QUANTIFYING BEHAVIORAL ISOLATION BETWEEN CLOSELY RELATED SPECIES OF MICE

By

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QUANTIFYING BEHAVIORAL ISOLATION BETWEEN CLOSELY RELATED SPECIES OF MICE

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Abstract: Mate choice, the propensity of one individual to preferentially mate with another individual that expresses certain phenotypic values, can be a strong force in promoting or limiting gene flow between species. Individuals use sensory signals to identify conspecifics and to assess their potential as mates. Signals from heterospecifics may be less attractive or even aversive. Assortative mating in favor of conspecifics, a key component of behavioral isolation, is critical to maintaining species boundaries. However, species boundaries can be permeable between closely related taxa; in sympatry, interspecific overlap in signals used in mate attraction and choice may lead to hybridization. Determining the strength of premating isolation can shed light on the contribution of behavior to the evolution and maintenance of species. Moreover, interspecific gene flow can be affected by mate choice by hybrids, a topic that is generally understudied. Despite evidence for historic gene flow between house mice (*Mus musculus domesticus*) and Algerian mice (*M. spretus*), hybrids are not found in nature. However, *M. m. domesticus* females will mate with *M. spretus* males in the lab, whereas the reciprocal cross is challenging. This suggests asymmetry in the strength of behavioral isolation. Surprisingly, mate preference is untested in this well-studied species pair. We used a mate choice experiment to quantify the strength of behavioral isolation between *M. m. domesticus* and *M. spretus*, and mate preference in their F_1 hybrid. Females had free access to a male of each species for 24hrs. *M. m. domesticus* females spent more time with heterospecific males than *M. spretus* females, suggesting that the strength of behavioral isolation is indeed asymmetric. Hybrids showed no preference, suggesting that hybrid females may recognize males of both species as potential mates. Combined analysis of mate choice in hybrids and parental species promises novel insight into the behavioral mechanisms that limit gene flow between sympatric congeners.

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CHAPTER I
INTRODUCTION

Behavioral isolation is a strong evolutionary mechanism that can limit gene flow to the point of initiating and maintaining reproductive separation between species (Coyne and Orr 2004). Behavioral isolation is a common barrier that separates closely related species in sympatry, which might otherwise be genetically compatible to interbreed. Assortative mating, assessing and preferentially mating with individuals expressing similar phenotypic values, is one behavior that can reproductively divide species within a community and maintain reproductive barriers between closely related species in secondary contact (Via 2001, Jiang et al. 2013).

Mate choice, the propensity to mate with one individual rather than another based on phenotype, can help an individual to avoid unfit mates and the production of unfit offspring (Jennions and Petrie 1997). Females are typically the choosier sex, due to the increased energetic demands of reproduction (Trivers 1972). In vertebrate mate choice experiments, females that reproduce with preferred males can have higher fitness than females that reproduce with nonpreferred males (Welch et al. 1998, Drickamer et al. 2000). For example, female mice produced more litters when mated with a preferred male than with a nonpreferred male and adult offspring from preferred-mated females had increased survival, established larger home ranges, and were more socially dominant than adult offspring from nonpreferred-mated females (Drickamer et al. 2000).

Across species, mate choice is based on a wide variety of visual, olfactory, tactile, and acoustic signals and cues. Sensory signals can convey a wide range of information, from sex and species to individual identity, competitive ability, and heterozygosity. Using these cues, a female can differentiate mates to assess which individual exhibits the most desirable traits (Jennions and Petri 1997, Ptacek 2000). In mice, for example, major urinary proteins encode information on individual identity, health, and social status (Kavaliers et al. 2003, Bimova et al. 2009, Hurst 2009, Lopes and König 2016).

Signal detection theory, the concept that organisms must discern relevant signals from irrelevant environmental noise, provides a framework to conceptualize mate choice. Noise can be anything that obscures the perception or interpretation of a signal (Reichert and Ronacher 2015). In the context of behavioral isolation, relevant signals convey information about the suitability of a conspecific individual as a mate while cues from closely related heterospecific individuals act as noise that may confound signal intepretation. Cues from potentially reproductively incompatible heterospecifics are common noise when closely related species live in sympatry (Wiley 1994, Shettleworth 2010).

If strong postzygotic isolating mechanisms exist between species, selection may favor females with sharper discrimination against heterospecific mating (i.e. reducing overlap in Fig. 1). Character displacement, the phenotypic divergence of closely related species in sympatry due to competition or reproductive interference, is one mechanism that can produce sharper species discrimination (Gröning and Hochkirch. 2008, Hamel et al. 2015). However, when male phenotypes overlap between species, females may incorrectly reject conspecific mates (‘missed mating’ in Fig. 1) by increasing their discrimination threshold to avoid heterospecific mating (‘infertile mating’ in Fig. 1). Thus, selection favors a female’s threshold of acceptance that optimizes the balance between maximizing conspecific mating and minimizing heterospecific mating.

In contrast, when postzygotic isolation is weak or absent (i.e. hybrids are viable and fertile), the cost of interspecific mating is less and whether or not gene flow occurs depends primarily on preexisting female preferences and the degree of overlap in male signals (illustrated by Fig. 1). Under these conditions, various interspecific mating patterns may arise (Willis 2013). When, for example, members of one species do not discriminate against heterospecific cues while members of the other species do, an asymmetric pattern of assortative mating could develop. Alternatively, but rarely, members of both species may have equal preference for conspecific and heterospecific mates. In either case, incomplete species discrimination could lead to gene flow between the species through the process of hybridization.

