompatibility complex (MHC) beeinflussen den Geruch und spielen eine wichtige Rolle, neben der Immunabwehr, in der Partnerwahl. Drei Hypothesen, die sich nicht gegenseitig ausschließen, erklären den Vorteil von MHC-abhängiger Partnerwahl. Erstens kann durch die Wahl eines MHC-unähnlichen Partners heterozygoter Nachwuchs produziert werden, die vom „Heterozygoten Vorteil“ profitieren. Weiters ergibt sich durch die Partnerwahl ein coevolutionärer Vorteil gegenüber Parasiten gegeben, die sich an das Immunsystem des Wirten anpassen. Drittens dienen MHC Gene zur Verwandtschaftserkennung und durch MHC-abhängige Partnerwahl kann Inzucht vermieden werden.

Für diese Studie wurde MHC-abhängige Partnerwahl bei wilden Hausmäusen untersucht. Einerseits in Geruchspräferenztest und andererseits in Verpaarungsexperimenten. Weibchen hatten die Wahl zwischen zwei Brüdern, die sich in zwei MHC Loci unterschieden. Ein Bruder war dem Genotyp des Weibchens ähnlicher und der andere unterschied sich vom weiblichen MHC. Die Geruchspräferenz wurde in einer Y-Röhre ermittelt, in denen das Weibchen zwischen den urinmarkierten Filterpapieren wählte. Sie bevorzugten signifikant den Geruch von MHC-unähnlichen als von MHC-ähnlichen Männchen. Die Verpaarungsexperimente fanden in großen Gehegen statt, in denen die Männchen mit Halsbändern auf ihren Käfig beschränkt waren. Die Weibchen hatten die freie Wahl mit welchem Männchen sie sich verpaarten. Anschließend wurden Vaterschaftsanalysen von den Würfe gemacht. Es konnte ein Trend gezeigt werden, dass sich die Weibchen bevorzugt mit MHC-unähnlichen Männchen verpaaren, doch die Ergebnisse waren unschlüssig. Weiters wurden überdurchschnittlich viele multiple Vaterschaften gefunden, die auf laborbedingte Artefakte durch den Versuchsaufbau hinweisen.

Zudem wurden unterschiedliche Ergebnisse gefunden abhängig von der Analysemethode. Einerseits wurde der Genotyp des Weibchen mit den männlichen Genotypen verglichen, andererseits der elterliche Genotyp als Referenz verwendet. Das Signifikanzlevel steigt wenn der elterliche Genotyp als Referenz verwendet wird. Dieses Ergebnis unterstützt die „familial imprinting“ Hypothese, die besagt, dass Mäuse den eigenen MHC-Typ von ihrer Familie lernen und auf diesen geprägt werden. Dadurch ist eine bessere Vermeidung von Inzucht gegeben, da nicht nur der eigene Genotyp mit potentiellen Sexualpartnern verglichen wird. Außerdem verändert sich das Signifikanzlevel je nach Berücksichtigung von gemeinsamen und neuen Allelen. der Gemeinsamen Allele sind die Anzahl von Allelen, die sich Weibchen und Männchen teilen, neue Allele sind die Anzahl der Allele, die sich Weibchen und Männchen nicht teilen. Die Analyse mit neuen Allelen bringt eine höhere Signifikanz.

Diese Studie ist die erste, die MHC-abhängige Geruchspräferenz bei wilden Hausmäusen zeigt. Vorangegangene Studien, die den Einfluss von MHC Genen auf die Partnerwahl bei wilden Mäusen untersuchten und diese nicht unterstützen konnten, haben die Möglichkeit des „familial imprinting“ nicht in Betracht gezogen. Die negativen Ergebnisse der Verpaarungsexperimente sind nicht überzeugend, aufgrund der Laborartefakte.

ABSTRACT

Mate choice potentially enhances offspring fitness by choosing mates that provide direct (such as territories) and/or indirect benefits (such as genetic quality). The highly polymorphic genes of the major histocompatibility complex (MHC) are thought to play a role in odor-mediated mate choice which can potentially be explained by three non-exclusive hypotheses: MHC-disassortative mate choice (1) functions to increase offspring heterozygosity, (2) provides a "moving target" to counter parasites and/or (3) is used for kin recognition and inbreeding avoidance. There is much evidence that house mice (Mus musculus musculus) as well as other species, including humans, mate MHC-dependent. The best evidence for MHC-disassortative mate choice comes from cross-fostering experiments supporting the familial imprinting hypothesis. I used female odor and mate preference to test MHC-dependent mate choice in wild house mice. Females significantly preferred the odor of MHC-dissimilar than of MHC-similar males. The results of the mate choice experiments were inconclusive assuming laboratory artifacts.

1. INTRODUCTION

Mate choice can potentially enhance offspring fitness by enabling individuals to obtain mates that provide indirect, genetic benefits (Trivers 1972; Andersson 1994). In house mice (*Mus musculus)* females prefer socially dominant, territorial males over subordinates, which they can distinguish through males' urinary odor and scent marking (Desjardins et al. 1973; Drickamer 1992). This preference likely provides direct fitness benefits (i.e., better territorial quality and protection of offspring), and perhaps also indirect benefits since females prefer the scent of the sons of dominant males and dominance may be heritable (Dewsbury 1990). Moreover, when females are experimentally mated to males they prefer, it increases their offspring viability compared to controls (Drickamer 1992; Gowaty et al. 2003), supporting the idea that choosey females can obtain genetic benefits. It is unclear how this occurs, but mate choice may function to increase the genetic quality or compatibility of potential mates (Neff & Pitcher 2005). For example, female mice are attracted to the scent of healthy versus infected males (Kavaliers & Colwell 1995; Penn & Potts 1998; Zala 2004), and males genetically resistant to infectious diseases (Zala et al. 2008). House mice prefer to avoid interspecific hybridization (Smadja & Ganem 2002), and close inbreeding (Barnard & Fitzsimons 1989; Krackow & Matuschak 1991), which both increase genetic compatibility. The genes of the major histocompatibility complex (MHC; Box 1) may play a role in odor-mediated mating preference to increase genetic compatibility, and the aim of my study was to experimentally test whether these highly polymorphic genes influence odor and mating preferences in wild house mice *(Mus musculus musculus).*

There is much evidence that MHC genes influence odor and mate choice (Yamazaki et al. 1988; see below), and three hypotheses have been proposed to explain the adaptive function of this behavior (Penn & Potts 1999). (1) MHC-dissasortative mating preference might function to increase or optimize MHC heterozygosity of offspring. This idea is supported by evidence that MHC heterozygotes are usually more resistant to infectious diseases than MHC homozygotes (heterozygote advantage) (Penn 2002; Penn et al. 2002). (2) This

mating preference may provide a “moving target” to counter parasites that rapidly adapt to host genotypes and evolve the ability to escape recognition of the host’s immune system (Penn & Potts 1999). This idea is supported by evidence that MHC-heterozygosity increases resistance particularly to pathogens adapted to parental genotypes (McClelland et al. 2003). (3) MHC genes may play a role in kin recognition and mating preferences may function to avoid inbreeding. This hypothesis is supported by evidence that MHC genes play a role in kin recognition (Yamazaki et al. 2000) and by cross-fostering studies showing that individuals avoid mating with individuals sharing familial MHC-genes from the foster family (Yamazaki et al. 1994; Penn & Potts 1999). These hypotheses are not mutually exclusive because mate choice potentially includes all of the functions.

