The role of sexual imprinting in speciation: lessons from deer mice (genus Peromyscus)

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The role of sexual imprinting in speciation: lessons from deer mice (genus *Peromyscus*)

presented by Emily Ho Kay

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Signature _____

Typed name: Professor Hopi E. Hoekstra

Signature _____

Typed name: Professor James Mallet

Signature _____

Typed name: Professor Scott V. Edwards

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The role of sexual imprinting in speciation:

lessons from deer mice (genus *Peromyscus*)

A dissertation presented

by

Emily Ho Kay

to

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The role of sexual imprinting in speciation: lessons from deer mice (genus *Peromyscus*)

ABSTRACT

Sexual imprinting, the process of learning mate preferences at a young age, could promote speciation by reducing attraction to individuals from divergent populations or species, consequently creating or maintaining reproductive isolation. Yet, despite the documentation of sexual imprinting in many taxa, its connection to speciation has been understudied. I chose to explore the potential link between sexual imprinting and reproductive isolation and in two North American rodents—the white-footed mouse (*Peromyscus leucopus*) and its sister species, the cotton mouse (*Peromyscus gossypinus*). These species have overlapping distributions in nature, possibly allowing interbreeding and admixture. In Chapter 1, I used double-digest restriction-associated DNA sequencing to test for hybridization in sympatric natural populations and found that 1.5% of sampled individuals showed evidence of admixture yet the species have maintained genetic distinctness in sympatry. In the lab, the species hybridize when given no choice of mates but mate more readily with conspecifics, suggesting that mating preferences may prevent hybridization in the wild. In Chapter 2, I tested whether mating preferences create significant reproductive isolation. I measured mating preferences in controlled laboratory conditions and found that both species and sexes preferred conspecific to heterospecific mates in 85% of trials. I then raised offspring with foster parents of the opposite species and found that *P. leucopus* has a genetically-determined preference while *P. gossypinus* learns its preference. In Chapter 3, I tested whether sexual imprinting on parental diet could generate assortative mating within a species. I tested this hypothesis by feeding *P. gossypinus* parents either orange- or garlic-

flavored water, thereby exposing their offspring to these flavors through their parents until weaning. I tested the preferences of these offspring as adults and found that *P. gossypinus*, especially females, had strong assortative mating preferences. This implies that at least females learn parental dietary information and that assortative mating could evolve within a single generation. Together, my results confirm that sexual imprinting on parental traits—possibly mediated through dietary differences—can create assortative mating capable of generating sexual isolation and reducing gene flow between species. My research supports the importance of mating preferences and learning in speciation.

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INTRODUCTION

Sexual isolation is arguably one of the most important reproductive barriers that can arise between species. It can evolve early and act strongly, and thus may create reproductive isolation that facilitates further species divergence. Comparative studies from fruit flies (Coyne and Orr 1989, 1997) and darter fish (Mendelson 2003) and have shown that sexual isolation arises more rapidly than reproductive barriers that act after mating: hybrid sterility and hybrid inviability. Sexual isolation also arises faster in sympatry, suggesting that it either is required for species to co-exist or is strongly selected for in sympatry through reinforcement (Coyne and Orr 1997). In a few extreme cases, sexual isolation may be the only reproductive barrier preventing hybridization between sympatric species (e.g., Seehausen 1997; Fisher et al. 2006). Thus, because sexual isolation is an effective barrier that appears to form early during the speciation process, it is important to understand *how* and *why* sexual isolation evolves to better understand the evolution of new species.

In a series of classic experiments in fruit flies, researchers discovered that sexual isolation could evolve during adaptation to new environments. After adapting populations to new media for several generations, flies no longer mated randomly (Dodd 1989; Rundle et al. 2005; Sharon et al. 2010). Instead, flies preferred to mate with individuals of the same population, creating a sexual reproductive barrier between populations raised on different media. Surprisingly, this pattern of concurrent adaptation and sexual isolation—when mating preferences increase attraction to individuals from the same population and reduce attraction to individuals from other populations—emerged between populations in just a few generations. Rapid sexual isolation during adaptation is not unique to laboratory populations of flies; it has been observed in natural

populations of additional taxa, including insects and fish (Funk 1998; Nosil et al. 2002; Mckinnon et al. 2004; Langerhans et al. 2007).

The emerging field of "ecological speciation", which focuses on the role of ecology in speciation, has argued that most sexual isolation is incidental. That is, sexual isolation is thought to evolve as a by-product of divergent natural selection (toward different optima) acting on either mating signals or preferences. In one scenario, divergent natural selection on mating signals could induce preferences for those traits to also diverge. In a second scenario, divergent natural selection on mating preferences (e.g. selection on the sensory apparatus to detect mating signals in different signaling environments) could generate sensory drive and cause mating signals to evolve better detectability. For example, if the environment influenced female perception of male mating signals, as has been shown to be the case for male cichlid color at varying water depths (Seehausen et al. 2008), male traits may evolve to be more conspicuous to females. There is strong support for both scenarios, implying a coupling between traits and preferences for those traits that can be affected by divergent natural selection (Nosil 2012).

The ability of divergent selection to act on ecological traits to produce sexual isolation will depend on the underlying genetic mechanisms. At the genetic level, divergent selection on a trait can be coupled to mating preference in two ways. First, linkage disequilibrium between a trait locus and preference locus will effectively allow selection on the two loci together. Examples from finches and flycatchers suggest that sexual preferences for male traits and preferences for them are co-inherited through the Z chromosome (Saether et al. 2007; Pryke 2010). However, linkage disequilibrium between separate loci, unless physically close or "locked" in an inversion, will be sensitive to gene flow because recombination between trait and preference alleles would erode sexual isolation (Servedio et al. 2011). An alternate, genetic

mechanism for establishing a link between traits and preferences for them is if the trait and preference are controlled by the same gene—i.e. pleiotropy. Pleiotropic genes undergoing divergent natural selection that consequently causes non-random mating will create "magic traits", those traits that are involved in both environmental adaptation and mate choice. Although there are several examples of such magic traits (e.g. mimicry color pattern in butterflies: Jiggins et al. 2001; body size in fish: Mckinnon et al. 2004), only one locus—YELLOW UPPER (*YUP*) in *Mimulus* monkeyflowers—thus far satisfies the dual criteria of experiencing divergent natural selection and causing assortative mating (Bradshaw and Schemske 2003). Although magic traits may not be as rare as previously thought (Servedio et al. 2011), magic genes still appear too seldomly to fully explain the frequent phenomenon of by-product sexual isolation.

A third, much less appreciated mechanism that could create "incidental" sexual isolation is sexual imprinting. In many ways, sexual imprinting is arguably better at translating divergent natural selection into reproductive isolation than the above-mentioned genetic mechanisms. Sexual imprinting, the process of forming mating preferences for parental traits at a young age, is immune to genetic recombination because learned mating preferences are automatically "inherited" with a given trait locus. Several theoretical models have shown that learned mating preferences via sexual imprinting will maintain sexual isolation much longer in populations experiencing gene flow than if mating preferences had a genetic basis (Laland 1994; Verzijden et al. 2005). Additionally, sexual imprinting often lowers the amount of divergent natural selection needed to maintain reproductively isolation groups (Verzijden et al. 2005). Thus, sexual isolation can also form as a result of divergence in a trait that is subject to sexual imprinting.

Despite the early documentation of sexual imprinting as a phenomenon within species during the 1960s, its role between species has only come into focus during the last two decades

(Verzijden et al. 2012). Sexual imprinting has the potential to establish sexual isolation, maintain strong sexual isolation in the presence of gene flow (Laland 1994; Verzijden et al. 2005), generate divergence for mating preferences that are learned asymmetrically (ten Cate et al. 2006), and be reinforced in sympatry (Servedio et al. 2007), but few empirical studies have examined the role of sexual imprinting on sexual isolation (Verzijden et al. 2012). One promising study in benthic and limnetic sticklebacks showed that sexual imprinting for paternal cues under ecologically divergent selection—odor and red throat coloration—created significant sexual isolation between the two forms (Kozak et al. 2011). However, more studies are needed to answer questions about how often sexual imprinting causes sexual isolation, and whether sexual imprinting on ecologically divergent traits can instigate speciation.

For my Ph.D., I tested whether learned mating preferences cause sexual isolation in a mammalian species pair. I focused my research on two North American mice—the white-footed mouse (*Peromyscus leucopus*) and its closest relative, the cotton mouse (*Peromyscus gossypinus*)—because these sister species show behavioral isolation but few other reproductive barriers. In the laboratory, the two species can be crossed to produce viable, fertile offspring when given no choice of mate, suggesting a lack of postzygotic hybrid sterility and inviability barriers (Dice 1937). When given a choice of mates, however, the species mate assortatively (Bradshaw 1968). This species pair thus presents an ideal study system for assessing the function of mating preferences in preventing or limiting hybridization—especially in the absence of other reproductive barriers—and the potential for sexual imprinting to cause sexual isolation.

In Chapter 1, I inferred the strength of reproductive isolation from hybridization rates in natural populations of sympatric *P. gossypinus* and *P. leucopus*. I used a double-digest restriction enzyme associated DNA (ddRAD; Peterson et al. 2013) sequencing approach to generate ~3,000

single nucleotide polymorphisms (SNPs) across the genome, and found that natural hybrids are extremely rare. I then used laboratory crosses to show that backcrossing is readily possible, indicating that the observed rates of hybridization in the wild are substantially lower than expected based on no-choice behavioral assays. Because both species remained genetically distinct where they co-occur, the presence of substantial reproductive isolation is affirmed.

In Chapter 2, I examined the extent to which reproductive isolation can be created through mating preferences. I tested conspecific mating preferences in laboratory colonies of each of the two focal species and found that both species and sexes exhibited a nearly complete preference for mates of the same species. I then raised offspring from birth to weaning with foster parents of the second species and found that one species (*P. leucopus*) has genetically-determined preferences while the other species (*P. gossypinus*) learns its preferences. This research demonstrates that sexual isolation is incomplete but strong, and that preferences are formed via sexual imprinting in at least one species.

In Chapter 3, I tested whether learned mating preferences for dietary cues can create significant sexual isolation. I exposed juvenile *P. gossypinus* (which I showed to learn its mating preferences in Chapter 2) to novel dietary cues in their natal environment by feeding their parents garlic- or orange-flavored water. I predicted that offspring would prefer mates of the same flavor if sexually imprinting occurred on these divergent diets. I found that *P. gossypinus* females strongly preferred males on the same diet as their parents. The strong assortative preferences of garlic- and orange-exposed females suggest sexual imprinting on diet could generate sexual isolation even stronger than what I have detected between *P. gossypinus* and its sister species, *P. leucopus*. I did not, however, find this pattern for males: males of both treatments preferred garlic-fed females, suggesting that though this species sexually imprints,

other factors likely influence mating preferences. My results confirm that *P. gossypinus* females, and possibly males, are capable of learning dietary cues that later influence mate preference.

Together, these studies are among some of the first to establish a connection between sexual imprinting and speciation. My research suggests that learned and potentially innate mating preferences create a sexual barrier between *P. leucopus* and *P. gossypinus*, two mammalian sister species that co-exist in sympatry without hybridizing. I further showed that the mechanism for learning in one of these species—*P. gossypinus*—could generate assortative mating within a species in one generation if the cues subject to sexual imprinting diverge. My research demonstrates that divergent adaptation and sexual imprinting are capable of generating by-product sexual isolation, and that this could be a powerful force during incipient speciation.

CHAPTER 1:

Hybridization and reproductive isolation between two sympatric sister species of North American deer mice (genus *Peromyscus*)

ABSTRACT

The presence of strong reproductive barriers can be inferred when sympatric species pairs can, but do not, hybridize. We examined the degree of reproductive isolation between two co-occurring, inter-fertile species of North American mice long thought to be reproductively isolated because of mating preferences: the white-footed mouse (*Peromyscus leucopus*) and its sister species, the cotton mouse (*Peromyscus gossypinus*). Using laboratory crosses, we compared the relative success of conspecific and heterospecific mating pairs and confirmed that heterospecific pairs can produce viable offspring, but they take an average of five more days to mate compared to conspecific pairs. Additionally, we showed that F1 hybrids backcross to *P. leucopus* and *P. gossypinus* with similar latencies as conspecific pairs, suggesting that hybrids can successfully reproduce. Yet, despite the ability of *P. leucopus* and *P. gossypinus* to produce fit hybrids in captivity, we find little evidence of admixture in natural sympatric populations. We found four putative hybrids from three sympatric sites, but our genomic data indicate that the species from these sites have nonetheless maintained genetic distinctness, implying the existence of strong reproductive barriers between *P. leucopus* and *P. gossypinus*. Reproductive barriers are likely to be both pre- and post-mating. The longer latency to mating in heterospecific pairs than conspecific pairs reflects a potential mating bias even within a no-choice trial assay, and we suggest that mating preferences and propensity may contribute to some of the sexual isolation we have observed between the species.