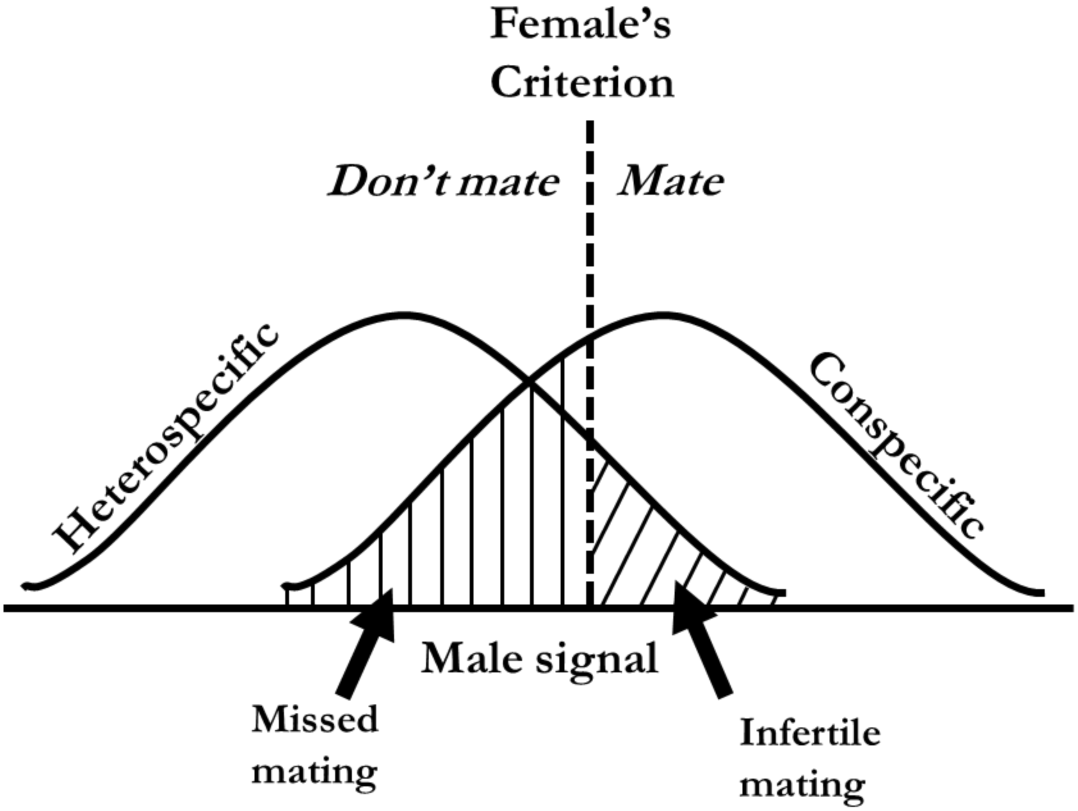

Figure 1. Signal detection theory curves applied to interspecific mate choice. Female's criterion is the threshold between mating and not mating in relation to males' signals or trait values. Missed mating occurs when females do not mate with conspecifics and infertile mating occurs when females mate with heterospecifics (after Shettleworth 2010, Fig. 3.9; see also Wiley 1994).

When interspecific mating occurs and an F_1 generation is produced, hybrids may express maladaptive traits, including reduced viability, infertility, or decreased success in a parental niche (Arnold 1997, Mallet 2008, Nosil 2012, Abbott et al. 2013, Willis 2013). Moreover, hybrids may exhibit maladaptive behaviors, such as failing to respond to species recognition cues and ceasing reproductive behaviors. Relative to inviability and sterility, maladaptive hybrid behavior has received less attention (e.g., Ptacek 2002; Hochkirch and Lemke 2011; Delmore and Irwin 2014, Schmidt and Pfennig 2016). While a few studies have tested hybrid mate preferences, results vary among taxonomic groups.

Thus, more work is needed to elucidate the role of hybrid behavior in promoting or limiting interspecific gene flow.

Hybrid mate choice can maintain species boundaries if hybrids mate with other F_1 hybrids. Hybrid sterility typically arises first in the heterogametic sex (e.g. XY males in mammals; Haldane 1992). Therefore, a fertile hybrid female's preference for a sterile hybrid male would halt further gene flow between species. This maladaptive behavior is seen in field crickets (*Teleogryllus*), in which female hybrids prefer the songs of male hybrids over the song of either parental species (Hoy et al. 1977). Alternatively, when both sexes of hybrids are fertile, hybrid preference for hybrids can facilitate hybrid speciation. Within one generation of experimental hybridization of African cichlid fish, female hybrids had a significantly higher preference for the novel phenotypes of hybrid males than the phenotypes of males from either parental species (Selz et al. 2014). Selz and colleagues' findings suggest that hybrid speciation can occur rapidly. Strengthened behavioral barriers can arise between parental species if hybrids exhibit an intermediate preference. Here, intermediate preference is defined as preference for an intermediate suite of traits that are not fully expressed in either parental species, which often manifests as a preference for other F_1 hybrids. For example, hybrid female brush crickets (*Ephippiger*), prefer synthetic male calling songs with an intermediate number of syllables relative to those of their parental species (Ritchie 2000). Furthermore, hybrids might not backcross in either direction due to the parental species' phenotypes being too extreme for the hybrids' intermediate preference (Ritchie 2000).

Conversely, hybrid mate choice can homogenize species through the process of introgression, or hybrid-mediated gene flow. For bidirectional gene flow to occur, fertile hybrids must readily mate with either parental species. Bidirectional gene flow may happen when hybrids exhibit no preference for individuals of either parental species. For example, F_1 hybrid female grasshoppers (*Chorthippus*) discriminated against hybrid males (Bridle et al. 2006) but expressed no preference for one parental species over the other, allowing introgression to occur in either direction (Hochkirch and Lemke 2011). A potential explanation for this apparent lack of preference is that both parental species' mating signals, while not a perfect match, are the phenotypes closest to the hybrids' novel preference that is not otherwise satisfied (i.e. making the best of bad conditions, Koprowski 1993, Barrera-Guzmán 2018). Under these conditions, hybrid behavior can weaken reproductive boundaries and reduce diversity (Gröning and Hochkirch 2008).

Lastly, asymmetry in interspecific hybrid mate choice may lead to unidirectional introgression gene flow (Abt and Reyer 1993). In situations where one species gained a fitness advantage in allopatry, introgression can transmit the beneficial allele to the second species, favoring individuals from the second species that interbreed. When the other species receives no benefit from interbreeding the strength of behavioral isolation can be asymmetric (Abt and Reyer 1993). While presumed to be rare, adaptive introgression has been demonstrated in a few empirical studies (Song et al. 2011, While et al. 2015).

Notably, current understanding of hybrid-mediated gene flow and hybrid mating preference comes from work in taxa with conspicuous auditory and/or visual mating signals, such as invertebrates, birds, anurans, and fish (e.g. Doherty and Gerhardt 1983, Noor 1997, Veen et al. 2001, Ptacek 2002, Stein and Uy 2006, Melo et al. 2009, Hochkirch and Lemke 2011, Schimdt and Pfennig 2016). To my knowledge, hybrid mating preferences are unstudied in mammals.

Here, I investigated the mating preference patterns of the house mouse (*Mus musculus domesticus*) and the closely related Algerian mouse (*Mus spretus*), a well-studied system in mammalian speciation genetics. *M. m. domesticus* and *M. spretus* are sympatric in Southwestern Europe and North Africa. *M. spretus* individuals pair bond and males exhibit paternal care for pups, while *M. m. domesticus* is a polygamous species (Cassaing and et al. 2010). Evidence for historic gene flow exists (Rikke et al. 1995, Green-Till et al. 2000, Song et al. 2011, Jones and Searle 2015, Liu et al. 2015) and laboratory crosses can produce viable hybrids (Zechner et al. 1996). However, there is no evidence for ongoing hybridization in nature (Dejager et al. 2009, Boursot et al. 2012). Prior laboratory studies provide anecdotal evidence for asymmetric assortative mating: *M. m. domesticus* females will mate with *M. spretus* males, but the reciprocal cross is very challenging (Zechner et al. 1996).