There is evidence for MHC-dependent mate choice in several species: house mice (Yamazaki et al. 1976; Yamazaki et al. 1988; Egid & Brown 1989; Potts et al. 1991; Penn & Potts 1998; Roberts & Gosling 2003), fish (Landry et al. 2001; Milinski 2003), birds (Richardson et al. 2005), reptiles (Olsson et al. 2003) and humans (Wedekind et al. 1995; Ober et al. 1997; 1997; Jacob et al. 2002; Roberts et al. 2008). For example in humans, Wedekind et al. (1995; Wedekind & Furi 1997) found an odor preference for MHC-dissimilar individuals, and others provide evidence for disassortative mating preferences, at least in some populations (Ober et al. 1997; Chaix et al. 2008). However, negative results are reported in several other studies: mice (Ehman & Scott 2001; Sherborne et al. 2007), Malagasy giant jumping rat (Sommer 2005); great reed warbler (Westerdahl 2004); great snipe (Ekblom et al. 2004); soay sheep (Paterson & Pemberton 1997) and humans (see review Havlicek & Roberts 2009). Thus, the evidence is mixed, and the reasons for the differences are unclear.

How do house mice recognize MHC identity? Many studies show that MHC genes influence odor (see review Penn & Potts 1998). There is experimental evidence with laboratory strains that males (Yamazaki 1976) and females (Egid & Brown) prefer to mate with MHC-dissimilar individuals and that females prefer the scent of MHC-dissimilar males (Egid; Roberts). Using congenic strains helps to

control for potential effects from non-MHC genes, but perhaps MHC effects on odor are only detectable because this is the only genetic difference in these mice. Also, Roberts & Gosling found differences only if the variation in the quantity of scent-marking is controlled, which suggests that normal variation in marking may completely override any bias for dissimilar males. Therefore, it is crucial to test wild house mice that are genetically heterozygous and show variation in scent marking. Two studies on semiwild mice (from crosses of laboratory x wild mice) in population enclosures found evidence for MHC-disassortative preferences (Potts; Penn & Potts). However, a recent observational study with wild-derived mice in enclosures found none (Sherborne 2007), but they did not consider familial imprinting, which as I explain below, may be crucial.

The best evidence for MHC-disassortative mate choice comes from cross-fostering studies showing that mice avoid mating with individuals carrying familial MHC genes (familial imprinting hypothesis). It indicates that mice learn the MHC type of their family and later mate with individuals whose MHC type differ from MHC of foster parents, regardless of their own MHC type (Yamazaki et al. 1988; Penn & Potts 1998). These results imply that mice use familial imprinting rather than self inspection, which may provide a more effective mechanism to reduce inbreeding (Figure 1).Thus, experimental tests with wild mice are needed that consider familial imprinting.

To determine if female wild-derived mice prefer mating with MHC-dissimilar males, I conducted odor preference tests in Y-mazes and mate preference tests in enclosures. Additionally, I used genetic paternity analyses of the females' offspring to determine their actual mate choice. If females prefer the odor as well as mating with MHC-dissimilar males, then this would indicate that females maximize heterozygosity in offspring. However, if the parental MHC genotype influence mate choice decision, inbreeding avoidance is the more probable explanation

Box 1: MHC background

The genes of the major histocompatibility complex (MHC) are highly polymorphic, with a high number of alleles (>100 alleles at a single locus) and they play an important role in immune response in vertebrates. They encode cell surface glycoproteins (class I & II MHC molecules). Foreign proteins enter cells either by infection or by phagocytosis. The specific MHC molecules bind these small foreign peptides and present them to T lymphocytes and subsequently initiate all specific immune responses. Ultimately, MHC genes are responsible for immune self/non-self discrimination.

The MHC region is divided into two groups, called class I and II genes (Figure A). In contrast to class I molecules which are expressed on the surface of all nucleated cells (except sperm cells and some neurons), class II molecules are present on antigen-presenting cells, like macrophages and lymphocytes. Hence, class II molecules are associated with immune insults derived from extracellular parasites and pathogens, such as bacteria or helminths whereas class I molecules are primarily responsible for defense against intracellular pathogens, such as viruses (Piertney & Oliver 2006). Despite these differences, class I and class II genes are closely linked in a single gene complex and inherited as a unit (haplotype) in mammals, but not in fish and frogs (Klein 1986; Penn 2002). A haplotype is the particular combination of alleles that is inherited as a linkage group.

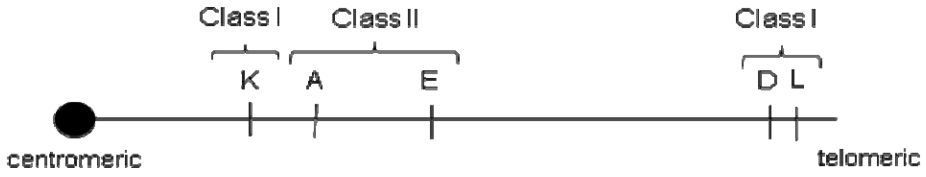


Figure A: In house mice, there are five loci of MHC class I and II with over 100 alleles per locus (after Penn & Potts 1999).

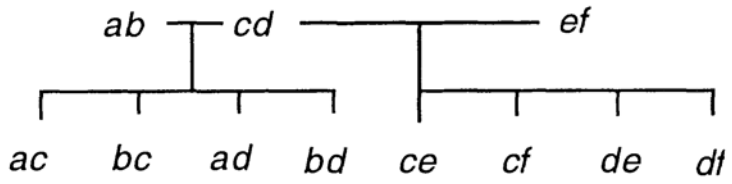


Figure 1. This figure shows a pedigree typical for wild mice and illustrates why familial imprinting would help reduce inbreeding. If individual *ac* would use self-inspection, it would risk mating with one fourth of her full siblings (*bd*) and one half of her half-siblings (*de, df*). On the other hand, using familial imprinting prevent individual *ac* to mate with all full siblings (*bc, ad, bd*), all half-siblings (*ce, cf, de, df*) and half of all cousins. It could avoid mating with MHC-similar and dissimilar kin (Penn & Potts 1998). Additionally, mice are often reared in communal nests with their aunts (Wilkinson & Baker 1988). Hence, familial imprinting allows an individual to avoid mating with most first-cousins (after Penn & Potts 1998).