INTRODUCTION

Mammalogists have long thought sexual isolation to be the primary reproductive barrier between two sister species of North American mice, the white-footed mouse (*P. leucopus*) and its closest relative, the cotton mouse (*P. gossypinus*) (Dice 1940b). These species can be crossed in the lab to produce viable, fertile hybrids when given no choice of mates (Dice 1937); however, when given a choice of mates, the species do not interbreed (Bradshaw 1968). The species also interact more positively with conspecifics than heterospecifics (Bradshaw, 1965). For these reasons, mammalogists have considered *P. leucopus* and *P. gossypinus* to be reproductively isolated because of mating preferences.

However, the degree to which sexual isolation prevents hybridization in natural populations is unclear. *P. leucopus* and *P. gossypinus* are sympatric throughout the southeastern United States where they breed year-round (Wolfe and Linzey 1977; Lackey et al. 1985). Both *P. leucopus* and *P. gossypinus* occupy upland and bottomland habitat in deciduous hardwood forests (McCarley 1963), potentially providing ample opportunity for hybridization. The evidence for hybridization in natural populations, though, is equivocal. In Dismal Swamp, Virginia, Dice and concluded based on morphological measurements that the species did not hybridize (Dice 1940a). By contrast, McCarley found evidence of two putative hybrids out of a sample of 400 in Louisiana (McCarley 1954).

Because the species overlap in body size and few measurements distinguish them (Dice 1940a), it is possible that morphological analyses may be inadequate to identify hybrids. A couple of studies have tested for hybridization between these species using one or a few allozyme loci, and they have also come to different conclusions: one group of researchers found a single hybrid in Southern Illinois (Barko and Feldhamer 2002) while others found no hybrid

individuals and high genetic differentiation among individuals collected across the southeastern United States (Price and Kennedy 1980). Here, we returned to the question of how much hybridization occurs between *P. leucopus* and *P. gossypinus* using a genomic approach. We collected mice from southeastern United States where the species have overlapping distributions and applied a new genotyping method, double-digest restriction-associated DNA (ddRAD) sequencing, to answer: (1) how genetically differentiated are *P. leucopus* and *P. gossypinus*, and (2) how much do the species hybridize in nature, given that they can cross in the lab and produce viable, fertile offspring? We also compared the mating success of conspecific, heterospecific, and backcross mating pairs to determine if heterospecific pairs and backcross mice suffered a disadvantage compared to conspecific mating pairs between species. Our laboratory crosses and genomic analyses showed that hybrids can be formed and backcross to both species, but that a lack of admixture and the preservation of genetic differentiation in sympatry indicates the presence of strong reproductive isolation between *P. leucopus* and *P. gossypinus.*

METHODS

Detection of hybrids

Sampling locations

During April 2008 and January-February of 2010 and 2011, we collected mice and from nine allopatric and 14 sympatric locations. We trapped mice with live Sherman traps and collected liver or tail tissue that we stored in 100% ethanol for DNA extraction. We augmented our sampling with tissues from specimens collected from additional allopatric and sympatric sites loaned from the Harvard Museum of Comparative Zoology, Oklahoma Museum of Natural History, San Noble Museum of Natural History, and the Museum of Texas Tech University.

ddRADseq library construction and genotyping

We extracted genomic DNA from 376 individuals using an Autogen kit and AutoGenprep 965 instrument. We prepared ddRAD tags for sequencing from each individual following the protocol described in Peterson et al. (2012). Briefly, we digested 100-200 ng of DNA from each individual with two restriction enzymes, EcoRI-HF and MspI (New England Biolabs) and purified the reactions with an Agencourt AMPure XP purification system (Beckman Coulter Genomics). Next, we quantified the cleaned, digested product on a spectrophotometer plate reader (SpectraMax Gemini XS Plate Reader) and ligated at least 50 ng of digested DNA to uniquely barcoded P1 adapters (EcoRI cut site specific) and P2 adapters (MspI cut site specific) in a 40 µl reaction volume with T4 DNA ligase (New England Biolabs) following the New England Biolabs ligation protocol. We pooled equal amounts of 48 ligated samples and used two rounds of AMPure XP purification to reduce the total pooled volume of to 30 µl. We loaded each ligation pool onto a 2% agarose Pippin Prep cassette (Sage Science) and selected fragments with a mean size of 300 ± 35 bp. We then ran five replicate Phusion PCRs according to the Finnizyme kit directions (Thermo Scientific) using 5 µl of eluted Pippin Prep product as template for 12 cycles of PCR. Each PCR was indexed using a unique reverse primer (primer and index sequences from Peterson et al., 2012). Following PCR, we pooled all replicate reactions and used a single AMPure XP purification step to concentrate each ddRAD library. We multiplexed ddRAD libraries in equimolar ratios and sequenced 50 bp single reads on an Illumina Genome Analzyer II or HiSeq (2000 or 2500).

We demultiplexed and aligned reads by sample to the draft genome sequence of *Peromyscus maniculatus* (NCBI: GCA_000500345.1) with STAMPY run in hybrid mode using the BWA mem algorithm (Lunter and Goodson 2011) with default parameters. We identified and

removed adapter sequences with Picard-tool 1.100 (http://picard.sourceforge.net). We realigned potential indels with the Genome Analysis Tool Kit (GATK) (McKenna et al. 2010) and performed SNP discovery across all samples simultaneously with the Unified Genotyper (DePristo et al. 2011). We filtered ddRAD alignments, keeping regions with 100 or more total reads and an average base quality of 20 or higher. We retained bi-allelic sites with a minimum mapping quality of 30 that were present in at least 90% of our individuals at a depth of 4 or greater. To reduce linkage among SNPs in our dataset, we identified "clusters" of SNPs within 100 bp of each other and more than 100 bp from another SNP; we randomly selected one SNP per cluster. Our final dataset contained 2,864 SNPs genotyped in 376 mice: 22 allopatric or lab *P. leucopus*, 47 allopatric or lab *P. gossypinus*, 2 known lab hybrids (one F1 and one *P. gossypinus* backcross hybrid), and 305 mice of unknown identity from sympatry.

Identification of hybrids

Our ddRAD dataset had missing genotypes (Figure 1.1), ranging from 13 to 2863 (out of 2864 sites) with a median of 299 missing genotypes. This amount of missing data is not unexpected, as our samples were collected during three field trips and sequenced with the best available Illumina sequencing technology (Genome Analyzer II, HiSeq 2000 and 2500) to ensure the most

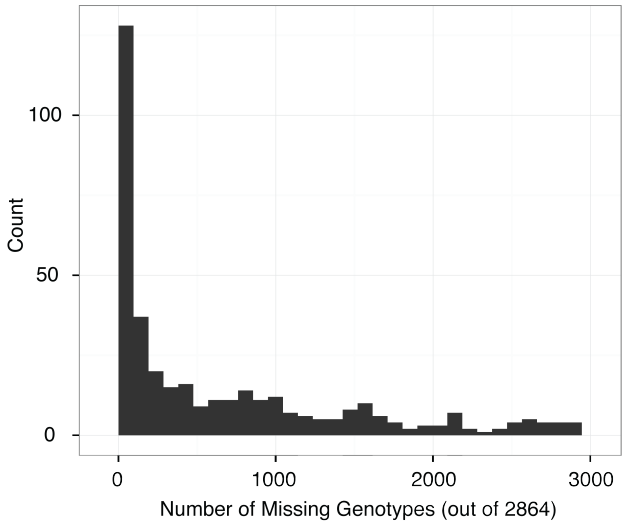

Figure 1.1. Number of missing genotypes (out of 2684 sites) in our ddRAD dataset.

sequencing reads were captured. However, this does introduce the possibility that our ability to identify hybrids may be affected by missing data. We addressed this possibility by including all individuals in a Bayesian model-based clustering analysis (implemented in STRUCTURE v. 2.3.1) and using a distance metric, Kullback-Leibler divergence (hereafter referred to as "KL divergence"; Kullback and Leibler 1951), to identify putative hybrids with near complete genotyping.

We first estimated the optimal number of genetic clusters in our allopatric and sympatric mice with STRUCTURE. We ran five independent, randomly initiated Markov chain Monte Carlo (MCMC) runs for each K (range 1-8) with a burn-in period of 50,000 iterations followed by a sampling period of 50,000 iterations. We tested admixture models with correlated allele frequencies and a migration prior of 0.05, but did not include location information in our estimation of clusters. All other parameters were set to default values. We used the ΔK method in Structure Harvester to determine the optimal number of clusters (Evanno et al. 2005; Earl and VonHoldt 2011), and then used CLUMPP v. 1.1.2 (Jakobsson and Rosenberg 2007) to combine data from the five independent runs and DISTRUCT v. 1.1. to visualize our results (Rosenberg 2004).

Afterward, we identified putative hybrids using ancestry proportions and KL divergence to remove individuals that may appear admixed because of missing data. We detected two genetic clusters within our data (see Results) and assigned individuals to these clusters based on their maximum ancestry proportion. We considered individuals to be putative hybrids if they: (1) had ancestry proportions belonging to both clusters, and (2) the proportion of ancestry for the second cluster had a 95% Highest Posterior Density (HPD) interval that did not overlap zero. We then calculated KL divergence for all individuals. KL divergence is a non-symmetric statistic

that quantifies the distance between two probability distributions—in this case, weighting each genotype by the difference in allele frequencies between the two genetic clusters identified by STRUCTURE. We calculated the sum of KL divergence in both directions (from cluster 1 to cluster 2, and cluster 2 to cluster 1) to estimate a symmetric KL divergence estimate for each individual. KL divergence ranges from 0 (no informative genotypes) to 1 (no missing data at informative positions), with the scale between these points representing the proportion of information, relative to a sample with no missing data, available to distinguish cluster membership based on allele frequencies.

Genomic differentiation between *P. leucopus* and *P. gossypinus*

We estimated F_{ST} between the species with mice sampled from a single site, Leflore, Oklahoma. We used this population because it had large numbers of individuals from both species known *a priori* from morphological data (28 *P. gossypinus*, 19 *P. leucopus*). We estimated F_{ST} with the Weir & Cockerham method (Weir and Cockerham 1984) implemented in VCFTOOLS (Danecek et al. 2011).

Mating success comparison among conspecific, heterospecific, and backcross mating pairs

To evaluate if mating between species or with hybrids was less successful than mating within species, we compared the: (1) proportion of mating successes and (2) average latency to successful mating among conspecific, heterospecific, and backcross mating pairs. We obtained outbred *P. leucopus* animals from the *Peromyscus* Genetic Stock Center (University of South Carolina), and derived a breeding colony of *P. gossypinus* animals from *P. gossypinus* trapped from Washington and Jackson counties in Florida during 2009. We set up no-choice trials with

all four combinations of conspecific and heterospecific mating pairs (L♀ x L♂, L♀ x G♂, G♀ x L♂, G♀ x G♂) and each type of backcross mating pair (L♀ x F1 hybrid ♂, G♀ x F1 hybrid ♂, F1 hybrid ♀ x L♂, F1 hybrid ♀ x G♂) by adding a sexually receptive virgin female to the cage of a virgin, sexually mature male. We determined female sexual receptivity through vaginal lavage and considered a female to be receptive between proestrus and estrus. The "F1" category in backcross mating pairs represents offspring from both reciprocal crosses (L♀ x G♂ and G♀ x L♂); we pooled F1 hybrids from both crosses because we did not have enough trials to analyze backcross mating success (defined as the presence or absence of a litter) by type of F1 hybrid. We set up approximately 30 conspecific and heterospecific crosses and 10 of each type of hybrid backcross. We allowed pairs 60 days to produce a litter, which is approximately 12 estrous cycles (mean estrous cycle length for both species is 5-6 days; Dewsbury et al. 1977) and recorded: (1) whether the pair produced offspring, and (2) if so, the number of days to parturition. Because we paired females when they were sexually receptive, we estimated the latency to successful mating by subtracting the average gestation period for both species (23 days; Lackey, Huckaby, Ormiston, James, and Lackey, 1985; Wolfe and Linzey, 1977) from the number of days it took each pair to give birth to a litter.