Hybrid male offspring are sterile in both directions of the cross (Matsuda et al. 1991). Thus, females and males of both species face fitness costs when mating with heterospecific individuals. Hybrid sterility alone, however, is not necessarily enough to prevent hybridization in the wild (e.g. McKean et al. 2016). Therefore, the apparent absence of hybrids suggests that there are strong behavioral barriers to gene flow between *M. m. domesticus* and *M. spretus* in nature. Within house mice, subspecies discrimination between *M. m. domesticus* and *M. m. musculus* has been the focus of many studies (e.g. Latour et al. 2014, Latour and Ganem 2017). However, species discrimination between *M. m. domesticus* and *M. spretus* is unstudied. Moreoever, F_1 hybrid behavior, including mate choice, is unstudied in this system. Combined analysis of mate choice in hybrids, and in parental species, can provide novel insight into the strength of barriers that limit interspecific gene flow between closely related sympatric species.

I used a laboratory mate choice experiment to examine mating preferences and quantify the strength of premating isolation between wild-derived inbred strains of *M. m. domesticus* and *M. spretus.* Given the evidence for behavioral isolation in nature, I predicted that females of both species would have a stronger preference for conspecific males over heterospecific males. Given the anecdotal evidence for asymmetric behavioral isolation in the lab (Zechner et al. 1996) and that *M. spretus* males provide paternal care while *M. m. domesticus males* do not (Cassaing et al. 2010), I also predicted that *M. spretus* females would have a significantly stronger preference for conspecific males than *M. m. domesticus* females. In the case of asymmetric mate preference, *M. m. domesticus* females may have a less discriminatory mate recognition system that leads to more heterospecific mating (see Fig. 2a), while *M. spretus* females may have a higher mating threshold for con- and heterospecific males, which would reduce heterospecific mating (see Fig. 2b). Finally, I predicted that F_1 hybrid females would not have a significant preference for males of either parental species (Fig. 2c), which is a relatively common pattern in non-mammalian hybrids (Hoy et al. 1977, Hochkirch and Lemke 2011, Selz 2014). To the best of my knowledge, this is the first experimental test of hybrid female mate preference in a mammal.

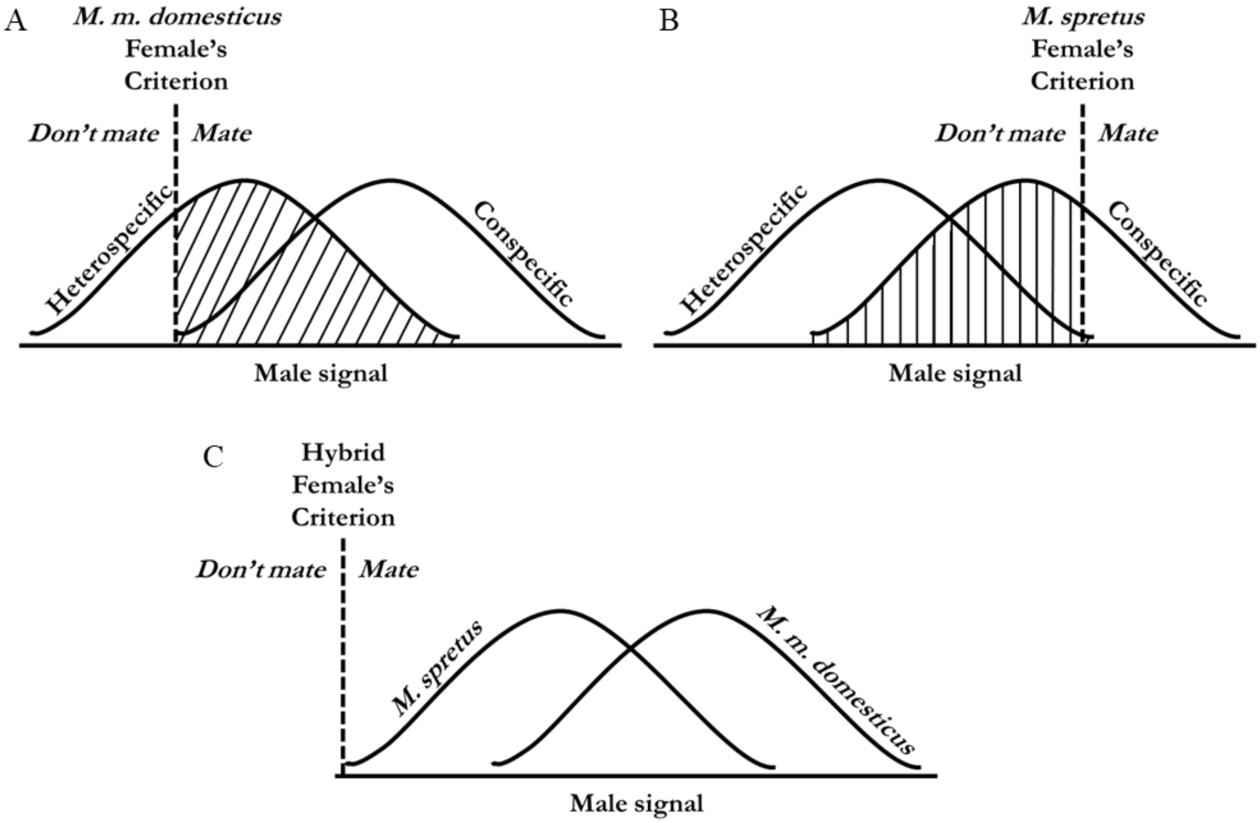

Figure 2. Hypothesized mate acceptance thresholds for female (A) *M. m. domesticus,* (B) *M. spretus* and (C) F_1 hybrids. Vertical dotted line indicates a female's acceptance threshold in relation to overlapping heterospecific and conspecific male signals. Vertical solid lines indicate incorrect rejections of conspecific males. Diagonal lines indicate incorrect acceptance of heterospecific males. I hypothesize that *M. m. domesticus* females are more likely to mate with *M. spretus* males than *M. spretus* females are to mate with *M. m. domesticus* males, and that hybrid females will mate with males of either parental species with equal probability.