2. GENERAL METHODS

2.1 Animals and housing

I used 50 adult female and 100 adult male mice of the outbred F1–3 generation of wild house mice *(Mus musculus musculus)* caught in Gänserndorf, Austria for the experiments. All subjects were raised in mixed sex family groups until weaning at age of 21 days. At weaning, males were housed individually whereas females were either kept individually or as sister pairs in type II cages (size: 26.5×20.5×18 cm, plus high stainless steel covers, mesh width 1 cm) with bedding and nesting material (Abedd). Home cages were kept in an air-conditioned animal room with a mean temperature of 20±1 °C and a 12:12 h light:dark cycle (lights on at 7:00 a.m.). Food (Altromin, Germany) and water were provided ad libitum. Female's age ranged from 2 – 12 months with an average age of 5.2 months and male's age ranged from 1.9 – 7.9 with an average age of 5.2 months at time of testing the odor preference. At the time of mate preference test, female's age ranged from 2.8 – 14.8 with an average of 6.4 +/-2.8 months and male's age ranged from 3.9 – 13.8 with an average age of 7.3 +/-2.3 months.

For preference experiments I chose 50 triplets, one female, two males that were MHC matched to the female's genotype (similar and dissimilar). Males were always full-brothers who differed in MHC genotype and were not related to female. For mate preference experiments 9 triplets were excluded because at least one mouse of the triplet died in the meantime and thus only 41 triplets were used.

2.2 MHC typing

I screened two class II MHC loci (Aα and Eβ) on chromosome 17. First, I extracted genomic DNA from tissue samples taken from the experimental mice using a standard protocol (Sambrook et al. 1989). Second, I used a two-step PCR to amplify the samples. The PCR amplifications were carried out on a Biometra-T1 thermocycler using the following program for Aα: a 94 °C initial denaturation step for 2 min; followed by 10 cycles of denaturation at 94 °C for 30 s, 59 °C annealing for 30 s, and 72 °C extension for 1 min; followed by 25 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s, 72 °C extension for 1 min and a 72 °C final extension for 10 min. The amplification for Eβ differed in the annealing temperature of 53 °C (step 1) and 48 °C (step 2). The 10 µl reaction mixture contained 1 µl of extracted genomic DNA, 1x B-buffer, 0.2 µM dNTPs, 3.75 µM $MgCl_2$, 1U Taq-Polymerase and 3 pmol of each primer for Aα, respectively 5 pmol for Eβ. I used the following primer for the Aα locus: Aα-F: 5'-ACCATTGGTAGCTGGGGTG-3'; Aα-R: 5'-CTAAATCCATCAGCCGACC-3'. For the amplification of the Eβ locus, I used JS1 5'-GAGTGTCATTTCTACAACGGGACG-3' and JS2 5'-GATCTCATAGTTGTGTCTGCA-3' primer (modified after Schad et al. 2004). The PCR products were electrophoresed on 1% agarose gels (TAE buffer) containing ethidium bromide for visualization to verify successful amplification.

For the CE-SSCP analyses, 1 µl diluted PCR product (dilution Aα 1:60; Eβ 1:50) were combined with 14 µL loading dye mix [13.75 µL Hi-Di™ formamide, 0.25 µL GeneScan ROX 350 standard (Applied Biosystems)]. The mixture was denatured for 6 min at 95 °C, immediately chilled on ice for 2 min and analyzed

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 run 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. I used the GeneMapper software packages 4.05 from Applied Biosystems to analyze the SSCP data.

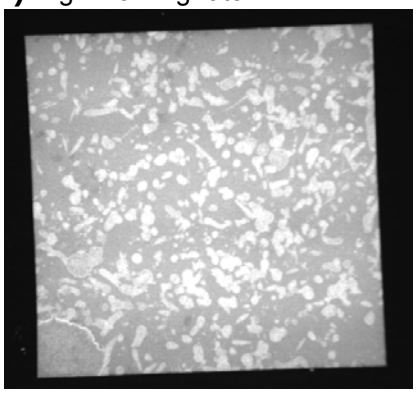
2.3 Scent mark collection

I collected scent marks overnight by putting filter papers (Whatman® chromatography papers 3 mm Chr; first 20 trials size: 10 x 10 cm; last 30 trials size: 5.5 x 6.5 cm) in the male's home cages (average duration 19 h). Males mainly scent mark with urine but also small amounts of salvia and feces can be found. Additionally, I put a stimulus paper (1 x 1 cm) containing 10 µl of female urine into the male's cages as this has been shown to increase male markings and females prefer scents of sexually stimulated males (Zala 2004). The stimulus urine was a mixture of 10 matured females, and I collected each female's urine on three different days to control for variation due to cycle stage (females were placed onto clean tinfoil, the urine was pipetted up into Eppendorf tubes and stored at -80°C until needed). The male scent marks were stored individually in Ziploc® plastic bags (Toppits, Allround Zipper, 3 l) at -80°C until used in the female odor preference tests. I always used clean gloves and forceps when handling the filter papers.

After using the scent marks in odor preference tests, I took photographs of each scent marked filter paper under ultraviolet light where urinary proteins glow and compared the scent marks of brothers (Figure 2). I estimated (1) the coverage and (2) the number of spots of the scent marks visually, and four persons rated the filter papers with the following ranks: (1) 0 = less, 1 = more marked; (2) 0 ≤ 10, 1 ≤ 20, 2 ≤ 50 and 3 ≥ 50 spots. The mean values of the

ratings were used for statistical analyses. Cases with equal ratings were excluded from statistics because they were assumed to have no impact on potential scent-mark dependent behavior.

(A) High marking rate



(B) Low marking rate

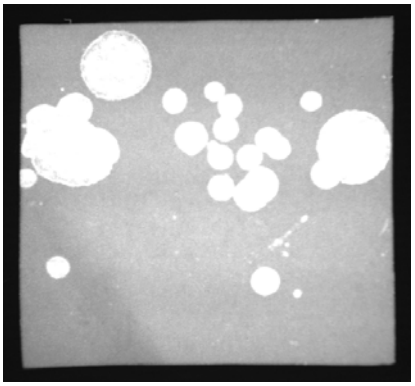


Figure 2. Photographs of scent-marked filter papers under UV-light showing (A) high marking and (B) low marking rate.