We compared proportion of mating successes by calculating the proportion of conspecific and heterospecific pairs that produced offspring and calculated the 95% HPD intervals for this estimate assuming a Beta(0.1,0.1) prior distribution for the proportion. We evaluated whether the success of a mating pair (i.e. the presence or absence of offspring within 60 days of pairing) was determined by the species of the female, the species of the male, or the combination of female and male species (a significant interaction term would indicate assortative or disassortative mating) using logistic regression and a backward stepwise AIC algorithm to select a minimal

model from our full model. Limiting our analysis to pairs that produced offspring, we also tested whether heterospecific and backcross pairs took longer to mate (i.e. had greater latency to mating) than conspecific pairs using Kruskal-Wallis non-parametric analysis of variance and Wilcoxon rank sum tests with a Bonferroni correction for multiple pairwise testing.

RESULTS

Detection of hybrids

We identified $K = 2$ as the most likely number of clusters in our samples collected from allopatry and sympatry (Figure 1.2a). This matches our expectation of two species with hierarchical clustering within each species. We verified that the two clusters detected in our data corresponded to *P. leucopus* and *P. gossypinus* in a known sample of lab-reared animals of mice. Allopatric and lab individuals of *P. leucopus* and *P. gossypinus* were assigned to discrete clusters (Figure 1.2b); thus, we considered these clusters to represent each species.

We validated our method of identifying hybrids with a known F1 hybrid and backcross mouse from our laboratory colonies. The F1 hybrid and *P. leucopus* backcrossed mouse had *P. leucopus* ancestry coefficients of 0.45 and 0.24, respectively ("Lab" in Figure 1.2b). Additionally, we had one sample that was contaminated with DNA from both species during DNA extraction; we called this sample a "fake hybrid", and confirmed that STRUCTURE correctly identified this mouse as admixed (not shown in Figure 1.2b). These verifications confirm that we have correctly identified two groups within our data, one corresponding to *P. leucopus* and the other to *P. gossypinus*, and that known hybrids are correctly identified as admixed.

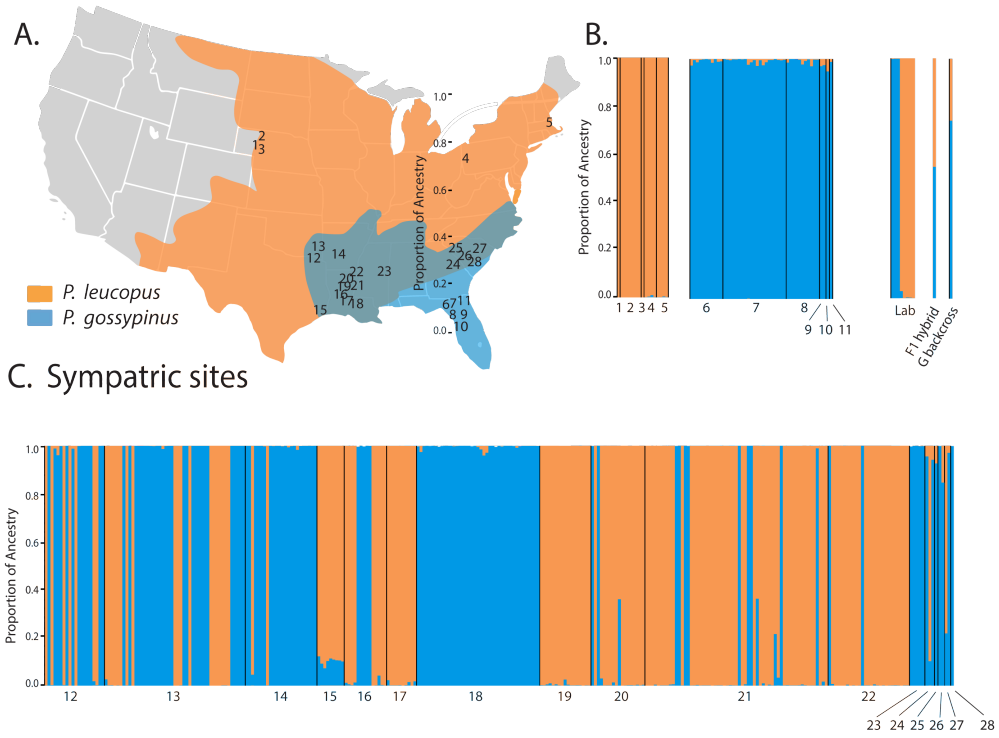

Figure 1.2. Sampling locations and ancestry proportions for 376 mice in our study. (a) Sampling localities with numbers representing their approximate location, shifted to be visible on our map. (b) Ancestry proportions for allopatric *P. leucopus* (orange), allopatric *P. gossypinus* (blue), lab-reared *P. leucopus* and *P. gossypinus*, and an F1 hybrid and *P. gossypinus* backcross mouse ("lab" and hybrids, far right). Each bar represents an individual and its proportion of ancestry belonging to each species. (c) Ancestry proportions for sympatric mice collected from the purple region in panel a. Sites 24 and 27 each had one putative hybrid and site 21 had two putative hybrids with a KL divergence >= 0.5.

We then identified putative hybrids that had ancestry from both species and 95% HPD intervals that did not overlap a zero value. In other words, we looked for mice that were unlikely to be pure *P. leucopus* or *P. gossypinus*. (All of our allopatric or pure lab-reared mice had 95% HPD intervals that overlapped with zero). 13 putative hybrids were identified in addition to our known set of known lab hybrids.

To evaluate if any of the 13 hybrids are likely to be the result of incomplete genotyping, we calculated KL divergence for each individual in our ddRAD dataset. We discovered that many of the putative hybrids had low KL divergence (e.g. KL divergence < 0.50; Figure 1.3) caused by both few genotypes and/or genotypes at uninformative sites that did not differentiate

the species. As expected, with low information it is difficult to definitely assign an individual to either cluster, and so individuals with few genotypes can appear as hybrids (a reflection of the prior assumption of no information). Putative hybrids with high KL divergence are likely real hybrids, as they have much more information than many individuals that were confidently assigned to either group. If we consider individuals above a KL divergence of 0.5 or greater to be real hybrids, then we have found five out of 247 sympatric mice that show some admixture. Of these five, one was from a site in eastern Texas (Figure 1.2a, site 15) where other individuals were identified as putative hybrids but had low KL divergence scores (Figure 1.3, red dots on bottom right). Thus, although we identified one putative hybrid from this Texas population, we exclude it because other individuals with similar ancestry proportions have missing data and/or genotyping at fewer informative sites. Interestingly, the four remaining hybrids all have larger proportions of *P.*

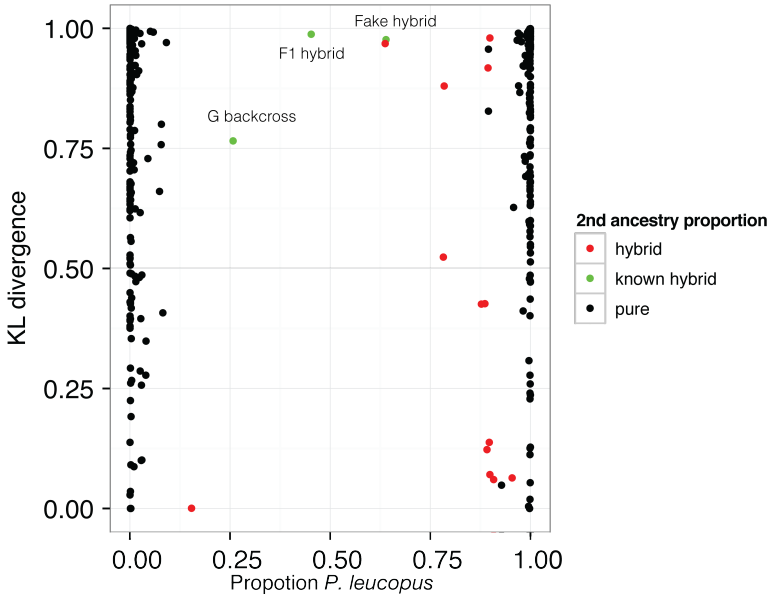

Figure 1.3. KL divergence by proportion of *P. leucopus* ancestry for pure individuals, known hybrids, and putative hybrids. Individuals on the left are *P. gossypinus*, and individuals on the right are *P. leucopus*. Each dot represents a sample. Black dots represent individuals with 2nd ancestry proportions overlapping zero, red dots represent putative hybrids based on their non-zero 2nd ancestry proportions, and green dots represent known hybrid individuals from the lab and a "fake hybrid" with contaminated DNA. We considered red dots above a KL divergence threshold of 0.5 to be real hybrids; red dots below this threshold have insufficient data and are less likely to be true hybrids.

leucopus ancestry than *P. gossypinus* ancestry indicating that hybrids backcross more often to *P. leucopus*.

Although our ability to detect admixture beyond six generations of admixture is limited, we can at least conclude that there is a paucity of recent hybrids in sympatry. Our hybrid analysis identified seven out of 16 sympatric sites with both species (Figure 1.2c). When the two species co-occurred, there was variation in the amount of hybridization present. For example, site 21 (Figure 1.2c) had two hybrids while the majority of two-species sampling sites did not have any hybrid individuals. A patchy distribution of the species and hybrids among sampling locations indicates that *P. leucopus* and *P. gossypinus* have a mosaic hybrid zone.

Genetic differentiation

We estimated mean F_{ST} between the two species sampled from Leflore, Oklahoma to be 0.218 (Weir and Cockerham weighted estimate = 0.550).

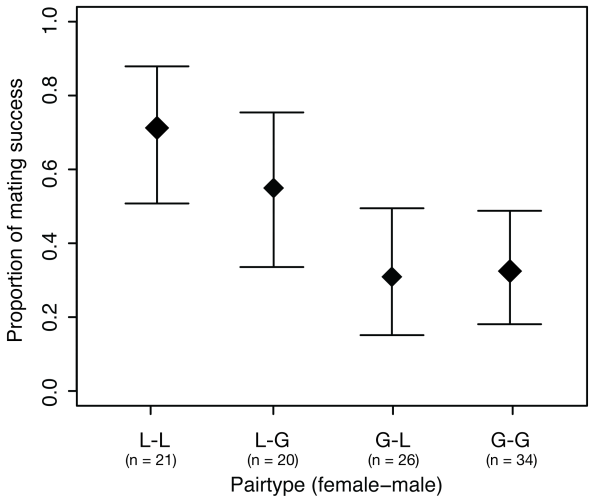

Figure 1.4. Proportion of pairs that produced offspring in a 60-day no-choice trial (diamond symbol) with 95% highest posterior density intervals. Number of pairs tested for each type are listed in parentheses.

Female species determines mating success

We used logistic regression to test whether mating success was predicted by the species of the female, species of the male, or the interaction between them. Using a backward stepwise AIC algorithm, we found only one significant term: female species (estimate = 1.4588, standard error = 0.4140, $Z = 3.524$, $p = 0.0004$). The proportion of

mating success—defined as the presence or absence of offspring—differed between pairs. We found that pairs with *P. leucopus* females had a higher rate of mating success than pairs with *P. gossypinus* females (Figure 1.4). Surprisingly, this meant that *P. gossypinus* males sired more offspring with *P. leucopus* females than females of their own species. This suggests that the female species determines whether or not a mating pair will produce offspring. The failure to detect a significant interaction between female species*male species indicates that we did not detect significant assortative or disassortative mating between species in our no-choice trials.