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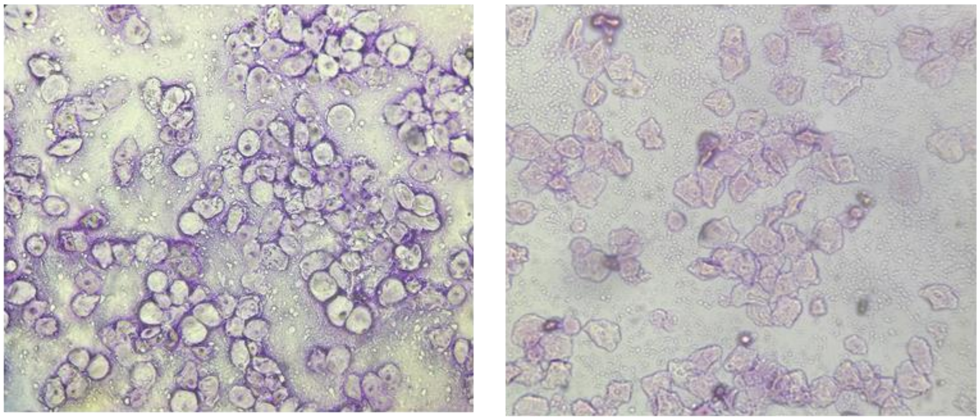
CHAPTER II
METHODS

Animals - *Mus musculus domesticus* (WSB/EiJ) were purchased from the Jackson Laboratory (ME, U.S.A.). The *Mus spretus* strain (SFM) used in this study was developed at the Montpellier Wild Mice Genetic Repository. Animals in the Campbell lab breeding colony were obtained from the Good lab at the University of Montana. All individuals were housed in standard polycarbonate cages bedded with Sanichips (Harlan Teklad, WI, U.S.A.) and were provided with nesting material (cotton nestlets and alfalfa hay) and *ad libitum* chow (LabDiet® Rodent Diet 5001) and water. Mice were maintained on a 12:12 light:dark cycle with lights on at 0900 hours. Pups were weaned at approximately postnatal day 28. Weanlings were housed with same-sex littermates; males were singly housed after postnatal day 60 to avoid fighting. F_1 hybrids were produced by crossing *M. m. domesticus* females to *M. spretus* males. Including hybrids from the reciprocal cross (female *M. spretus* x male *M. m. domesticus*) would have been desirable, but all attempted pairings failed to produce pups, further motivating this study. Female mice used in the experiment were 2-6 months old, sexually naïve, and in proestus at the start of each trial (see below). Each female was used only once. Stimulus males were 2-9 months old and sexually experienced to maximize the probability of mating behaviors. Stimulus males were age-matched with each other (± 2 weeks). To reduce the number of total experimental mice used, each stimulus male participated in three trials, one for each female genotype.

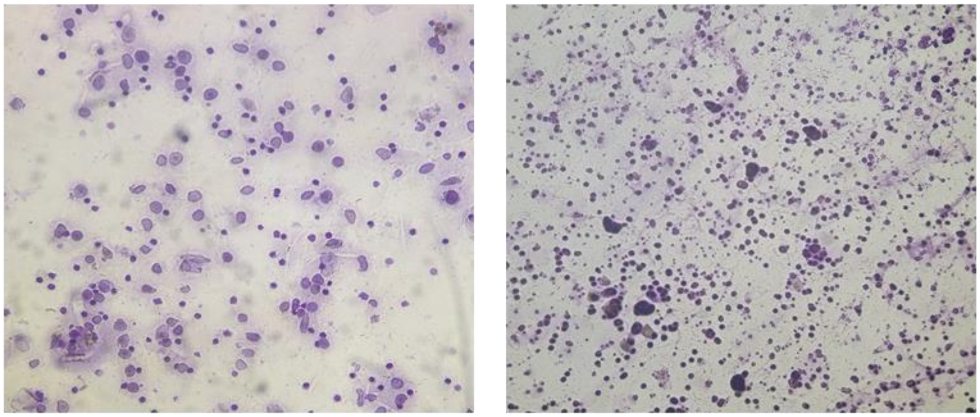
A microtransponder (1.4 x 9 mm, Planet ID GmbH) for individual identification was implanted subcutaneously into each experimental mouse via needle injection. All mice resumed normal behaviors immediately following injection. All animal procedures were in accordance with the Oklahoma State University Institutional Animal Care and Use Committee (protocol AS-14-1).

Cytological Assays – The reproductive status of female mice was determined with a simple, minimally invasive cytological assessment of the predominant cell type in vaginal smears (McLean et al. 2012). Each female's vaginal canal was gently flushed with approximately 0.1 mL of distilled water. Fluid containing vaginal tissue was expelled onto a glass microscope slide. Once dried, the slides were incubated for two minutes in a 0.1% hematoxylin (Fischer Chemical) solution and washed with distilled water for one minute to remove excess stain. Cytology was evaluated using light microscopy (Fisherbrand Micromaster™, 10x). Estrous cycle phases can be discriminated by the relative ratio of nucleated epithelial cells, cornified squamous cells, and leukocytes (see Fig. 3). The onset of behavioral estrus (the display of precopulatory and copulatory behaviors) coincides with proestrus (Byers et al. 2012). Females used in the experiment were assayed daily at 1700 hours. Mate choice trials were started when vaginal cytology was consistent with proestrus (Fig. 3a).



A. Proestrus

B. Estrus



C. Metestrus

D. Diestrus

Figure 3. Cell type differs based on phase of the estrous cycle. (A) Nucleated epithelial cells are prevalent during proestrus. (B) Cornified squamous epithelial cells lacking a nucleus are present during estrus. (C) Cornified squamous epithelial cells and leukocytes are relatively evenly distributed during metestrus. (D) Leukocytes outnumber nucleated epithelial cells during diestrus (McLean et al. 2012).

Mate Choice Trials – A two-way choice paradigm (Fig. 4) was used, in which the focal female mouse began in the middle cage and had free access to a *M. m. domesticus* male in one end cage and a *M. spretus* male in the other. The species identify of males in the left vs. right end cages was alternated between trials within each female genotype to control for potential female side bias. Trials started at the beginning of the 12-hour dark period (approximately 2100 hours) and were run for 24 hours during which time the mice were left undisturbed. Behaviors exhibited during trials were recorded with Raspberry Pi NoIR camera modules and videos were scored manually (see below). Infrared lamps (Towallmark 48-LED CCTV) produced light (invisible to mice) over each male's cage so that the camera modules could capture video during the dark phase.

The apparatus comprised three clear polycarbonate cages (26 x 16 x 48 cm) connected by tunnels (4 cm diameter, 15 cm long) containing automated gates (FBI Science GmbH; black vertical lines in Fig. 4a). Ring antennae (5.5 cm diameter, dotted lines in Fig. 4a) fitted around the tunnels read microtransponder IDs. The software OLCUS ID (FBI Science GmbH) was used to program the gates and record the ID number and timestamp each time a mouse with a microtransponder implant passed through a ring antenna.