2.4 Female odor preference assays

I tested female's preference for male scent marks during estrus, determined by examining vaginal smears under a phase contrast microscope (Flowerdew 1987), to ensure they were sexually receptive. I placed male's soiled bedding into the female's home cages three days before testing since it has been reported that male's bedding induces estrus (Cheetham et al. 2007). The Y-maze apparatus for the odor preference tests was composed of acrylic, and divided into a start chamber (6 x 14 x 6 cm) at the proximal end, where the mice were placed first, followed by neutral zone (6 x 24 x 6 cm) and ending into two arms of choice zones (6 x 8 x 6 cm) at the distal end. The chambers containing the filter papers (6 x 13.5 x 6 cm) were separated with wire-mesh dividers for the first 20 trials (Figure 3). The dividers prevented the females from touching or chewing the filter papers. I placed an air pump (Sera Air 110) and the scent marked filter papers at the end of the chambers to ensure a constant airflow of volatiles through the maze. For the last 30 trials, the scent marks were attached to the wire-mesh dividers. I did not use the air pump for these trials anymore. Females had either

access only to the volatiles evaporating from the scent marks (first 20 trials) or direct contact with the paper including volatiles and non-volatiles (last 30 trials).

At the start of each trial, I placed a female in the start chamber for 5 minutes to habituate to the maze and to the air pump (first 20 trials). Afterwards, I placed the scent-marked filter papers in the maze and released the female from the start chamber. I recorded the trials for 15 minutes on videotape (Sony Handycam DCR-SR 30E) and later analyzed 10 minutes (Observer software, Noldus, Version 7.0) from the point the female entered the neutral zone. I measured the total duration a female spent in the choice zone of each side of the y-maze and separately analyzed the duration of sniffing and gnawing on the dividers respectively the filter papers. I assessed the proportion of sniffing and gnawing per total time females spent on each side. I assigned the filter papers haphazardly to the sides. After each trial, I cleaned the Y-maze with ethanol to remove scents from previous trials. Each female and filter paper was used only once (except two pairs of filter papers were used twice). I conducted the experiments in the afternoon starting at 15:00 always in the same experimental room.

As positive control to confirm that scent marks attract female's interest, I tested a scent mark versus a blank filter paper. The procedure and analysis were the same as described above. Eleven females, not used in preference tests, from three different populations (Safaripark, Reitschule Schottenhof; Veterinary University, Vienna) were used as controls with an age range from 3.2 – 10 and an average age of 6.1 months. Males from Safaripark population were used with an age range from 2.5 – 8.9 and an average age of 5.6 months.

Since the results did not differ dependent on placing the scent marks either behind or attached on the dividers, I combined both approaches in the analyses. From the 50 trials, I excluded a various number of trials because either females did not leave the start chamber or I could not clearly match female's, maternal, paternal or parental with male's genotypes.

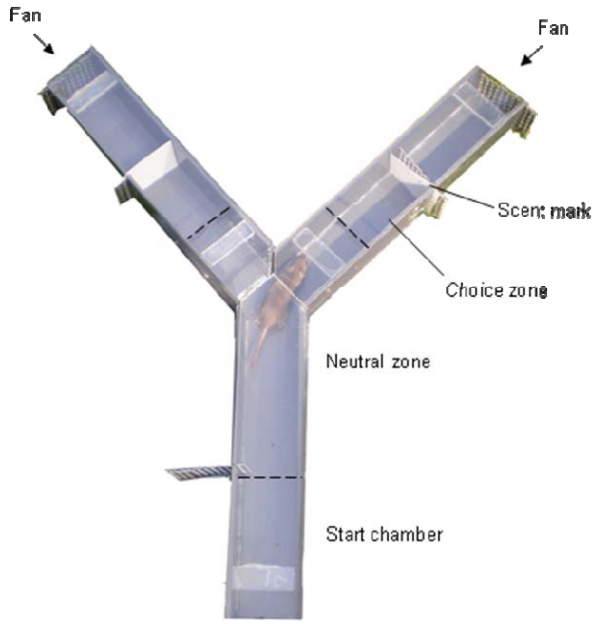


Figure 3. Females were placed in the start chamber (6 x 14 x 6 cm) of the y-maze and released into the neutral zone (6x24x6 cm). The mice had the choice between two filter papers, scent marked by brothers that were either (1) placed behind the dividers or (2) attached on the wire-mesh in the choice zone (6 x 8 x 6 cm). In the first 20 trials, a fan was fixed at each end of the y-maze.

2.5 Mate preference assays

After the odor preference tests, I used the same triplets (1 female, 2 males) for a mate preference test. I conducted 41 experiments in 12 different large enclosures of similar sizes [A: 6 enclosures 2.68 x 1.8 m (4.8 m^2) respectively B: 6 enclosures 4.5 x 1.5 m (6.8 m^2); Figure 2]. The males were restricted to their cages (Type II, size: 26.5 x 20.5 x 18 cm) with collars to avoid any interactions between the males. The collars were made of plastic ties and commercial florist wire. Each cage was opened with a tube which only the females could pass through. Consequently, the female had free choice between the two. The enclosures were subdivided into two territories, territory boarders were created by placing bedding from the male's homecage. The size, resources (water, food) and shelters were equally distributed. In each "territory" were two shelters (S1 and S2) and the male's cage (Figure 4). Food (Altromin, Germany) and water

was provided ad libitum. The males' cages were haphazardly distributed to avoid any side bias.

I conducted daily observations over four weeks to record the female's position with particular focus on her association with either male. After this period, experiments were terminated and mice returned to the animal room. Females were weighed regularly for three weeks to check for pregnancies. In case of litters, I weighed the offspring at the age of three and four weeks and took tissue for paternity analyses.

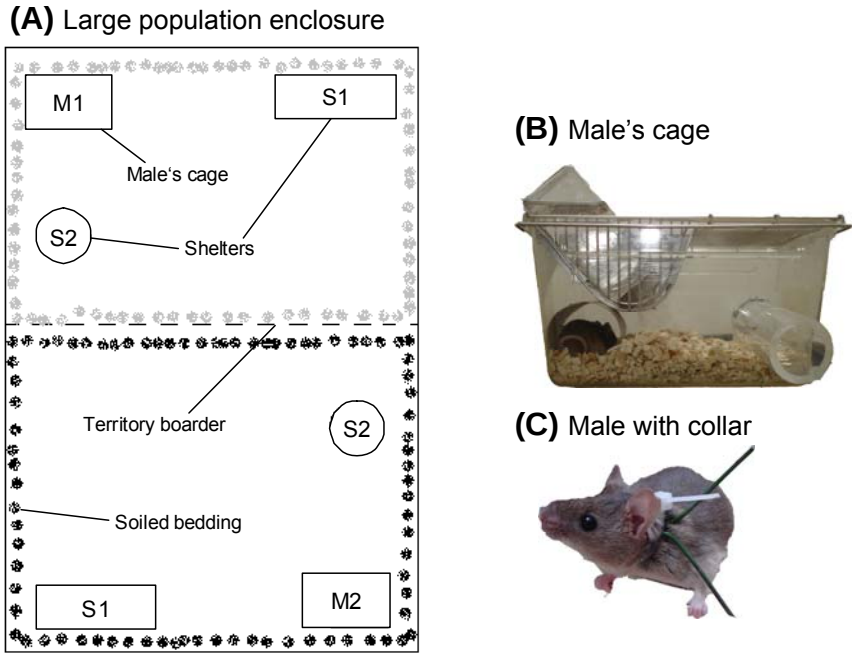


Figure 4. (A) Large population enclosure, where the mate preference experiments were conducted (4.8 m^2 and 6.8 m^2). Territory borders were marked with soiled bedding from each male's cage. Each territory contained of two shelters for the female and (B) one male cage (Type II, size: 26.5 x 20.5 x 18 cm) was placed in each corner and were opened with a tube (diameter 5 cm); (C) males were restricted to their cages with collars whereas females had free access.