Lower latency of heterospecific pairs compared to conspecific and backcross pairs

Limiting our analysis to the pairs that produced offspring, we also compared the inferred latency to successful mating among the three categories of pairs: conspecific, heterospecific, and backcross. Not all pair types mated as quickly (Figure 1.5; Kruskal-Wallis test, X^2= 7.084, df = 2, p = 0.0290). Pairwise comparisons between the groups revealed that heterospecific pairs took an average of 5 days longer to produce a litter than conspecific pairs (two-tailed Wilcoxon rank sum test, p = 0.0030; significant after Bonferroni correction), but that backcross pairs were

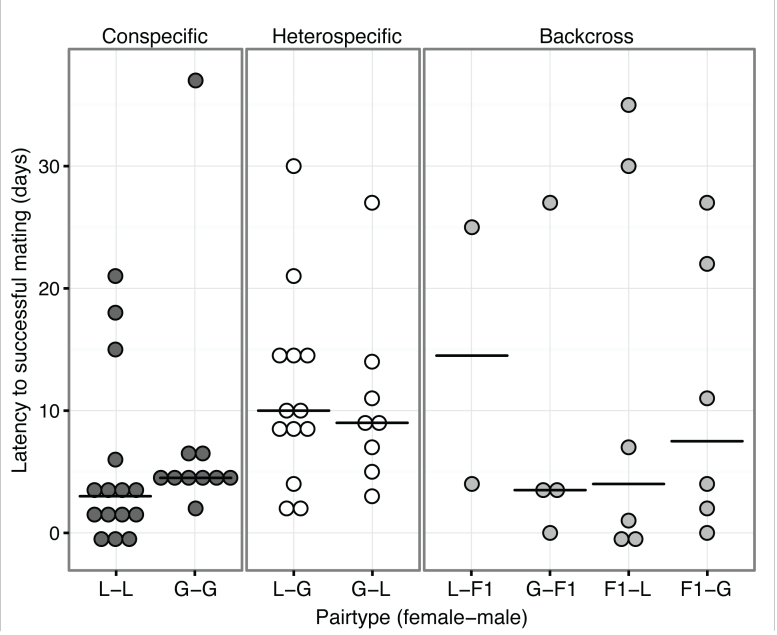

Figure 1.5. Inferred latency to successful mating for conspecific, heterospecific, and backcross pairs in 60-day no-choice trials. Trials are represented by dots, with lines showing median values for each pair type.

not significantly different from either conspecific (two-tailed Wilcoxon rank sum test, p = 0.5357) or heterospecific latencies (two-tailed Wilcoxon rank sum test, p = 0.2963).

DISCUSSION

We revisited the long-standing question of whether *P. leucopus* and its sister species, *P. gossypinus*, hybridize using genomic data. Our analysis corroborates both McCarley's and Dice's conclusions about hybridization: we found some populations with hybrids and others without. We estimated that ~1.5% of sympatric individuals are hybrids (4/247 sympatric individuals with KL divergence >= 0.5), but these hybrids were limited to three of the 16 sympatric sites we sampled. Other sympatric sites, particularly those in Oklahoma, had no hybrids. Thus, our study, in combination with previous genetic studies (e.g. Price and Kennedy 1980; Robbins et al. 1985; Barko and Feldhamer 2002), confirms that the species are patchily distribution hybridization is varied but generally rare. We conclude that *P. leucopus* and *P. gossypinus* have mosaic hybrid zone across the southeastern United States.

Although rare, hybrids could cause gene flow if they backcrossed to the parental species. We did not have enough trials to compare the mating successes and failures for each type of backcross mating pairs, but we were able to estimate the latency to mating for all backcross pairs combined. We found F1 hybrids mated as quickly as conspecific pairs, suggesting that backcrosses are not disadvantaged in terms of latency to fertilization (at least in the lab). F1 hybrids have also been reported to have similar copulatory behavior to both *P. leucopus* and *P. gossypinus* (Lovecky et al. 1979). Together, these results suggest that hybrids are capable of backcrossing.

Our genomic analysis of sympatric mice also supports this conclusion. We identified four putative hybrids that all had larger proportions of *P. leucopus* ancestry, indicating that backcrossing has occurred and was biased toward *P. leucopus*. We suspect that *P. leucopus* females may facilitate backcrossing because we found them to appear less choosy and/or more fertile than *P. gossypinus* females. It is possible this result is an artifact of our laboratory colonies: the *P. leucopus* in our experiment come from the *Peromyscus* Genetic Stock Center where they have bred in captivity for nearly 40 generations, during which they could have experienced laboratory selection for low choosiness and high fecundity. By contrast, we established our breeding colony of *P. gossypinus* in 2009 and have only bred them for 3-4 generations, thus giving them less opportunity for laboratory selection. However, other researchers have also found differences in the fecundity of these species indicating that these differences could be real (Bradshaw 1968). If indeed *P. leucopus* females are less discriminating or more fertile than *P. gossypinus*, our no-choice trials lead us to predict that hybrids may reproduce more successfully with *P. leucopus* females. We had too few L♀ x F1 hybrid ♂ trials to confirm this prediction, but future studies should test whether introgression from *P. gossypinus* into *P. leucopus* is facilitated through female *P. leucopus*.

Despite some apparent introgression from *P. gossypinus* into *P. leucopus*, we were surprised to find that the species were very genetically distinct ($F_{ST} = 0.550$). The persistence of two distinct genetic clusters among sympatric mice suggests the presence of significant and strong reproductive isolation between the species. We speculate that several plausible premating (habitat or sexual) and postmating reproductive barriers (sexual selection against hybrids, or reduced fertility) may account for the lack of admixture we have observed between the species. We describe the potential for these barriers below.

Although both species can use the same habitats, *P. leucopus* and *P. gossypinus* have been trapped in different habitats within the same site (Calhoun 1941) suggesting that they may either prefer or use different habitats whens sympatric. A capture and release study suggests that habitat segregation in sympatry might be a consequence of competitive displacement. McCarley (1963) released mice onto an experimental plot and found that *P. leucopus* initially occupied upland habitat and *P. gossypinus* occupied bottomland habitat. Over the course of a three years, however, *P. gossypinus* had moved into upland habitat appearing to displace *P. leucopus* (McCarley 1963). As *P. gossypinus* has been to be more shown to be more aggressive and dominating in interactions with *P. leucopus*, aggression and competition might create habitat separation reducing the probability of coexistence, and thus also the probability of interbreeding.

Sexual isolation may also prevent hybridization. We found heterospecific mating pairs (L♀ x G♂ and G♀ x L♂) took an average of five days to mate successfully in no-choice trials, which is significantly longer than conspecific and backcross pairs. A delay of five days is equivalent to one estrous cycle in females, and thus may be meaningful in natural populations where the mice are not confined together. If this delay was caused by strong conspecific mating preferences, sexual isolation might be an important barrier as mammalogists have long thought. In Chapter 2, we investigate whether the species strongly prefer conspecific mates when a choice is provided.

A five-day latency might also reflect early mating but delayed fertilization. Sperm competition is likely in these mice because females are thought to be polyandrous: multiple paternity litters have been found in *P. leucopus* (Xia and Millar 1991) and *P. gossypinus* does not form stable pair bonds, indicative of a promiscuous mating system (McCarley 1959). Thus, if conspecific male sperm outcompetes heterospecific male sperm for fertilization, hybrid mating

may occur but rarely result in offspring. This reproductive barrier, known as conspecific sperm precedence, has been detected between in several species (e.g. house mice: Dean and Nachman 2009; fruit flies: Price 1997; beetles: Matsubayashi and Katakura 2009). No studies have yet tested for conspecific sperm precedence in *P. leucopus* and *P. gossypinus*, but differences in fertilization rate could explain the presence of hybrids but rare evidence for admixture between *P. leucopus* and *P. gossypinus*.

Finally, postmating barriers may prevent hybrids from backcrossing more often. Although F1 and F2 hybrids are fertile (Dice 1937), hybrids may be unfit in other ways. First, hybrids may be unattractive. This pattern has been found in several species, suggesting that hybrids with intermediate mating signals might be disadvantaged compared to the pure species (e.g. sticklebacks: Vamosi et al. 1999; butterflies: Naisbit et al. 2001; flycatchers: Svedin et al. 2008). Additionally, hybrids might be fertile in no-choice, non-competitive lab crosses but have reduced fertility relative to the male *P. leucopus* or *P gossypinus*. A survey of house mice hybrids found that sperm quality was variable throughout a hybrid zone, suggesting that hybrid male subfertility could reduce fertilization success in competitive contexts (Turner et al. 2011). Such postmating reproductive barriers could resolve the presence of hybrids yet the absence of more introgression between the species.

In summary, we have inferred a paradoxical pattern: we have found *P. leucopus* and *P. gossypinus* will hybridize but they remain genetically distinct in sympatry. Their patchy distribution certainly reduces opportunity for hybridization, but we expect other reproductive barriers such as sexual isolation, sperm precedence, sexual selection against hybrids, or weakened fertility may additionally reduce hybridization and introgression between the species. The barriers acting to reduce hybridization should be investigated. Species pairs like *P. leucopus*

and *P. gossypinus* that can, but do not, hybridize will continue to prove extremely useful for studying the evolution of reproductive barriers. These systems will allow researchers to study reproductive barriers in the absence of others and improve the ability to identify which reproductive barriers tend to evolve first.

CHAPTER 2:

Sexual isolation via sexual imprinting between *P. leucopus* & *P. gossypinus*

ABSTRACT

Sexual imprinting, the process of learning parental cues at a young age that are later used in mate choice, is thought to promote speciation by forming an association between traits and preferences for them. This association can generate assortative mating that contributes to or results in sexual isolation between incipient species. Here, we quantified sexual isolation between two sympatric sister species of mice—the white-footed mouse (*Peromyscus leucopus*) and its closest relative, the cotton mouse (*P. gossypinus*)—and tested whether sexual imprinting contributes to reproductive isolation. We used a novel electronically-gated apparatus to test mating preferences in two-way choice trials, and showed that both species and sexes preferred conspecific to heterospecifc mates, creating incomplete but strong reproductive isolation. We then tested the preferences of individuals that were cross-fostered to parents of the opposite species and surprisingly found that *P. gossypinus* reversed their mating preferences when raised by heterospecific parents while *P. leucopus* did not. Video analysis of the behavioral interactions between conspecifics and heterospecifics during these choice trials revealed that mice interacted more positively with their preferred stimulus mouse and did not appear influenced by negative interactions with the stimulus mice. Together, our results indicate that *P. leucopus* and *P. gossypinus* have strong mating preferences between them and that learning contributes to their sexual isolation. This study shows that sexual imprinting in species that learn their mating preferences can generate significant behavioral isolation and supports a role for sexual imprinting in speciation.

INTRODUCTION

Sexual isolation is thought to be an important reproductive barrier caused by divergent mate preferences, but how it evolves remains unclear. Comparative studies suggest that sexual isolation can arise quickly compared to other intrinsic postzygotic barriers (Coyne and Orr 1989; Gleason and Ritchie 1998; Mendelson 2003), and thus may be integral to the early stages of speciation. Importantly, sexual isolation has been observed more often in sympatric species pairs than allopatric pairs (Coyne and Orr 1989); in some extreme cases, it appears to be the primary reproductive barrier preserving species boundaries of sympatric species (e.g. cichlids: Seehausen 1997; swordtails: Fisher et al. 2006, fruit flies: Doi et al. 2001). Thus, elevated sexual isolation in sympatry may indicate that this reproductive barrier may be important, or even required, for species to coexist. However, the forces that cause sexual isolation to evolve quickly and act strongly in sympatry are not well understood.

One mechanism that can generate sexual isolation is sexual imprinting. Sexual imprinting, the process of learning parental cues at a young age that are used later to select mates, can cause associations between traits and preferences that can arise instantaneously. This process has been demonstrated to create associations so rapidly that, should a novel trait arise in a population, learned preferences could allow the trait to persist. For example, studies with finches and mannikins suggest that novel colored crest feathers introduced into a population were sexually imprinted and preferred during mate selection within a single generation (Witte et al. 2000; Witte and Sawka 2003). Because sexual isolation creates powerful associations between traits and preferences, if sexually imprinted traits—whether visual, auditory, or olfactory—were divergent between populations, sexually imprinted preferences for these traits could yield sexual isolation as a by-product (e.g. Kozak et al. 2011).

The tight associations between trait and preferences that sexual imprinting creates also allows sexual imprinting to maintain sexual isolation in the presence of gene flow (Verzijden et al. 2005). When sexually imprinting species hybridize, they can maintain sexual isolation because traits and preferences for them are automatically coupled (no genetic linkage is required). By contrast, when species with genetically determined mating preferences hybridize, recombination can erode linkage between a trait locus and a preference locus that together cause sexual isolation. Models of parental sexual imprinting have been shown to maintain stable associations between traits and preferences permitting significant sexual isolation to persist under scenarios of gene flow (Verzijden et al. 2005).