Gates were programmed to open when a female's transponder passed through a ring antenna, giving her access to a male's cage. Male transponders, when read by the ring antennae, signaled the gate to close if opened, or remain shut. Thus, stimulus males were restricted to their respective cages while the focal female was free to move between them. Cages were bedded with Sanichips and each contained a cotton nestlet, a cardboard mouse hut, and equal amounts of food and water. Prior to being used in a trial, each female was given two twelve-hour exposure periods to the apparatus to acclimate to the door-opening process. Males were acclimated to the apparatus for ten minutes immediately before a trial. The apparatus was thoroughly cleaned with warm, soapy water between trials.

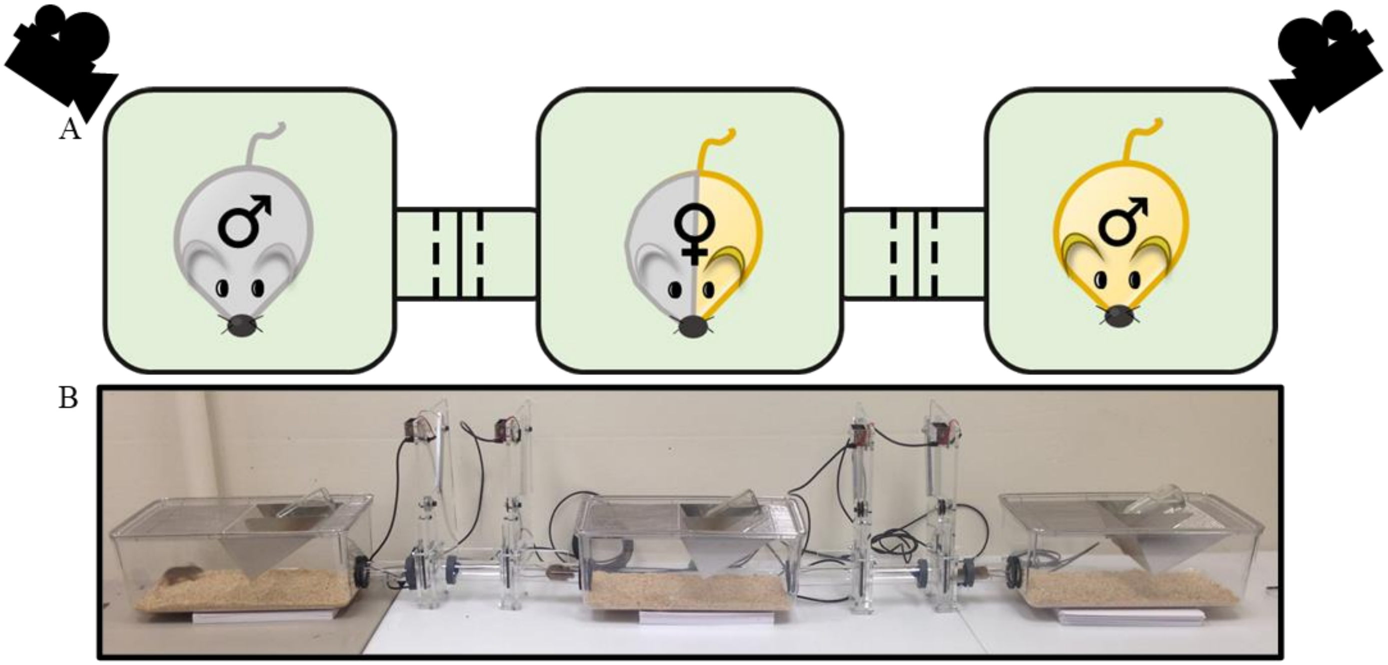


Figure 4. Two-way mate choice apparatus, in which the focal female mouse (the chooser) begins in the middle cage and has access to a conspecific male in one neighboring stimulus cage and a heterospecific male in the other. (A) Top-down schematic. Dotted vertical lines represent ring antennae and solid vertical lines represent automated gates. Gray and yellow mice represent *M. m. domesticus* and *M. spretus,* respectively; a hybrid female is shown in the center cage. All activity in males' cages was recorded with Raspberry Pi cameras. (B) Side view of mate choice apparatus with mice.

Behavioral Repertoire - Male mice perform anogenital investigations on females to stimulate sexual responses (Pankevich et al. 2004). Vaginal sniffing can elicit aggression or copulation in females. Successful mating (ejaculation achieved) cannot occur if the female does not assume a receptive posture. Consequently, successful copulation is a strong index of female mate preference in mice. Behaviors scored as increased preference towards a male included copulation, time spent with a particular stimulus male, frequency of visits to a male’s cage, and frequency of affiliative interactions (Table 1; Ivantcheva and Cassaing 1999). The frequency of agonistic behaviors directed towards a male was treated as an index of decreased preference for that male (Table 1; Ivantcheva and Cassaing 1999, Koolhaas et al. 2013). The number of times a female visited a given male and the time she spent in his cage were calculated from timestamps when her microtransponder passed through an antenna as she entered and left a male’s cage. All other female behaviors were scored manually from videos by me.

Table 1
Suite of behaviors scored as affiliative or agonistic, used to represent increased and decreased preference, respectively

Affiliative Behaviors	Agonistic Behaviors
Olfactory investigation (smelling/nose-to-nose contact)	Wrestling/biting
Side-by-side contact sleeping	Chasing
Successful copulation attempts from males	Resisting copulation attempts from males
Allogrooming	Defensive postures in response to male-initiated investigation
Directly approaching males	
Copulation	

Statistical Analysis – Within the three female genotypes, differences in time spent, and in number of visits, affiliative interactions, and agonisitic interactions with conspecific vs. heterospecific males were tested with paired *t*-tests (Vamosi and Schluter 1999, Pilakouta et al. 2017).

To control for among-female variation in overall activity levels, I normalized the data by calculating strength of preference (SOP) scores for each parameter that indicated increased preference (Sobel and Chen 2014). To normalize behaviors that indicated decreased preference, a strength of agonism (SOA) score was calculated. SOP and SOA scores were quantified using the equation of Stalker (1942):

$$SOP = \frac{freq.\,of\ conspecific\ directed\ behaviors - freq.\,of\ heterospecific\ directed\ behaviors}{freq.\,of\ conspecific\ directed\ behaviors + freq.\,of\ heterospecific\ directed\ behaviors}$$

Which can be abbreviated as:

$$SOP = \frac{Con - Het}{Con + Het}$$

Scores can range from -1 (all behaviors directed towards heterospecifics) to 1 (all behaviors directed towards conspecifics), with 0 (behavior directed equally toward con- and heterospecifics) indicating no preference. One-sample *t*-tests were used to test whether SOP and SOA scores for each female genotype were significantly different from zero. Among-genotype differences in SOP and SOA scores were tested with analysis of variance (ANOVA). Statistical analyses were carried out using R (version 3.4.3) and Microsoft Excel.