2.6 Paternity analyses

I used a standard protocol (Sambrook et al. 1989) to extract DNA from tissue of litters. I used Multiplex PCR-amplification, where I used 3 sets of primers and a Qiagen multiplex kit and 6 pairs of primers (each 10µM, Table 1). The thermal

cycling profile for the PCR consisted of initial denaturation at 94°C for 15 min, followed by 31 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 90 s, extension at 72°C for 1 min and ending with a 10 min extension step at 72°C. If the paternity was still unclear, I used a second set of primers [MM252-F and R; Mit138-1 and 2; Mit16-1 and 2; MM25-F and R; MM39-F and R; MM227-F and R; MM456-F and R (each 10µM)]. For the analysis I used Beckman Coulter (CEQ 8000) and the BECKMAN Coulter CEQ 8000 fragment analysis software.

Table 1. Microsatellite markers used for parentage assignment

Primer name	Forward primer	Reverse primer
D5-Mit25	5'-AACACACCTCCATACTGGTCG-3'	5'-GGCTAACTGAAATTGTTTTGTGC-3'
D2-Mit252	5'-CAGTGCCGTGGAGAAGAAGT-3'	5'-AGTCATCAAGAGATTGACATTACACA-3'
D7-Mit227	5'-GAGTCCTCAGCAGATATTACTCAGC-3'	5'-CTGATGTCTCATCATTTGGGG-3'
D2-Mit380	5'-CCTCAGGTCTGAAATGAGGTG-3'	5'-AATGATGTGCATGTGCGC-3'
D1-Mit456	5'-TGGCTTCCACAGGAATGAG-3'	5'-GCCAGTACAGATGCACAGACA-3'
D1-Mit404	5'-AGGAATAGAAAAATCAGCAAGCC-3'	5'-CCATTGCCCTTGCTTTAGAA-3'
D6-Mit138	5'-GCTCTTATTAATGAAGAAGAAGGAGG-3'	5'-CAAAGAAAGCATTTCAAGACTGC-3'
D9-Mit34	5'-AGTTTTAGGCTAGTATAGGTT-3'	5'-ACTGGAACCTTAGAGCATGAG-3'
D9-Mit135	5'-ATTACATAGTCACTCTGAATG-3'	5'-ACTTTTAGCAATTAGTAATTC-3'
D10-Mit20	5'-CACCCTCACACAGATATGCG-3'	5'-GCATTGGGAAGTCCATGAGT-3'
D11-Mit150	5'-GTACTAAAACGTCTACAAGTGG-3'	5'-GCGGATATATATGCAGCAGAG-3'
D15-Mit16	5'-AGACTCAGAGGGCAAAATAAAGC-3'	5'-TCGGCTTTTGTCTGTCTGTC-3'
D17-Saha	5'-CGACTGTAGAACCTTAGCCTG-3'	5'-TGGAGCTGTCCTCCTTGTAG-3'
D17-Mit28	5'-ATCCAGGACTCAGAATGAAGATCC-3'	5'-ATTCCTACATCAAAACTCTCTCCC-3'
D19-Mit39	5'-GGAGGTCTCAGGAAATATTACTCC-5'	5'-ATTCCTGTGTAAAGGTGGATGG-3'
D17-Mit21	5'-TAACACCAGACATTGACCTC-3'	5'-AGTCTAGATATGTGTCTCCC-3'

2.7 Genetic analyses for MHC-dependent mate choice

I analyzed the preference results in two different ways: first I looked how many new MHC alleles males provide compared to the females, and second how many MHC alleles are shared between females and potential mates. Unexpectedly, the results can differ with these two methods. For example, a female with genotype **A2E3** shares one allele with male 1 (**D4F**2) and none with male 2 (**D4D4**), hence she potentially prefers male 2, but the number of new alleles (non shared) is 3 for male 1 (D4F2) and 2 for male 2 because of his homozygosity (D4D4). Thus, she prefers male 1 when looking at new alleles. Additionally, I used not only the female's MHC genotype, but also maternal and paternal as a referent for

comparison with each male's genotype to test for familial imprinting (Yamazaki et al. 1988; Penn & Potts 1998).

2.8 Statistical analyses

I tested the data for the assumption of normality (Shapiro-Wilkinson test) before conducting parametric tests (SPSS version 15.0). If these assumptions were not met, I calculated the ratio (similar/dissimilar), log-transformed the data to get normal distribution and performed a one-sample t-test. If the data was still not normal distributed, I performed nonparametric tests (Wilcoxon Signed Rank tests). I used directed tests when the direction of a test could be described *a priori* (Rice & Gaines 1994). In all cases I considered $p \leq 0.05$ to be statistically significant.

3. RESULTS

3.1 MHC typing

I identified six different alleles at the Aα locus and six different alleles at the Eβ locus and 20 different genotypes.

3.2 Scent mark variation

Scent marking behavior varied between males, some urine marked with high rate and others with a lower rate (Figure 2). However, variation in marking behavior of brother pairs did not influence the females' preference in the Y-maze: the variation of the degree of coverage between male pairs ($n = 35$, $t = -1.152$, $p = 0.257$) as well as the different number of spots ($n = 46$, $z = -1.633$, $p=0.103$) had no effect on the females' choice.

3.3 Female odor preference

In the positive control, females stayed significantly longer on the side of the Y-maze with the scent marked filter papers than at the blank ones ($n = 8$, $Z = -2.1$,

p = 0.036, p_{dir} = 0.023) confirming that male's odors attract female's interest (Zala 2004).

The preference of females for male's scent varied significantly between MHC-similar and dissimilar males. In general, female mice spend more time at the scent marks of MHC-dissimilar than similar males, though the results differ dependent on analysis type. When considering the number of new alleles females spent significantly more time at the MHC-dissimilar scent mark consistent with all referents [(1) female's genotype (n = 31, p_{dir} = 0.029), (2) maternal genotype (n = 27, p_{dir} = 0.039), (3) paternal genotype (n = 28, p_{dir} = 0.01) and (4) parental genotype (n = 29, p_{dir} = 0.009) (Figure 5A)]. When considering the number of shared alleles the preference for MHC-dissimilar males was significant only when using the father's genotype whereas the other comparisons only showed trends [(1) the female's genotype (n = 30, p_{dir} = 0.163), (2) maternal genotype (n = 25, p_{dir} = 0.065), (3) paternal genotype (n = 24, p_{dir} = 0.021) and (4) parental genotype (n = 27, p_{dir} = 0.07) (Figure 5B)].