Despite the theoretical impact of sexual imprinting on speciation, studies rarely test the role of sexual imprinting in sexual isolation. Sexual imprinting has been documented in more than 100 taxa across at least 30 orders in birds, mammals, fish, and insects—but few of these empirical studies have explored the connection of sexual imprinting to speciation (Verzijden et al. 2012; but see Kozak et al. 2011). Here, we assess the strength of sexual isolation and test for sexual imprinting in two species of sympatric North American mice: the white-footed mouse (*Peromyscus leucopus*) and its closest relative, the cotton mouse (*P. gossypinus*). Previous studies suggested that these species hybridize successfully in the lab when given no choice of mates (Chapter 1; Dice 1937), but that they mate with conspecifics when given a choice of multiple mates (Bradshaw 1968). We aimed to quantify the strength of sexual isolation between these mice and test if learning contributes to this reproductive barrier.

METHODS

We quantified conspecific mating preferences in laboratory strains of each species. We obtained outbred *P. leucopus* animals from the *Peromyscus* Genetic Stock Center (University of South Carolina), and derived a breeding colony of *P. gossypinus* animals from *P. gossypinus* trapped from Washington and Jackson counties in Florida during 2009.

To quantify mating preferences, we developed an electronically-controlled gated mate-choice assay that reliably indicates a mouse's mating preference. We then applied this assay to test mating preferences of pure *P. leucopus* and *P. gossypinus* and determine if their preferences established are strong enough to create sexual isolation. Finally, we used cross-fostering within and between species to assess to what degree mating preferences in these species are formed through sexual imprinting on parents. We analyzed video data from all trials to confirm that behavior of the two stimulus mice did not strongly influence the mate preference of the chooser mouse.

Does association time reflect mating preference?

We measured the strength of conspecific mating preferences in a two-way, electronically-controlled, gated mate choice apparatus that consisted of three collinear rat cages, each pair of cages separated by two RFID antennae and gates (FBI Science Gmbh; Figure 2.1). Each pair of gates separating chambers was programed to allow passage depending on the identity of the mouse. Specifically, for each trial we implanted three mice with small

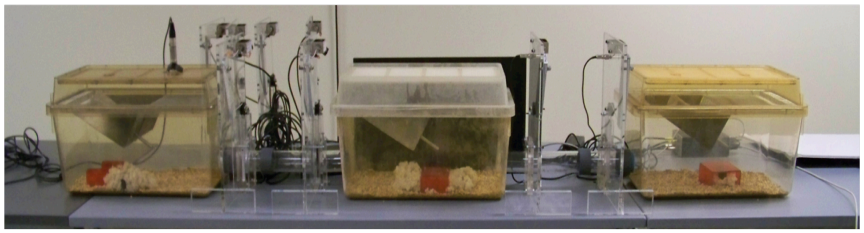

Figure 2.1. Photograph of the electronically-controlled gated mate choice apparatus.

transponders (1.4 mm x 9 mm, ISO FDX-B, Planet ID Gmbh) in the interscapular region using a sterile hypodermic implanter and then programmed the gates to allow the designated "chooser" mouse (i.e. the individual whose preference we tested) to pass freely through all three cages while constraining each "stimulus" mouse to the left or right cage, respectively.

For each trial, we tested chooser preferences for opposite sex *P. leucopus* and *P. gossypinus* stimulus mice. We allowed the chooser mouse—either a sexually receptive virgin female (in proestrus or estrus as determined by vaginal lavage) or a sexually mature virgin male—to acclimate to the apparatus for a day, adding food, water, and used nesting material and a hut from each stimulus mouse's colony housing cage to the flanking cages of the apparatus. At approximately 1:00 pm the following day, we returned the chooser mouse to the center cage (if it had not already nested there), closed all gates, and added stimulus mice to the flanking cages to give them two to four hours to acclimate to their new environment. At lights out (4:00 pm; 16:8 hours light:dark cycle), we re-opened the gates and recorded RFID readings at all antennae as well as webcam video streams from each flanking cage for two nights (~44 hours) (camera model: DLINK DCS-942L). Each chooser mouse was tested once.

At the end of each trial, we parsed a log file of RFID readings and calculated preference for a stimulus as the proportion of time spent with that stimulus divided by the time spent with both stimuli (i.e. a ratio of association time between the stimuli). We analyzed only trials in which the chooser mouse investigated both cages during the acclimation, spent at least 10 minutes investigating stimulus mice during the trial, and where the stimulus mice were in their cages at least 75% of the trial period (in 11% of trials, at least one stimulus mouse escaped).

To confirm that our assay accurately measures mating preference, we performed two additional tests. First, we tested whether we quantified a side bias in our choice apparatus (i.e. for

the left or right flanking cage) or a preference for the stimulus and its cage. In a subset of trials ($N = 8$), we swapped the positions of the stimulus mice and their cages half way through the two-day trial period. We predicted that if a chooser preferred a particular stimulus or its cage, the chooser would track its preferred stimulus and switch which side it most frequently visited. We used a one-tailed binomial test to assess whether the chooser mouse continued to prefer the same individual on day 2 after we switched stimuli positions, assuming equally likely preferences for either side as a null model. Second, we tested whether our assumed metric of preference—the proportion of association time with a stimulus mouse—indeed reflects a mating preference. We identified trials with successful mating events by the presence of sperm in a female reproductive tract at the end of a trial or the birth of a litter three weeks later. If a female choice trial resulted in offspring, we determined the identity of the father by genotyping both male stimuli and the pups at two to three microsatellite markers (locus 14, 35, and 80 from Weber et al. 2010) following the protocol described in Weber et al. 2010 ($N = 15$ trials) or watching video data for copulation events (one trial). In choice trials in which the chooser was male, no genotyping was necessary because the father's identity was known. We combined data from 16 trials with successful mating events and tested whether the mate was the most preferred individual (as determined by the greatest proportion of association time) using a two-tailed binomial test under the random expectation that each stimulus had an equal chance of being preferred. These two tests allowed us to rule out side bias in our apparatus and determine that association time is an accurate predictor of mating preference.

Do mice of each species prefer conspecific mates?

We measured the preferences of 10-15 adults of each species and sex for conspecific and heterospecific stimulus mice of the opposite sex. We tested chooser mice at 9-14 weeks of age. To quantify the preferences of each species and sex, we tested virgin female preferences using either: (1) pairs of sexually experienced males that had successfully sired offspring with a conspecific female prior to use in the two-way choice trials (*P. leucopus*, $N = 8$ trials; *P. gossypinus*, $N = 6$ trials), or (2) pairs of virgin males as stimuli (*P. leucopus*, $N = 7$ trials; *P. gossypinus*, $N = 7$ trials). In male choice trials, we used only virgin females as stimuli. We did not detect a difference between female preferences from trials with experienced males or virgin males as stimuli (Wilcoxon rank sum test, *P. gossypinus* females: $p = 0.4634$; *P. leucopus* females: $p = 0.4634$); we therefore combined female preference data from trials with sexually experienced and virgin male stimuli. We compared the mating preferences of both species and sexes using Wilcoxon rank sum tests.

It is important to note that because of the structure of the breeding colony used for the experiments, some of the tested mice were siblings (we tested an average of 1.79 chooser mice per breeding pair). To control for any possible family effects, we verified that all results reported as statistically significant were still significant ($p < 0.05$) when comparing the Wilcoxon rank sum U statistic to 10,000 random permutations that reassigned sibling mice to different treatments as a group (in contrast to the standard Wilcoxon rank sum test, in which the permutations could assign different siblings to different groups).

Are the species sexually isolated?

To measure the strength of sexual isolation between the species, we used a joint isolation index, I_{PSI} (Rolán-Alvarez and Caballero 2000). This index compares observed and expected mating pairs (assuming random mating among individuals) among the four possible pair types (L♀ x L♂, L♀ x G♂, G♀ x L♂, and G♀ x G♂, in which "L" represents *P. leucopus* and "G" represents *P. gossypinus*) and reflects the amount of interbreeding between two groups or species. The index ranges from -1 (all pairing occurs between species) to +1 (all pairing is within species), with a value of 0 indicating equal pairing among pair types. Although we measured association time in our trials, we considered a chooser mouse's most preferred individual to be a "mate" because association time predicts mating in our assay. We counted the number of conspecific and heterospecific mates preferred for each species and estimated I_{PSI} for each sex separately in JMATING v. 1.0.8 (Carvajal-Rodriguez and Rolán-Alvarez 2006). We calculated separate isolation indices for each sex because it is possible that female stimuli behaved differently from male stimuli in our trials. We also tested for asymmetry in observed putative mating pairs (IA_{PSI}), which would occur if one species or sex strongly preferred conspecific mates but the other did not. We used 10,000 bootstrap replicates to estimate the isolation indices, their standard deviation, and to test the hypothesis that our estimates of isolation deviated significantly from zero.

Are mating preferences learned?

To test whether mating preferences could be learned in the nest, we measured the preferences of mice from each species after they had been cross-fostered—raised from birth until weaning—to parents of the opposite species. We swapped whole litters at birth between breeding

pairs of *P. leucopus* and *P. gossypinus*, reducing litters to the same number of offspring if litters differed in number of pups. All cross-fostering attempts were successful, indicating that parents readily attended to unrelated offspring. We allowed cross-fostered offspring to remain with their foster parents until weaning (23 days old), when we separated offspring into same sex cages; this matches the life cycle of all other mice in our study. As a control, we also cross-fostered offspring within species (i.e. swapped litters between conspecific families) to partition the effects of litter transfer and foster parent species on mating preference. Although there is mixed (or incomplete) information for whether fathers contribute parental care in *P. leucopus* and *P. gossypinus* (Hartung and Dewsbury 1979; Schug et al. 1992), we maintain stable male-female breeding pairs in our laboratory colonies of *P. leucopus* and *P. gossypinus*, and thus we cross-fostered offspring with both parents present in the cage to be able to compare preferences between cross-fostered and non-cross-fostered species trials.

We tested the mating preferences of all cross-fostered mice in the two-way gated choice assay described above. We predicted that if young mice sexually imprint on their parents, cross-fostered mice raised with the opposite species should prefer heterospecific stimuli and exhibit a weaker preference for conspecifics compared to individuals raised by their biological parents or other unrelated conspecific parents. We used Wilcoxon rank sum tests and a Bonferroni correction to compare differences in mating preferences among mice raised by their biological parents, heterospecific foster parents, or conspecific foster parents.

Are preferences affected by the behavior of stimuli?

Mate choice is an interaction between males and females, and stimulus behavior may influence chooser preference in our trials. We thus analyzed video data from each trial to

compare the rates of positive, neutral, and negative interactions between choosers and the stimulus mice. We predicted that if preferences are affected by the differences in behavior between the stimuli—for example, if the heterospecific stimulus responded more aggressively towards the chooser than the conspecific stimulus—then we might see an effect on which mate (conspecific of heterospecific) was preferred.

For each trial, we randomly selected 100 unique, one-minute video clips of the chooser mouse with each stimulus from the set of video clips in which the chooser and stimulus occupied the same chamber. If the chooser spent fewer than 100 minutes with a stimulus, we scored all of the one-minute video clips. To limit biases in scoring, we scored the interactions between the chooser and stimulus without knowing the identity of the stimulus mouse, and we never scored video data from sides of the same trial in sequential order. For each one-minute clip, we recorded the number of bouts of positive (male mounting or copulation, male pursuing, grooming, and nesting together), neutral (no interaction), and negative interactions (fighting, upright posture, threat, chasing) between the chooser and stimulus (following Eisenberg 1962). We calculated the rate of behavioral bouts per minute of video scored for each category—positive, neutral, or negative—to estimate rates of each. With these data, we tested if a chooser mouse interacted more positively and neutrally, and less negatively with its preferred stimulus. We used a logistic regression to test whether the difference in positive, neutral, and negative interactions between the conspecific stimulus and the heterospecific stimulus predicts the chooser's preferred mate.

RESULTS

Mating assay accurately measures mating preference

We assessed the quality of our two-way electronically-gated mate choice assay in two ways: (1) for eight trials, we reversed the stimulus mice and their cages halfway through the 44-hour experiment to test whether choosers preferred the stimulus and its cage or simply a particular side of the apparatus (i.e. the left or right flanking cage), and (2) we tested for an association between time spent with a stimulus and successful copulation and/or mating events.