CHAPTER III
RESULTS

A total of 104 mice (62 females, 42 males) were used in 62 trials. 70.9% (44/62) of attempted trials were used in final analyses. 29.1% (18/62) of trials were excluded because the automated gate system malfunctioned and males escaped from their designated cages into the central cage for more than six hours. Of the 44 trials used in final analyses, males escaped to the central cage for ≤ six hours in 12 (27.2%). In these cases, males were out of their cages for an average of 136.8 minutes (± SD 113.1 min, range 1 – 360 min) and female behavior was not scored during that time. Final sample sizes for the three female genotypes were 16 *M. spretus* trials, 17 *M. m. domesticus* trials, and 11 hybrid trials.

Female preference for conspecific males is stronger in M. spretus *than* M. m. domesticus

M. spretus females exhibited significant preference for conspecific males over heterospecific males based on strongly positive strength of preference (SOP) scores (time spent: 0.62 ± 0.55; visits: 0.56 ± 0.47; affiliative interactions: 0.51 ± 0.49; mean ± SD). All SOP scores were significantly greater than zero, indicating a preference (time spent: t_{15} =3.588, p = 0.002; frequency of visits: t_{15} = 4.717, p = 0.0002; affiliative interactions: t_{15} = 2.74, p = 0.0007; see Fig. 5). *M. spretus* females also had a positive strength of agonism (SOA) score (0.38 ± 0.77), indicative of more agonisitic interactions with conspecific males. However, SOA score was not significantly different from zero (t_{15} = 1.945, p = 0.07). *M. spretus* females spent only marginally more time with conspecific males relative to heterospecific males (t_{15} = 1.894, p = 0.067), but visited conspecific males significantly more than heterospecific males (t_{15} = 2.161, p = 0.04), and had significantly more affiliative interactions with conspecifics than heterospecifics (t_{15} = 2.915, p = 0.01). There was no effect of male species on number of agonistic interactions with males (t_{15} = 1.738, p = 0.10; Fig. 6). Copulation was observed in 25% (4/16) of *M. spretus* trials. Due to the infrequency of mating in either species, copulation was included as one behavior in the suite of affiliative interactions but could not be analyzed separately. Of the 4 trials in which copulation was observed in *M. spretus*, females copulated with conspecific males in 3 trials and a heterospecific male in 1 trial.

M. m. domesticus females exhibited no significant preference for males of either species. SOP scores were weakly positive (time spent: 0.26 ± 0.74; visits: 0.12 ± 0.61; affiliative interactions: 0.14 ± 0.58), only marginally favoring conspecific males. While *M. m. domesticus* females had lower SOP scores than *M. spretus* females, the species difference was not significant (ANOVA: $F_{2,30}$=1.09; p = 0.38). Likewise, while all female *M. m. domesticus* SOP scores were positive (indicating more interactions with conspecific males), none were significantly different from zero (time spent: t_{16} = 1.457, p = 0.16; visits: t_{16} = 0.805, p = 0.43; affiliative interactions: t_{16} = 0.956, p = 0.35; agonistic interactions: t_{16} = 1.355, p = 0.19; Fig. 5). There was no effect of male species on time spent (t_{16} = -1.589, p = 0.13), frequency of visits (t_{16} = -0.548, p = 0.59), or affiliative interactions (t_{16} = 0.105, p = 0.92). Likewise, neither the weakly positive SOA score (0.19

± 0.59) nor the number of agonisitic interactions was significant ($t_{16} = -0.078$, $p = 0.94$; Fig. 6). Copulation was only observed in 11% (2/17) of *M. m. domesticus* trials. In both trials females copulated with heterospecific *M. spretus* males.

First generation female hybrids show no preference for males of either parental species

Hybrid females showed no preference for males of either species, based on neutral SOP scores for time spent (0.02 ± 0.89), frequency of visits (-0.03 ± 0.65), numbers of affiliative interactions (0.09 ± 0.64), and agonistic interactions (0.04 ± 0.68; Fig. 5). None of the hybrid SOP scores were significantly different from zero (time spent: $t_{10} = 0.057$, $p = 0.96$; visits: $t_{10} = -1.741$, $p = 0.87$; affiliative interactions: $t_{10} = 0.445$, $p = 0.67$). Similarly, the SOA score was not significantly different from zero ($t_{10} = 0.181$, $p = 0.86$). There was no effect of male species on the amount of time spent ($t_{10} = 1.175$, $p = 0.27$), frequency of visits ($t_{10} = -1.622$, $p = 0.14$), affiliative interactions ($t_{10} = -0.510$, $p = 0.62$) or agonistic interactions ($t_{10} = -0.317$, $p = 0.76$; Fig. 6). Hybrid females did not copulate with males of either parental species.