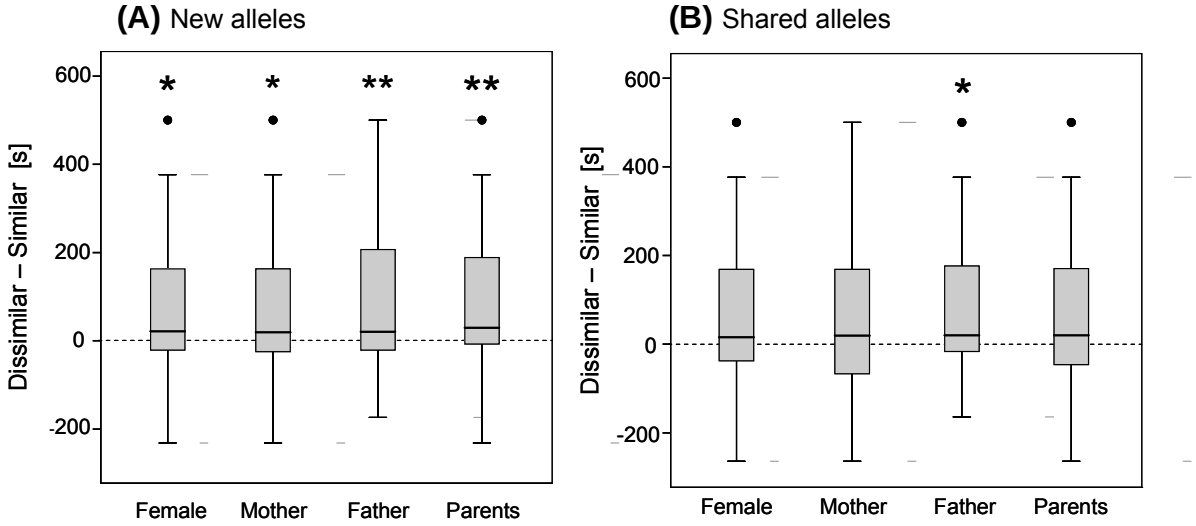


Figure 5. Female odor preference in the Y-maze showed as difference in time (s). (A) represents analysis of new alleles and (B) represents analysis of shared alleles. Positive scores indicate more time spent near the odor of MHC-dissimilar males, negative scores more time near the MHC-similar odor. Outliers are represented as black dots. Full range of data is displayed in the box plots. Single asterisks represent significance at a level of $p < 0.05$; double asterisks represent significance at a level of $p \leq 0.01$.

I did not find any difference in sniffing and gnawing behavior at the filter papers of MHC-similar versus dissimilar males (Table 2), except when analyzing new alleles and female's genotype. Here, females sniffed significantly longer at the MHC-similar scent marks.

Table 2. (A) represents the analysis approach of counting the number of new alleles; (B) represents the number of shared alleles. Mean time (s) of total duration, sniffing and gnawing at MHC-similar and dissimilar scent marks are displayed. P-values in bold represent significant differences, bold and italic values are significant but directed towards MHC-similar males.

		Mean time (s)					
		Similar	Dissimilar	N	Effect size	P-value	P_{dir}
(A)	Total Duration[1]	125,26	193,61	31	-2,079	**0,046**	**0,029**
	Sniffing/time[2]	0,34	0,26	31	-2,136	***0,033***	***0,021***
Female	Gnawing/time[2]	0,09	0,09	31	-0,08	0,936	0,585
	Total Duration[1]	139,56	189,87	30	-1,148	0,26	0,163
(B)	Sniffing/time[2]	0,33	0,27	30	1,44	0,161	0,1
	Gnawing/time[2]	0,08	0,09	30	-0,095	0,925	0,578
(A)	Total Duration[1]	137,29	204,53	27	-1,953	0,062	**0,039**
	Sniffing/time[1]	0,31	0,25	27	1,299	0,205	0,128
Mother	Gnawing/time[2]	0,1	0,11	27	-0,37	0,711	0,445
	Total Duration[1]	143,71	197,31	25	-1,69	0,104	0,065
(B)	Sniffing/time[1]	0,36	0,27	25	1,55	0,134	0,084
	Gnawing/time[2]	0,07	0,09	25	-0,026	0,979	0,612
(A)	Total Duration[1]	127,73	212,35	28	-2,539	**0.017**	**0.010**
	Sniffing/time[1]	0,33	0,24	28	1,637	0,113	0,071
Father	Gnawing/time[2]	0,08	0,09	28	-1,06	0,289	0,181
	Total Duration[1]	126,81	211,06	24	-2,257	**0,034**	**0,021**
(B)	Sniffing/time[1]	0,34	0,25	24	1,407	0,173	0,108
	Gnawing/time[2]	0,09	0,13	24	-0,738	0,46	0,288
(A)	Total Duration[1]	126,98	210,55	29	-2,628	**0,014**	**0,009**
	Sniffing/time[1]	0,32	0,24	29	1,539	0,135	0,084
Parents	Gnawing/time[2]	0,11	0,13	29	-0,604	0,546	0,341
	Total Duration[1]	133,58	191,97	27	-1,644	0,112	0,07
(B)	Sniffing/time[1]	0,36	0,27	27	1,283	0,211	0,132
	Gnawing/time[2]	0,07	0,11	27	-0,781	0,435	0,272

[1] = t-test, effect size t; [2] = Wilcoxon-Signed ranks test, effect size z

3.4 Mate preference and paternity

In the enclosures, females again showed social preference for MHC-dissimilar males. They spent significantly more time in MHC-dissimilar male's cages than in those of MHC-similar males, but only using shared alleles and maternal genotype as a referent. The mean time that females associated with MHC-dissimilar males is on average higher than with MHC-similar males, but with one exception (paternal genotype) (Table 3).

Table 3. Social preference of females in the enclosures: mean time (d) females spent either in the cage of MHC-similar or dissimilar male. Wilcoxon Signed Rank (effect size z) test was conducted for all analyses: using the number of (A) new and (B) shared alleles and comparing male's genotype with four different referents (female's, maternal, paternal and parental genotype).