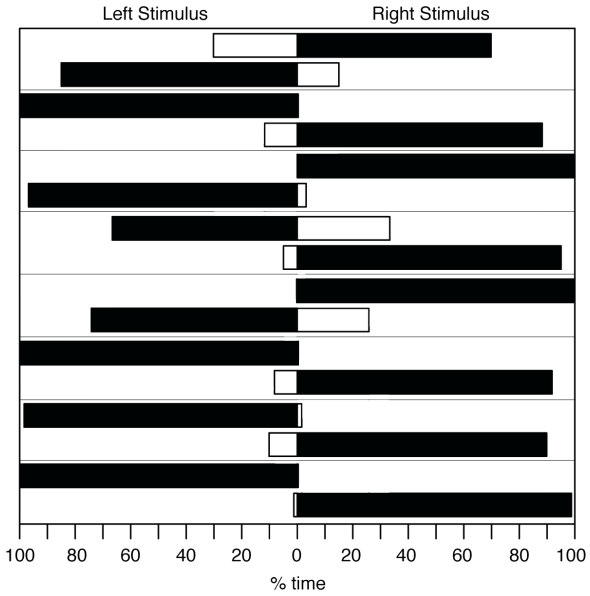

Figure 2.2. Preference of the chooser mouse in trials where the stimuli and their cages were swapped between the left and right sides halfway through a 44-hour trial ($N = 8$ trials). Each trial is represented in two rows: the first bar represents preference from night 1, and the second preference from night 2. Black bars indicate the proportion of time spent with the conspecific stimulus; white bars indicate percent of time with the heterospecific stimulus. All trials show that mice associate with the conspecific stimulus (or its cage), rather than showing a bias for either the left or right side of the testing apparatus.

Our first experiment indicated that choosers tracked their most preferred stimulus and its cage, following the stimulus to its new side after we had reversed the positions of mice and their cages (Figure 2.2). In all eight trials, the chooser switched sides to continue interacting with the individual it preferred on day 1 after the stimuli positions were reversed (one-sided binomial test: $p = 0.0039$). This suggests that our assay measures chooser preferences for stimuli and their cages, rather than preferences for a particular side of the apparatus.

Next, we wanted to confirm that the time spent with a particular stimulus mouse indeed reflected a mating preference. We detected successful mating in 16 out of 102 trials (female

chooser: $N = 7$; male chooser: $N = 9$). In only one case did a chooser (male) mate with both stimuli. In 15 out of 15 remaining trials, choosers mated with their most preferred stimulus (i.e. the individual with whom the chooser spent more time). We can therefore reject an assumption of random mating and conclude that time spent with a stimulus is a reliable indicator of mating preference (two-sided binomial test, $p < 0.0001$) in theses two species.

Both species prefer conspecific mates

The species differed in their preferences for *P. leucopus* stimuli (Wilcoxon rank sum test, $P < 0.0001$), such that both species spent more time with conspecific mates (Figure 2.3a). The median proportion of time spent with the *P. leucopus* stimulus was 0.88 for *P. leucopus* choosers and only 0.16 for *P. gossypinus*. We did not detect significant sex differences within either species (Wilcoxon rank sum test, *P. leucopus*: $p = 0.5065$; *P. gossypinus*: $p = 0.2405$).

Because of the structure of our breeding colony, we occasionally tested preferences of multiple siblings per family (range: 1-5 siblings tested per family, mean number offspring tested per family: 1.79). When we randomly assigned families to species, we found no evidence that families explain the difference in preference we observed between the species (permutation test, $p = 0$), thus we treated trials as independent.

Sexual isolation is strong

We calculated the joint isolation index (I_{PSI}) for each sex separately. In 27 female-choice trials, we calculated I_{PSI} as 0.65 (SD = 0.15, $p = 0.0004$); in 17 male-choice trials, we calculated I_{PSI} as 0.58 (SD = 0.20, $p = 0.0162$). Thus, in both cases, there is a statistically significant preference for conspecific mates. These indices suggest that, given equal access to conspecific and heterospecific mates, the majority of mating pairs will be conspecific. We did not detect any asymmetry in mating preference among the four possible pair types (IA_{PSI}: $p > 0.05$), suggesting that sexual isolation is not a result of strong preference in only one species or one sex.

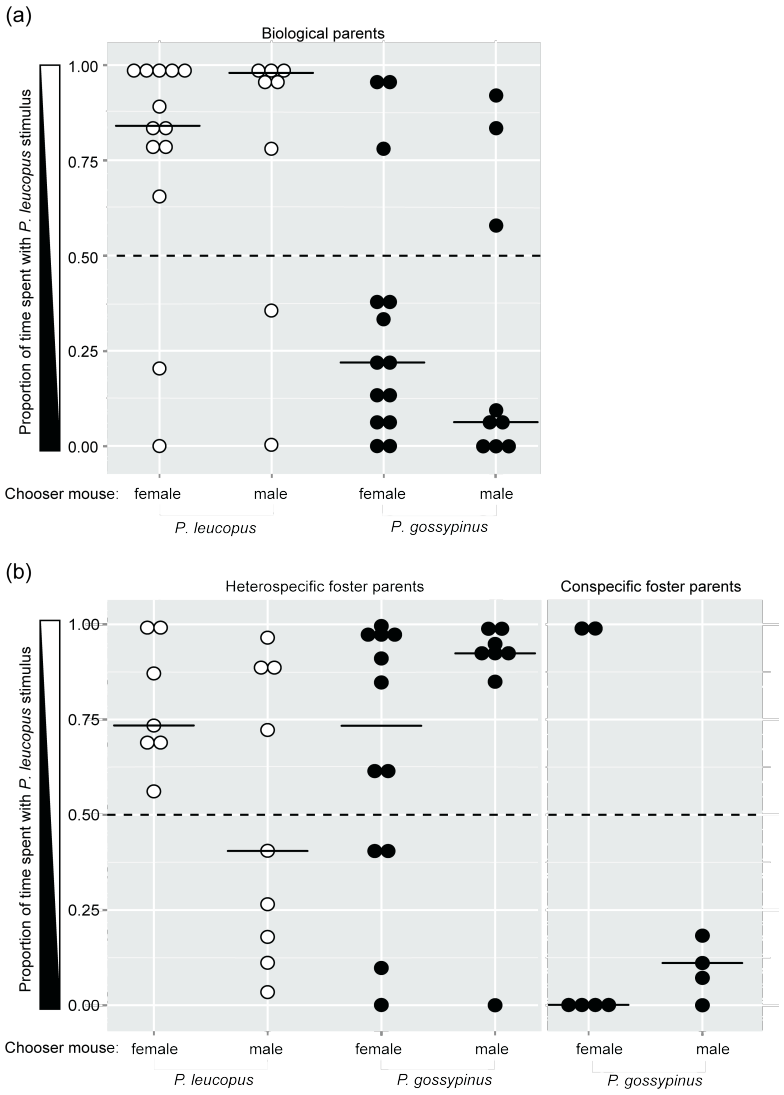

Figure 2.3. Mating preferences of each species and sex shown as proportion of time spent with one species, *P. leucopus*. The dashed line represents equal time spent with the *P. leucopus* and *P. gossypinus* stimulus, and solid lines represent median preferences. (a) Preferences from each species and sex (raised with own parents). (b) Preferences of each species and sex cross-fostered to the opposite species (heterospecific foster parents, left panel) or other families of the same species (conspecific foster parents, right panel).

Species differences in sexual imprinting

Cross-fostering offspring had different effects on mating preference in the two species (Figure 2.3b). First, *P. leucopus*, when raised with *P. gossypinus* parents, had weaker conspecific preference compared to *P. leucopus* raised by their biological parents (one-sided Wilcoxon rank sum test, $p = 0.0088$, significant after Bonferroni correction). To determine if this change in preference was sex-specific, we evaluated preferences by sex and found that neither cross-fostered *P. leucopus* females nor males showed any change in conspecific preference compared to those raised by their biological parents (one-sided Wilcoxon rank sum test, $p = 0.0956$), but males did (one-sided Wilcoxon rank sum test, $p = 0.0221$, not significant after Bonferroni correction). However, some *P. leucopus* males cross-fostered to *P. gossypinus* still preferred conspecific mates (4 out of 9), and the reduction in conspecific preferences was low (cross-fostered males spent an average of 16% less time with *P. leucopus* females). By contrast, *P. gossypinus* cross-fostered to *P. leucopus* parents, reversed their preferences from conspecifics to heterospecifics when compared to those raised by their biological parents (one-sided Wilcoxon rank sum test, $p = 0.0005$, significant after Bonferroni correction). This was true for both sexes (one-sided Wilcoxon rank sum test, females: $p = 0.0048$, males: $p = 0.0135$, both tests significant after Bonferroni correction)

We have assumed these comparisons—between mice raised by their biological parents and mice raised by heterospecific parents—reflect the effects parents (and their species) on preference. However, this pattern of weaker/reversed preferences in cross-fostered is confounded with presence or absence of a litter transfer to new parents, as offspring raised by their biological parents were never transferred between parents. To disentangle these effects, we cross-fostered *P. gossypinus*, the species that showed a sign of strongly learned preferences, to unrelated *P.*

gossypinus foster parents as a control. We found that mating preferences from *P. gossypinus* individuals raised by conspecific foster parents preferences did not differ from those raised by their biological parents (two sided Wilcoxon rank sum test, $p = 0.1257$) but did differ significantly from *P. gossypinus* fostered by heterospecific parents (two-sided Wilcoxon rank sum test, $p = 0.0027$, significant after Bonferroni correction; Figure 2.3b, left and right panels). These results are consistent with sexual imprinting in *P. gossypinus* and rule out the possibility the observed patterns are caused by the transfer litters to novel parents.

More positive interactions between choosers and their preferred stimulus

Mating involves an interaction between males and females, and it is likely that stimulus behavior could affect the mating preferences of the chooser. We tested whether the chooser's mate preference is influenced by its interactions with the stimuli mice in these species. For example, did the chooser prefer the stimulus with which it had a lower rate of negative interactions? Specifically, we predicted that choosers would have more positive (and less negative) interactions with their preferred stimulus. We used a logistic regression to test whether the difference in rate of interactions (scored as positive, neutral, or negative) between the chooser and the conspecific stimulus versus the chooser and the heterospecific stimulus predicted which mate the chooser preferred. Positive differences would indicate greater positive interactions with the conspecific stimulus than the heterospecific stimulus; negative differences would indicate more positive interactions with the heterospecific stimulus than the conspecific stimulus.

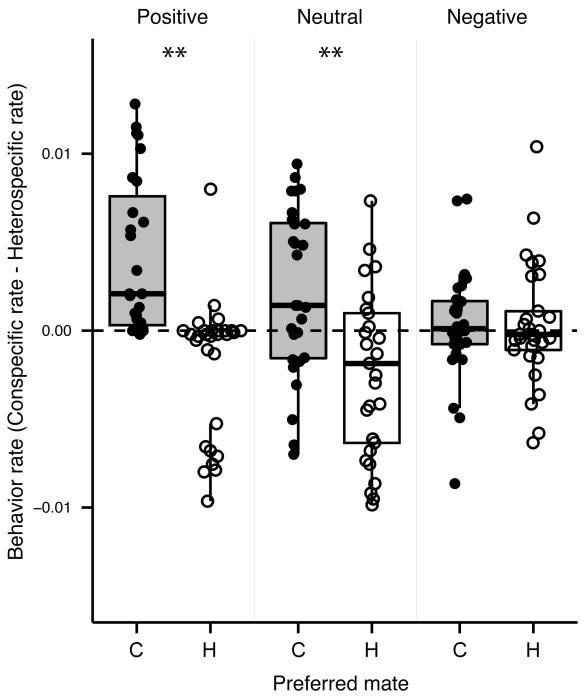

Figure 2.4. Differences in positive and neutral interactions between conspecific and heterospecific stimuli predict preferred mate (x-axis: C = conspecific or H = heterospecific). Negative interactions were not significant. $^{**}p < 0.01$.

We fitted a full model with all behavioral categories and used a backward stepwise algorithm to determine the best-fit model according to the Akaike Information Criterion (AIC). The model with the lowest AIC value included two terms, one for a difference in rates of positive interactions and one for the difference in rates of neutral interactions between the conspecific and heterospecific stimulus mice (Figure 2.4). Surprisingly, we did not detect an effect of the frequency of negative behavior, despite many observations of chasing and fighting in our video data. We attribute this to negative interactions from both stimuli, as the means of the differences between negative conspecific interactions and heterospecific interactions are centered at 0 (Figure 2.4). Thus, we found that the chooser mouse interacted more positively or neutrally with its preferred stimulus. Although we cannot determine from our video data whether more positive interactions between a chooser and stimulus occur because of the chooser's preference or the stimulus's preference, our video results support the conclusion that greater association time with a stimulus corresponds to more positive/neutral interactions.