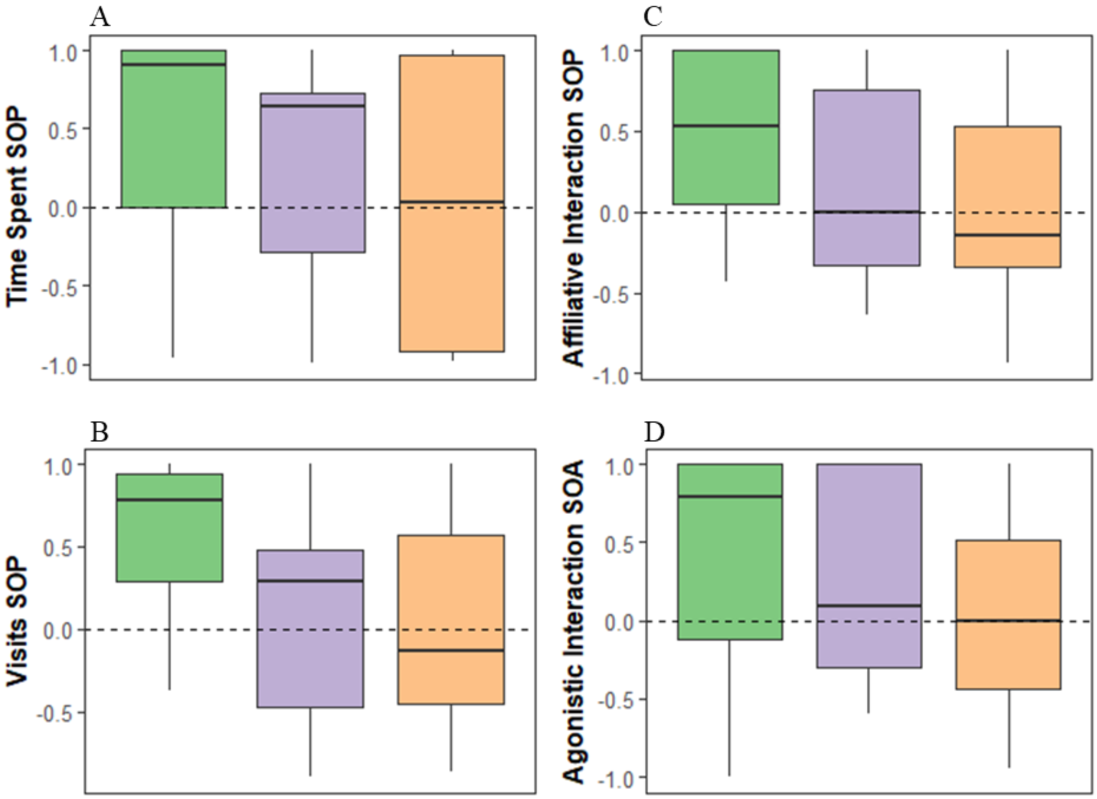


Figure 5. Strength of preference scores and strength of agonism score for female *M. spretus* (green), *M. m. domesticus* (purple) and hybrids (orange). An SOP score of 1 represents exclusive preference for conspecifics (arbitrarily set to *M. spretus* for hybrids) and -1 represents exclusive preference for heterospecifics (*M. m. domesticus* for hybrids). SOP scores based on (A) amount of time each female genotype spent with *M. spretus* and *M. m. domesticus*, (B) number of visits, (C) number of affiliative interactions, and SOA score based on (D) number of agonistic interactions. Genotypes did not significantly differ from one another in SOP scores for time spent ($F_{2,41} = 1.782$, $p = 0.18$), affiliative interactions ($F_{2,41} = 2.535$, $p = 0.09$), or agonistic interactions ($F_{2,41} = 0.823$, $p = 0.45$). Female *M. spretus* had a significantly higher SOP score for visits than female hybrids ($F_{2,41} = 4.115$; Tukey's HSD: $p = 0.03$).

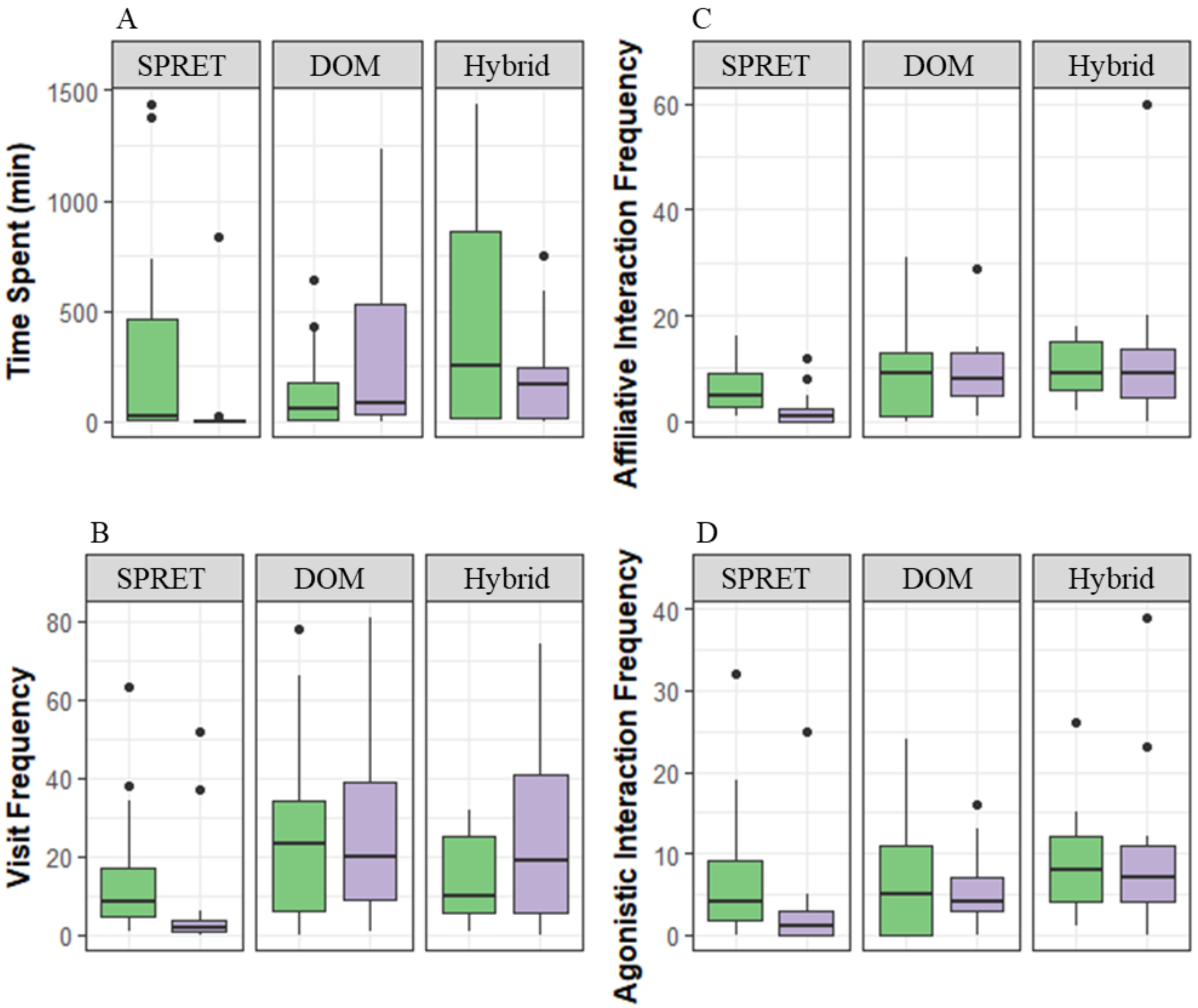


Figure 6. Raw count data for each female behavior parameter grouped by female genotype (*M. spretus*: SPRET, *M. m. domesticus*: DOM, F_1 hybrids: Hybrid). Boxplots represent 25th, 50th, and 75th quartiles. Dots represent outliers. Boxplots indicate female behavior directed towards *M. spretus* (green) or *M. m. domesticus* (purple) males during mate choice trials. (A) There was no difference in the amount of time spent with *M. spretus* vs. *M. m. domesticus* males for any female genotype. (B) Only *M. spretus* females visited conspecific males significantly more than heterospecific males (t_{15} = 2.161, p = 0.04), while the other two female genotypes exhibited no difference. (C) Only *M. spretus* females exhibited significantly more affiliative behaviors towards conspecifics than heterospecifics (t_{15} = 2.915, p = 0.01). (D) Male species had no effect on the number of agonistic interactions for any female genotype.