		Mean duration (d)					
		Similar	Dissimilar	**N**	**Effect size**	**P-value**	**P_{dir}**
Female	(A)	5.15	7.92	26	-1.487	0.137	0.086
	(B)	4.26	6.30	23	-1.083	0.279	0.174
Mother	(A)	5.27	6.73	22	-0.748	0.455	0.284
	(B)	4.32	7.59	22	-1.852	0.064	**0.04**
Father	(A)	6.8	5.64	25	-0.658	0.511	0.319
	(B)	6.13	5.46	24	-0.341	0.733	0.458
Parents	(A)	6.08	6.39	26	-0.148	0.882	0.551
	(B)	5.27	7.32	22	-1.089	0.276	0.173

From the 41 experimental females 24 reproduced (58.5 %) during the mate preference tests (up to two litters per female). Paternities of 7 females were unresolved and therefore excluded and hence only 17 females with full resolved paternities were included into analyses. Out of 17, 7 females mated monogamous and 10 mated multiply (58.8 %) and produced 24 litters. Out of 24, 14 litters were sired monogamous (58.3 %) and 10 litters were sired multiply (41.7 %). Overall, the average litter size of monogamous females (5.1) versus polyandrous females (5.0) did not differ. Mean age of monogamous females was 6.6 whereas polyandrous females were on average 5.6 months old.

When analyzing the number of litters, multiple paternity ("mixed paternity") was the highest with new alleles and females' and paternal genotype as referents (Figure 6A) and with shared alleles and females' and maternal genotype as referents (Figure 6B) (new alleles: female's genotype n=16, 56.3%, paternal genotype n=16, 50%; shared alleles: female's genotype n=19, 42.1%, maternal genotype n=15, 40%). In all other cases monogamous sired litters were higher (new alleles: maternal and parental genotype) than or as high as multiple sired litters (shared alleles: paternal and parental genotype). Monogamous sired litters were frequently sired by MHC-dissimilar males than by similar males (new alleles: maternal genotype n=15, dissimilar > similar 40.0 vs. 26.8 %, paternal genotype n=16, 31.3 vs. 18.8 %, parental genotype n=16, 43.8 vs. 18.8 %; shared: maternal genotype n=15, 33.3 vs. 26.7%, paternal genotype n=16, 27.5 vs. 25.0%, parental genotype n=14, 35.7 vs. 28.6%) except considering female's genotype where the higher number of litters is sired by MHC-similar males (new: n=16, dissimilar < similar 18.75 vs. 25.0%, shared: n=19, 26.3 vs. 31.8%; Figure 6A & B).

When analyzing the mean litter size, the size was larger when sired by MHC-dissimilar males than by MHC-similar males, except analyzing shared alleles and paternal genotype where MHC-similar males sired larger litters (new alleles: female's genotype: sim n = 5, $\bar{x}$ =3.6; dissim n=3, $\bar{x}$ =4.33; mixed n=8, $\bar{x}$ =5.25; maternal genotype: sim n=4, $\bar{x}$ =3.0; dissim n=7, $\bar{x}$ =5.86; mixed n=5, $\bar{x}$ =4.8; paternal genotype: sim n=2, $\bar{x}$ =4.5; dissim n=6, $\bar{x}$ =4.67; mixed n=7, $\bar{x}$ =4.86; parental genotype: sim n=2, $\bar{x}$ =4.5; dissim n=6, $\bar{x}$ =5.5; mixed n=5, $\bar{x}$ =5.2; Figure 6C) and (shared alleles: female's genotype: sim n=5, $\bar{x}$ =4.0; dissim n=6, $\bar{x}$ =4.83; mixed n=7, $\bar{x}$ = 4.86; maternal genotype: sim n=3, $\bar{x}$ =2.33; dissim n=6, $\bar{x}$ =5.5; mixed n=4, $\bar{x}$ =5.25; paternal genotype: sim n=4, $\bar{x}$ =5.25; dissim n=7, $\bar{x}$ =4.14; mixed n=6, $\bar{x}$ =5.0; parental genotype: sim n=4, $\bar{x}$ =5.25; dissim n=6, $\bar{x}$ =5.5; mixed n=5, $\bar{x}$ =6.0; Figure 6D).