DISCUSSION

Sexual imprinting is a potential mechanism for generating sexual isolation, a reproductive barrier created by divergent mating preferences between populations. Here, we tested whether sexual imprinting contributes to sexual isolation in two sympatric sister species of mice—*Peromyscus leucopus* and *P. gossypinus*—which we had previously shown to be genetically distinct and largely reproductively isolated in natural populations (Chapter 1).

Our choice trials revealed that both *P. leucopus* and *P. gossypinus* preferred conspecific mates. Their preferences created a significant amount of sexual isolation: we estimated the average sexual isolation index, I_{PSI}, from female- and male-choice trials to be 0.62 and likely driven by strong preferences in both species and sexes. A sexual isolation index of 0.62 is greater than what has been detected among many morphologically divergent populations (e.g. among cactophilic [Etges and Tripodi 2008] or cosmopolitan [Yukilevich and True 2008] populations of fruit flies, walking stick insect populations [Nosil et al. 2013], or between gold and wildtype color morphs of Nicaraguan cichlids [Elmer et al. 2009]), placing our study species farther along a speciation continuum. Because the *P. leucopus* and *P. gossypinus* interbreed in no-choice trials (Chapter 1), but show considerable sexual isolation in choice trials (present study), we conclude that mating preferences create strong reproductive isolation in this pair of sister species.

Different mechanisms, however, appear to explain the strong conspecific mating preferences we observed between these species. Our cross-fostering data clearly show that *P. gossypinus* males and females sexually imprint on their parents. They strongly preferred conspecific mates when raised by their own parents or with unrelated conspecific parents, but they switched preferences to heterospecific mates when raised by *P. leucopus* parents. This sharp reversal of preferences suggests that mate preference in *P. gossypinus*, at least to a large degree,

is learned. By contrast, we found only weak evidence for an effect of learning in *P. leucopus* males and no evidence for learning in female *P. leucopus*. A previous cross-fostering study between *P. leucopus* and *Onychomys torridus* found a similar sex bias in sexual imprinting for conspecific odors in *P. leucopus*: males switched species preferences after cross-fostering while females showed a reduced, but less significant, preference for conspecific odors (McCarty and Southwick 1977). Thus, it appears that male *P. leucopus* partially sexually imprint, while female mate preferences likely have a genetic basis.

What might account for the differences in preference development between the two species? One possibility is that mating preference in *P. leucopus*, particularly females, are truly innate. Evidence for genetically determined preferences have been identified in insects, birds, and fish (Bakker and Pomiankowski 1995). Because rodents are specifically sensitive to olfactory cues, preferences could be innate, for example, if species produced at least one unique odor or pheromone (e.g. in their urine, saliva, or sweat) and had specific olfactory receptors devoted to its detection. Recent work on vomeronasal organ receptors in house mice indicates species- and sex-specific receptors exist (Isogai et al. 2011). Mating preferences could be innate in *P. leucopus* if divergence in odorants and their detection was highly specialized and species-specific.

Another possibility is that *P. leucopus* also learn their preferences, but has a shifted sensitive period from *P. gossypinus*. The cross-fostering experiments we report here were all done postnatally by swapping offspring after birth. If *P. leucopus* sexually imprinted on parental cues prenatally, we would be unable to detect learning for this species in this study. Such *in utero* learning is plausible in mammals for a couple of reasons. First, there is evidence that humans can learn vocalizations *in utero* and respond to them after birth (Partanen et al. 2013).

Second, the olfactory system is functional in the late stages of embryogenesis, and could be active to learn maternal cues. If *P. leucopus* did indeed have a sensitive period earlier than *P. gossypinus*, a 2 x 2 factorial experiment with embryo-transfers within and between *P. leucopus* and *P. gossypinus* could help isolate the effects of *in utero* from postnatal cross-fostering effects. Although embryo transfers are unlikely to work between distantly related species, *P. leucopus* and *P. gossypinus* are sister species and thus create a ripe opportunity for testing the timing of sexual imprinting in two inter-fertile species (see Appendix).

Yet another possibility is that the species (and sexes, in the case of *P. leucopus*) use different imprinting sets—i.e. whether the mother, father, or even siblings are used as models during the learning phase. Male and female zebra finches, for example, appear to diverge in their imprinting sets. Female zebra finches maternally imprint, while males appear to imprint on both parents (Vos 1995). Female zebra finches learn to prefer maternal traits, while male zebra finches tend to learn a preference for maternal stimuli but learn to avoid paternal stimuli (Vos 1993). Thus, there may be both species and sex differences in the model used for imprinting—whether offspring imprint on their mother, father, siblings, or some combinations. As designed, we cannot identify the models used in imprinting because the mice in our experiment were all raised offspring with mothers and fathers. In addition, differences in the sensory modality—visual, auditory, or olfactory—may also be different between the species and sexes. Different emphasis or combinations of multiple cues could account for both the difference between *P. leucopus* and *P. gossypinus*, but also the difference between *P. leucopus* sexes.

Future experiments should be aimed at both confirming sex differences and attempting to distinguish between these alternative mechanisms for the establishment of differential sexual imprinting. Should these results be upheld, *P. leucopus* and *P. gossypinus* would make an ideal

species pair—because they are reciprocally interfertile (L♀ x G♂, G♀ x L♂)—in which to dissect the genetic basis of sexual imprinting and preference loci.

In addition to identifying sex-based differences in sexual imprinting in *P. leucopus*, we have clearly demonstrated that sexual imprinting in at least one species (*P. gossypinus*) greatly contributes to moderately strong sexual isolation between the two species. Specifically, we showed that sexual imprinting in *P. gossypinus* contributes to conspecific preferences. Learned mating preferences have been theorized to create sexual isolation, and learning has been documented in a wide variety of species, but sexual imprinting and reproductive isolation have not explicitly been connected in many studies. We show a clear link between learning and speciation and provide support for a role of sexual imprinting in the speciation process.

CHAPTER 3:

The role of sexual imprinting in diet-based assortative mating

ABSTRACT

Reproductive isolation may evolve as a by-product of adaptation to local environmental conditions. Such ecological speciation is assumed to be the result of genetic divergence, but sexual imprinting for traits under divergent natural selection could also spur rapid and significant sexual isolation between populations. We hypothesized that many cases of adaptation and sexual isolation might be explained by shifts in diet coupled with sexual imprinting. If populations shift their diet for any reason—for example, to utilize a novel food source or to reduce competition for specific food resources—this shift may be detectable and, in turn, sexual imprinting could produce diet-based assortative mating. We tested this hypothesis in a sexually imprinting species, the cotton mouse (*Peromyscus gossypinus*), by providing breeding pairs with either garlic- or orange-flavored water. We then tested whether their offspring, exposed to these flavors through their mothers *in utero* and both parents in the nest, later preferred mates with the same diet, and presumably their odor cues, as their parents. We found extremely strong support for assortative mating in females: females spent more time with males consuming the same flavored water as their parents. Males exposed to both garlic and orange flavors, however, appeared to prefer females fed a garlic diet, suggesting that garlic may be a more attractive scent to males than orange. Our data show that *P. gossypinus* (particularly females) are capable of sexually imprinting on dietary cues learned either *in utero* or postnatally, but that the sexes may differ in their preference for these flavors. Overall, our research demonstrates the ability of sexual imprinting to create diet-based assortative mating.

INTRODUCTION

It is becoming increasingly accepted that adaptation to divergent environments can produce reproductive isolation as a by-product, so-called 'ecological speciation' (Schluter 2001; Nosil 2012). Once considered to be rare, traits under divergent natural selection that also lead to assortative mating—referred to as "magic traits"—have been identified in a diversity of taxa (reviewed in Servedio et al. 2011). Reproductive isolation has evolved in response to differences in habitat use (Funk 1998; Mckinnon et al. 2004), competition (e.g. Calsbeek and Smith 2008; Hendry et al. 2009), and sexual selection (e.g. Boughman 2001; Seehausen et al. 2008). A meta-analysis of such studies found that differences in ecology were positively associated with the strength of reproductive isolation across multiple groups of organisms, indicating that reproductive isolation as a "by-product" may be quite common (Funk et al. 2006).

By-product reproductive isolation is thought to arise as a pleiotropic consequence of the genetic basis of traits undergoing divergent selection (Schluter 2001; Nosil 2012). Selection on a trait could contribute to or result in sexual isolation, for example, if that trait and a mating preference for that trait were genetically linked—either through physical linkage of two separate loci in the genome or through pleiotropic effects of a single gene. Divergent selection on such traits could then produce assortative mating if the underlying genetic mechanisms facilitated co-inheritance of traits and their preference.

However, there is an alternative, non-genetic mechanism that could also generate assortative mating: sexual imprinting. Sexual imprinting—the process by which young offspring learn parental traits and prefer them in future mates—forms associations between traits and preferences within a population. For example, if offspring from different populations sexually imprinted on traits under selection toward different phenotypic optima, sexual imprinting would

create reproductive isolation between populations that might look like a pleiotropic effect of adaptation. An example from benthic and limnetic sticklebacks supports this idea: sexual imprinting on male nuptial coloration and odor—two traits under divergent selection in benthic and limnetic habitats—enabled assortative mating between morphs (Kozak et al. 2011).

Sexual imprinting may explain some cases of diet-based assortative mating. One of the earliest classic examples of by-product reproductive isolation found significant diet-based assortative mating between populations of *Drosophila pseudoobscura* that had adapted to either starch or maltose media (Dodd 1989). Similar diet-based assortative mating has been detected in other laboratory experiments using *Drosophila* (Rundle et al. 2005; Sharon et al. 2010) as well as in natural populations of stickleback fishes (Snowberg and Bolnick 2008). Diet could be indirectly sexually imprinted upon if diet affected a mating signal, which was itself the object of sexual imprinting. This may be the case in Darwin's finches. It has been demonstrated that seed availability affects beak shape (Schluter and Grant 1984), which in turn affects song (Podos et al. 2004), and beak shape and song are both subject to sexual imprinting (Grant and Grant 1997). Thus, divergence in diet coupled with sexual imprinting may have contributed to the reproductive isolation of Darwin's finches. A more direct reflection of diet, however, might be through chemical odors or pheromones. Changes in diet are known to alter body odors in mammals (Ley et al. 2008) and even pheromone production in a number of taxa (e.g. rats: Leon 1975; swordtails: Fisher and Rosenthal 2006; fruit flies: Sharon et al. 2010). If offspring are able to detect dietary information from their parents, either directly or indirectly, it raises the possibility that diet-based assortative mating may arise.

We experimentally tested the hypothesis that changes in diet, when coupled with sexual imprinting, cause assortative mating by manipulating diet in cotton mice (*Peromyscus*

gossypinus), a species known to sexually imprint on parents (Chapter 2). Specifically, we fed breeding pairs either garlic- or orange-flavored water and allowed offspring to be exposed to these flavors during gestation up until weaning. Then, we tested if offspring learned to prefer the odors they experienced during pre- and post-natal development, thereby creating diet-based assortative mating in *P. gossypinus*.

METHODS

We tested whether imprinting on diet might create sexual isolation in a species known to sexually imprint: *Peromyscus gossypinus* (Chapter 2). Specifically, we manipulated parental diet and tested whether their offspring preferred mates of the same flavor type as their parents in electronically-controlled two-way choice assays (described below). Our laboratory colony of *P. gossypinus* was derived from wild-caught mice from Washington and Jackson counties, Florida, during 2009.

Diet manipulation

We maintained all mice on a regular Purina diet (Purina Iso Pro 5P76) but fed *P. gossypinus* parents either garlic- or orange flavored water. We diluted 2 µl of Chinese garlic or orange oil (Sigma Aldrich) into 400 ml of distilled water (0.0005% v/v) and mixed them together by shaking vigorously. We replaced the flavored water every 1.5 weeks to preserve freshness. Diet has been shown to affect urinary metabolites in rats (Bell et al. 1991; Phipps et al. 1998); thus, we expected garlic and orange flavors would be metabolized and be detectable through urine but possibly also feces, saliva, and sweat. In our experiment, offspring are exposed to these odors/flavors *in utero* (in rodents the olfactory system is functional before birth [Pedersen et al.

1983; Todrank et al. 2011]) through weaning, which occurs at 23 days of age. At weaning, we assigned offspring as either "stimulus" or "chooser"; stimulus mice were weaned and continued on the same flavored water diet as their parents, but chooser mice were weaned and returned to a diet of unflavored water.