CHAPTER IV
DISCUSSION

Here, I showed that *Mus spretus* females have stronger preference for conspecific males than heterospecific males. *M. m. domesticus* females had weak strength of preference scores that did not significantly differ from 0, indicating no preference. These results are broadly consistent with predicted asymmetry in the strength of behavioral isolation between *M. m. domesticus* and *M. spretus* (Fig. 2a and b). In contrast, F_1 hybrid females interacted equally with males of both parental species, suggesting that hybrids either do not discriminate between *M. m. domesticus* and *M. spretus* males, or lack preference for one over the other. This result is consistent with the predicted lack of preference in hybrids (Fig. 2c). Collectively, the results of this study suggest that female mate choice contributes to potentially asymmetric reproductive isolation between *M. m. domesticus* and *M. spretus* in sympatry, and provide the first index of hybrid female mate preference for a mammal.

Evidence for asymmetry in the strength of behavioral isolation

While copulation was rare in my experiment, spending time with and investigating males are important first steps of mate assessment and mate choice, and can be proxies for mate preference (Capittini et al. 2008, Costello et al. 2015). Similarly, frequently entering a male's cage can be interpreted as increased preference. Here, *M. spretus* females spent more time, visited more frequently, and regularly performed more affiliative behaviors towards conspecific males relative to heterospecific males (Fig. 5). Increased preference and assessment of conspecific males is reasonable, given that *M. spretus* males provide paternal care for offspring while *M. m. domesticus* males do not (Cassaing et al. 2010). Thus, *M. spretus* females would benefit from increasing investment into conspecific males over heterospecific males. In contrast, *M. m. domesticus* females showed no preference for conspecific or heterospecific males by interacting with males of both species equally (Fig. 5). *M. m. domesticus* females did not spend more time on average with males from one species over the other. Similarly, female *M. m. domesticus* visited and affiliated with heterospecific and conspecific males approximately equally (Fig. 6).

Female hybrids show no preference for either parental species

Much of the work on mammalian speciation has highlighted the differences between closely related species' mate choice (Theiler and Blanco 1996, Dempster et al. 1992, Latour et al. 2014) but hybrid mate preference has gone unstudied in mammals (Ptacek 2000). Studies of hybrid mate choice in other systems suggest that assortative mating patterns can vary among species (e.g. Abt and Reyer 1993, Selz et al. 2003, Hochkirch and Lemke 2011). Therefore, preference for one parental species over the other cannot be assumed. Here, I investigated mating preference in female F_1 hybrid mice. In accordance with my predictions, female hybrids lacked assortative mating preferences towards males of either parental species. Specifically, female hybrids exhibited large inter-individual variation and no detectable patterns in regards to time spent and frequency of visits with males of a given species (Fig. 6). Furthermore, hybrid females on average engaged in approximately equal amounts of affiliative and agonistic behaviors with males of both species. These results suggest that female hybrids do not have a preference. However, there are several potential causes for apparent lack of preference. First, F_1 hybrid females may not be able to discriminate accurately between *M. spretus* and *M. m. domesticus* males (Cáceres et al. 2009, Segura et al. 2011). Second, hybrids may discriminate between *M. spretus* and *M. m. domesticus* males but recognize both as potential mates (Parker 1983). Third, hybrid females may prefer novel or intermediate male phenotypic values that are missing in both parental species (Cáceres et al. 2009). Determining whether or not female hybrids can discriminate between *M. spretus*, *M. m. domesticus*, and F_1 hybrid males will be a critical next step towards understanding the causes of hybrid female lack of preference. Hybrid mate choice studies in other taxa found similar results, in which F_1 female grasshoppers (*Chorthippus*) showed no preference for males of either parental species. Lack of preference might be associated with lower acceptance thresholds (increasing the number of potential mates), or might reflect low motivation to mate (Brooks and Endler 2001). Finding which phenotypic values, if any, that increase responsiveness and motivation to mate in hybrids would aid in interpreting hybrids' lack of preference. Similarly, quantifying hybrid females' willingness to copulate, regardless of their mates' species, would shed light on a potentially maladaptive behavior.

Implications for gene flow

While *M. spretus* and *M. m. domesticus* can interbreed to produce viable hybrids (Zechner et al. 1996), previous work suggests that there is no ongoing gene flow between the two species in nature (Dejager et al. 2009, Boursot et al. 2012). I found that *M. spretus* females exhibited strong preferences for conspecific males, whereas *M. m. domesticus* females were more tolerant of heterospecific males. This behavioral asymmetry could affect the direction of occasional gene flow in nature. Indeed, the findings from Orth et al. (2002) support this notion. Orth and colleagues found evidence of historical introgression from *M. spretus* into *M. m. domesticus.*

Depending on the strength of preference towards parental species, hybrids can mediate the direction of introgression between two congeners in secondary contact. The results of my experiment suggest that female hybrid mice have no preference. Depending on the underlying cause of lack of preference, hybrid-mediated gene flow could occur bidirectionally or not at all. While copulation was rare in my study, it is worth noting that mating was observed in at least one trial for both *M. spretus* and *M. m. domesticus* females, but never for hybrid females. More importantly, the fact that F_1 hybrids are not found where *M. spretus* and *M. m. domesticus* co-occur in nature (Dejager et al. 2009, Boursot et al. 2012) suggests that behavioral isolation between parental species is the primary barrier to introgression in this system.

This study is limited in the fact that the species used were represented by inbred strains that have been in the laboratory for many generations. Future work should use the same mate choice paradigm with wild-caught *M. spretus* and *M. m. domesticus* from regions of sympatry. *M. m. domesticus* typically occur at high densities, allowing females to regularly assess potential mates, either through direct interaction, or indirectly through major urinary protein cues (DeLong 1967). Comparing females' mate choice from populations with high densities of conspecifics to those with high densities of heterospecifics would shed light on how social conditions might weaken or strengthen species selectivity.

Additionally, future work should quantify male mate choice in the same paradigm presented here. Identifying potential differences in males' motivation to mate with conspecific over heterospecific females would provide valuable insight into whether males contribute to behavioral isolation. For example, males might exhibit more aggression towards conspecific females than heterospecific females. Based on frequency of agonistic interactions data from this experiment, *M. spretus* males may be more aggressive with females than *M. m. domesticus* males (Fig.6). Additionally, to further quantify reproductive isolation between species, it would be of interest to investigate hybrid preference for other F_1 hybrids relative to parental species.

CHAPTER V
CONCLUSION

This study provides evidence for behavioral barriers between sympatric congeners that can be crossed in the laboratory but hybridize rarely in nature. Using *Mus spretus*, *Mus musculus domesticus* and their interspecific hybrid in a two-way mate choice paradigm, the results of this experiment suggest that female *M. spretus* strongly prefer conspecific males, but female *M. m. domesticus* show no preference between conspecific and heterospecific males. Likewise, female hybrids exhibited no preference for males of either parental species, suggesting potential hybrid-mediated gene flow could occur bidirectionally. My results suggest that the preference of *M. spretus* females for conspecific males may translate to behavioral barriers to the production of hybrids in nature. Ultimately, studying the strength of behavioral barriers to gene flow between closely related species can provide insight into the role of assortative mating in speciation.

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