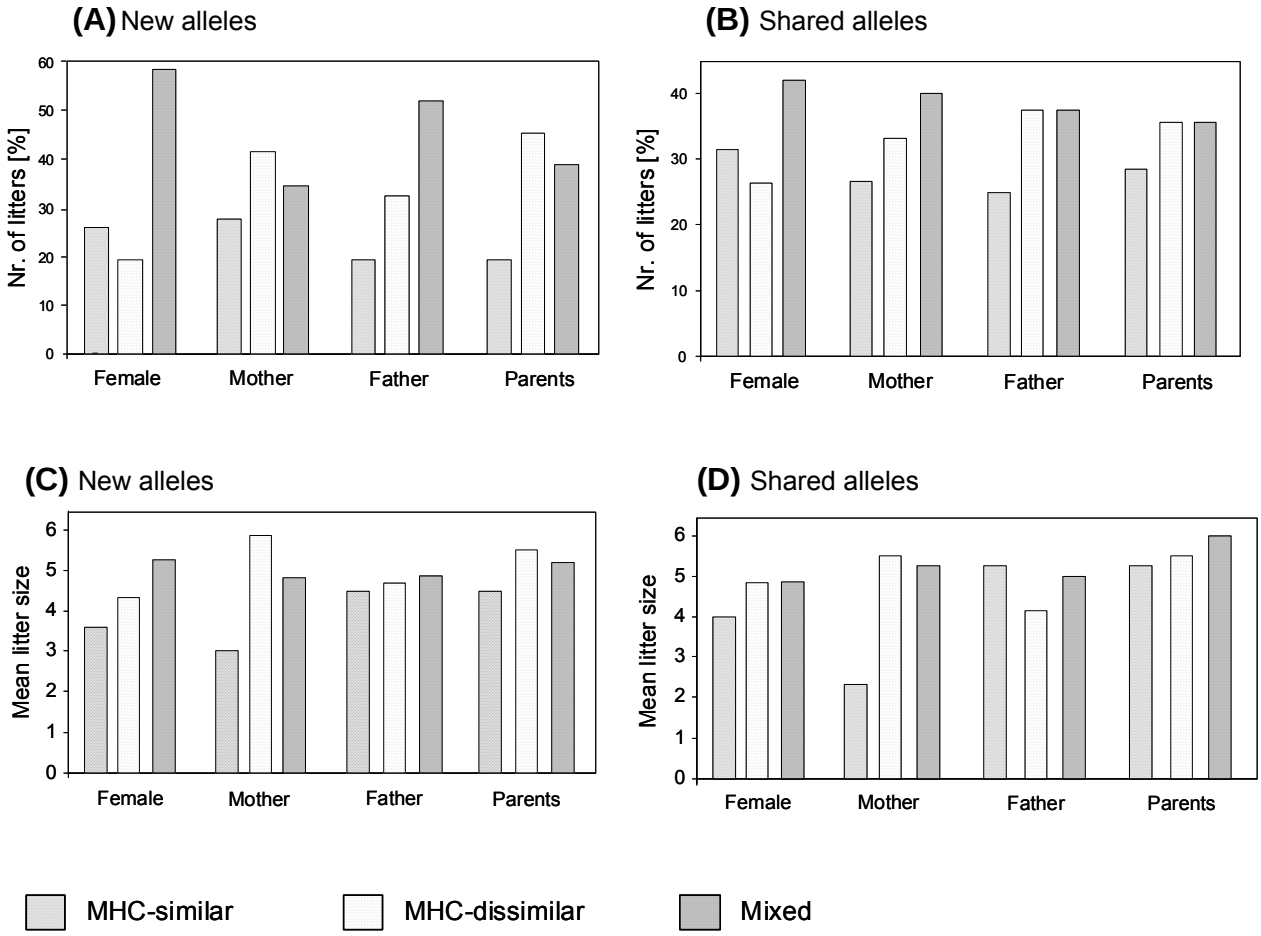


Figure 6. Relative mating success of MHC-similar vs. dissimilar males when considering (A) number of new alleles and (B) number of shared alleles. Mean litter size of MHC-similar, dissimilar or both (mixed) sires when considering (C) number of new alleles and (D) shared alleles. Four different analyses using female's, maternal, paternal and parental genotype as a referent to male's genotype.

4. DISCUSSION

My results provide evidence for the hypothesis that MHC genes influence females' odor preferences for males. As predicted, females spent more time in proximity of MHC-dissimilar odors in the Y-maze tests. MHC-similar scent marks induced more investigation behavior, such as sniffing and gnawing, suggesting that females needed longer to process more similar signals. Sniffing implies that animals did not get sufficient information and try to gain further information (Cheetham et al. 2007). Contrary to Roberts & Gosling (2003) females preferred MHC-dissimilar males despite variability of the males' scent marking.

Interestingly, females' preferences for MHC-dissimilar odor were significant regardless of the referent (females', maternal, paternal and parental genotypes) when using the number of new alleles as an indicator of similarity. But using the number of shared alleles, the preference for MHC-dissimilar odor was only significant when looking at paternal genotype compared with male's genotypes. This finding implies that males sharing MHC alleles with the female's father are less attractive and suggests that females learn the MHC type from their families. Jacob et al. (2002) found that a woman's preference for male odor is based on positive matches to the alleles inherited from the father. These findings suggest a particular importance of father's MHC genotype. My results are consistent with previous cross-fostering experiments showing familial imprinting (Yamazaki et al. 1988; Penn & Potts 1998). Familial imprinting suggests MHC-dependent mating functions to avoid inbreeding (Penn & Potts 1999) (Figure 1), and since mice live in high densities family-based social groups, it is essential to recognize kin and avoid inbreeding and its deleterious effects (Pusey & Wolf 1996). A previous study with wild mice potentially failed to detected MHC-dependent mate choice because it they did not consider parental genotypes as referent (Cheetham et al. 2007; Sherborne et al. 2007).

Previous studies on MHC-dependent mate choice usually examine the number of shared alleles (Roberts & Gosling 2003; Sherborne et al. 2007; Schwensow et al. 2008) versus new alleles (Sommer 2005). They did not consider the possibility that avoiding mates with shared alleles does not necessity increase offspring's MHC variability, whereas choosing males with higher numbers of new alleles will increase MHC variability. Thus, it might be that the number of new alleles is a more reliable indicator of a MHC-dissimilar mate.

Although I found females prefer the scent of MHC-dissimilar males, mate preference experiments were mixed and arguably inconclusive. Females showed social preferences for MHC-dissimilar males (at least for avoiding maternal MHC) but genetic paternity analyses indicated that females showed no significant bias for MHC-dissimilarity. Unexpectedly, females mated multiply at a higher rate (40.0%) than in the wild (10.5%) and in previous enclosure experiments (12.5%)

(Musolf et al. unpublished data). These results strongly suggest that the experiment had laboratory artifacts. Possibly, the enclosures were too small and females did not recognize two territories. Males were not able to establish territories on their own but boarders were artificially defined with soiled bedding. Further, the experimental set-up differs from previous studies because of using two brothers instead of unrelated males which might caused the unusual high number of multiple matings. When a female was confronted with two males, she was likely to mate with both, as previously suggested (Manning et al. 1992). This behavior likely functions to reduce the risk of infanticide since males kill females' pups if they have not mated recently (Perrigo & vom Saal 1994) Another non-exclusive possibility is that polyandrous matings function to increase genetic variability of offspring. Polyandrous females were on average 9 months younger than monogamous females, suggesting an age-dependent mating tactic. In lizards, younger and older females increased their fitness by being more polyandrous. Only intermediate aged females were monogamous which appeared to be their optimal performance. Younger and older animals appeared to choose apparently suboptimal strategies (Richard et al. 2005). It appears that only older females were monogamous and showed a bias. I found that the mean litter size of monogamous females was larger when sired by MHC-dissimilar male with one exception (paternal genotype in Figure 6D, but see 6C) which suggests that females increased their fitness by exclusively mating with MHC-dissimilar males.

To my knowledge, this is the first experimental evidence for MHC-dependent odor and social preference in wild mice. I have shown that female's odor choice is better explained using parental genotypes as a referent (familial imprinting) rather than only the females own (self-inspection). I have also shown that females prefer new alleles versus shared alleles. Although, I found that females prefer MHC-dissimilar males in social context, I found no mating preference for MHC-dissimilarity. Though, there were consistent trends in the expected direction. However, the negative results are not convincing due to apparent laboratory artifacts of unusual high polyandry.

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7. CURRICULUM VITAE

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ACADAMIC CAREER

10/ 2007 – pres.	**Diploma thesis at the Konrad Lorenz Institute for Ethology, Austrian Acadamy of Science (ÖAW), Vienna** Supervisor: Dr. Dustin Penn
05/ 2007 – 06/2007	**Field assistant: "Flycatcher project", Baltic island Öland, University Uppsala**
08/ 2006 – 05/ 2007	Student: **University Lund, Sweden** Department of Ecology
10/ 2005 – 07/ 2006	**Research assistant: Konrad Lorenz Institute for Ethology, ÖAW, Vienna**
10/ 2002 – pres.	**Study of biology, University of Vienna** **Zoology (Animal behavior)**
09/ 1994 – 06/ 2002	**Erich Fried Gymnasium, Vienna**

NON ACADAMIC OCCUPATIONS

03/ 2008 – pres	**Employment at the Zoo of Vienna** Guiding tours

GRANTS

2006 – 2007	**ERASMUS – Mobilitätsstipendium**

LANGUAGE SKILLS

German	mother tongue
English	excellent
French	basic
Italian	basic
Swedish	basic

COMPUTER SKILLS

Microsoft Office, Adobe Photoshop, SPSS, GeneMapper Software, Beckman Coulter CEQ Analysis Software, Noldus Observer XT