Quantification of mate preferences

We tested the mating preference of adult mice for opposite sex stimuli that were fed either garlic- or orange-flavored water. We tested mice that were at least 80 days old in an electronically-controlled gated choice apparatus following the testing protocol described in Chapter 2. In brief, we implanted three test mice with small transponders (1.4 mm x 9 mm, ISO FDX-B, Planet ID Gmbh) in the interscapular area and programmed the gates to allow the designated "chooser" mouse (i.e. the individual whose preference we tested) to pass freely through all three cages while constraining each "stimulus" mouse to the left or right cage. We tested individual preferences of approximately 10 chooser mice from each flavor and sex in the gated apparatus for an opposite sex mouse of the same and alternate flavor. For each trial, we added a sexually mature chooser—either a virgin female in proestrus/estrus or a mature virgin male—to the apparatus for a day to acclimate, adding used nesting material from the flavor-fed stimulus mice to the flanking cages. Although stimuli mice drank flavored water up until the trial, unflavored water was added to all cages during the trial under the assumption that the odors from garlic- and orange-fed stimulus mice would persist for the duration of the two-day trial; we did not add flavored water to the flanking cages to avoid confounding a stimulus preference with a flavored water preference. The next day, we added stimulus mice to the flanking cages to give them two to four hours to acclimate to their new environment before opening the gates at lights

out (4:00 pm; 16:8 hour light:dark cycle). We recorded RFID readings at all antennae and scored preference as the proportion of time spent with the garlic-treated stimulus mouse divided by the total time spent with both stimulus mice. We analyzed only trials in which the chooser mouse investigated both cages during the acclimation, spent at least 10 minutes investigating stimulus mice during the trial, and the stimulus mice were in their cages at least 75% of the trial period (in 34% of trials, at least one stimulus mouse escaped). We used Wilcoxon signed rank tests to assess whether garlic-exposed male and female choosers or orange-exposed male and female choosers differed in their preferences for mates of the same parental flavor.

Estimate of sexual isolation attributable to flavor cue

We calculated the strength of sexual isolation between each flavor treatment using a joint isolation index (I_{PSI}; Rolán-Alvarez and Caballero 2000), separately for female- and male-choice trials. We assumed the stimulus with which the chooser spent more time was the preferred mate and used these values to estimate the sexual isolation index in JMATING v. 1.0.8 (Carvajal-Rodriguez and Rolán-Alvarez 2006). The I_{PSI} index compares observed and expected mating pairs (assuming random mating among individuals) among the four possible pair types (garlic ♀ x garlic ♂, garlic ♀ x orange ♂, orange ♀ x garlic ♂, and orange ♀ x orange ♂) and reflects the amount of interbreeding between the two flavor types. A value of -1 indicates that all pairing occurs between flavor types, +1 indicates that all pairing occurs within each flavor type, and 0 indicates equal pairing among all four pair types. We also tested for asymmetry in observed mating frequencies (IA_{PSI}), which would occur if one only one flavor treatment or sex had strong preferences for mates of the same flavor, while the alternative treatment or sex did not. We used

10,000 bootstrap replicates to estimate the isolation indices, their standard deviation, and to test the hypothesis that our isolation estimate deviated significantly from zero.

RESULTS

We found evidence for strong assortative mating based on diet in females but mixed evidence in males (Figure 3.1). Females exposed to garlic consistently preferred garlic-treated to orange-treated males, significantly exceeding the null expectation of 50:50 (one-tailed binomial test, $p = 0.0078$). Females exposed to orange preferred orange males in 75% of trials, but this was not significantly different from a null expectation of 50:50 (one-tailed binomial test, $p = 0.1445$). Combined, however, mating preferences of garlic-exposed females and orange-exposed females create significant sexual isolation. We calculated I_{PSI} to be 0.72 (SD = 0.17, $p = 0.0024$) in female trials, which indicates extremely high assortative mating. We did not detect any evidence for asymmetry (IA_{PSI}: $p > 0.05$).

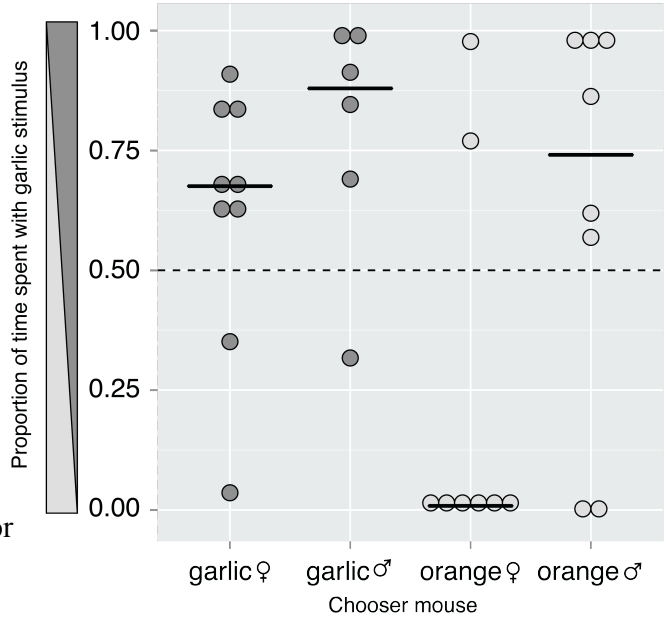

Figure 3.1. Mating preferences of *P. gossypinus* males and females for garlic stimuli. The dotted line represents equal time with both stimuli: above, the garlic stimulus was preferred, and below, the orange stimulus was preferred. Solid lines represent median preferences. Each dot represents the preference of an individual chooser that was raised with either garlic parents (dark gray) or orange parents (light gray).

By contrast, males showed mixed evidence for assortative mating. Garlic-exposed males strongly preferred garlic-fed females to orange-fed females, but orange-exposed males also preferred garlic-fed females. Sexual isolation between garlic- and

orange-exposed males in our experiment was not significantly different from zero (I_{PSI}: $p > 0.05$) driven by the fact that both treatments preferred garlic-fed females.

DISCUSSION

Diet-based assortative mating has been documented in laboratory populations of fruit flies and in natural populations of stickleback fishes, but we suspect it may be a more general consequence of divergence. If reproductive isolation evolved as a sort-of pleiotropic response to changes in diet in sexually imprinting species, populations that shifted to eat novel sources of food or changed their dietary niche to reduce competition with a sympatric species could potentially speciate. Assortative mating based on diet would preserve mating among members occupying similar niches (e.g. Snowberg and Bolnick 2006). Assortative mating would also allow for the coexistence of incipient (or even well-diverged) species in sympatry. Thus, any assortative mating that arises as a "by-product" of dietary change could influence the speciation process.

It has not been immediately clear, however, how mating preferences become coupled with diet unless sexual imprinting is invoked. By imprinting on dietary information—for example, if diet was detectable through changes to a trait's color, shape, or smell—we hypothesize that assortative mating could form based on differences in diet. Studies from a number of mammalian species including humans (Schaal et al. 2000; Mennella et al. 2001); European rabbits (Altbackek and Bilko 1995); spiny mice (Porter and Doane 1977); and rats (Galef and Henderson 1972; Sullivan et al. 1990) have demonstrated that young offspring are capable of learning dietary information from their mothers during gestation and while nursing, and that they prefer foods experienced through their mothers. Learning food preferences about

diet, at least in mammals, might also be extended to learned mating preferences if dietary cues are sexually imprinted.

We tested the hypothesis that sexual imprinting can create assortative mating for diet by manipulating diet in a species of mouse, *P. gossypinus*, which learns mating preferences (Chapter 2). We found strong evidence for diet-based assortative mating between garlic-exposed and orange-exposed *P. gossypinus* females ($I_{PSI} = 0.72$). This amount of isolation is in fact greater than what we have detected between *P. gossypinus* and its sister species, *P. leucopus* (Chapter 2). Because our experiment was done within a single species and chooser mice had limited exposure to garlic- and orange-diet cues, we can attribute the sexual isolation in our to sexual imprinting on parental diet cues.

We did not find the same degree of assortative mating preferences in males; in fact, males raised with orange parents demonstrated disassortative mating preferences, preferring garlic-fed females to orange-fed females. In Chapter 2, we found that *P. gossypinus* males who were raised by a different species (*P. leucopus*) strongly preferred females of their foster parent species to females of their own species, demonstrating a reversal of mating preferences because of early life experience. Because it is known that *P. gossypinus* males sexually imprint, it is likely that our detection of a general preference for garlic stimuli suggests that garlic may be more attractive than orange stimuli. This suggests that there may be hierarchical levels in the formation of mate preferences. For example, males may broadly imprint on parental traits that they later use to find mates—this could explain why *P. gossypinus* males raised with another species would prefer females of that species. However, once males have learned a search image for acceptable mates, they may be less discriminating within that group. *P. gossypinus* is thought to be promiscuous (McCarley 1959), and thus males may be less choosy than females if the cost

and benefits of mating are lower relative for males (Kokko et al. 2003). In the present study, both sexes may have imprinted on *P. gossypinus* cues as well as diet cues, but males may be either less discriminating or other factors may influence male choice such as female sexual receptivity or female tolerance. Nonetheless, our data suggest a trend toward assortative mating but more trials are be necessary to determine if a sex difference exists.

Overall, our ability to detect significant assortative mating by flavor type supports the possibility that dietary information can be learned and preferred in a mammalian species, and future experiments should continue testing the role of diet in speciation for *P. gossypinus* and other taxa. Our study is one of a few experiments explicitly testing the effects of sexual imprinting on diet. Our results suggest that sexual imprinting could be a by-product mechanism that produces assortative mating for traits (diet or otherwise) under divergent natural selection, and we suspect that traits reflecting diet such as metabolized pheromones, carotenoid-based colors, and protein excretion, coupled with sexual imprinting, could create diet-based assortative mating.

CONCLUSION

I have presented research on the role of sexual imprinting in the formation of sexual isolation in two sister species of mice, *P. leucopus* and *P. gossypinus*. I first established that *P. leucopus* and *P. gossypinus* are separated by strong reproductive isolation (Chapter 1). Specifically, using genomic data, I found that the two species are patchily distributed where their distributions overlap in the southeastern United States, possibly indicating a lack of a structured hybrid zone and a potentially low degree of contact between the two species. Although many sampling sites had only a single species, a subset of sites contained individuals of both species, thus providing opportunity for hybridization. In these, I identified some populations with hybrids and some without indicating that there is geographical variation in hybridization rate; however, Bayesian model-based clustering identified two distinct genetic clusters in my samples corresponding to each species. Thus, presence of hybrids but the lack of admixture between the species leads me to infer that strong postzygotic reproductive barriers separate *P. leucopus* and *P. gossypinus*.

In Chapter 2, I showed that mating preferences could reduce hybridization between the species. Both *P. leucopus* and *P. gossypinus* preferred conspecific mates in ~85% of two-way choice trials, creating a significant amount of sexual isolation between these two species (I_{PSI} = 0.62). I next tested whether conspecific mating preferences in *P. leucopus* and *P. gossypinus* are innate (i.e. genetically determined) or learned using a cross-fostering experiment. I found that one species learns its preference (*P. gossypinus*) while the other (*P. leucopus*) appears to have a genetically-determined preference. This difference in how mating preferences are acquired was unexpected because these mice are closely related; future research should exploit this result to

study how variation in mate preferences (learned vs. genetic) can arise. However, future research should rule out the possibility that *P. leucopus* mating preferences are learned earlier (i.e. *in utero*) than I tested. This possibility could be tested by *in utero* cross fostering (using the methods of superovulation [see Appendix] and embryo transfers) to quantify the relative effects of sexual imprinting before and after birth. Overall, while preferences in *P. leucopus*, especially females, are likely to be genetically determined, learned mating preference, or sexual imprinting, in *P. gossypinus* contributes to sexual isolation between *P. leucopus* and *P. gossypinus*.

To determine how much reproductive isolation sexual imprinting may create, I manipulated potential sexual imprinting cues within a single species that learns its preference, *P. gossypinus* (Chapter 3). I tested whether divergent diets could be sexually imprinted upon to form mating preferences strong enough to create diet-based assortative mating. I showed that *P. gossypinus* females, and perhaps males, are able to sexually imprint on dietary cues from their parents. These preferences created significant sexual isolation among females ($I_{PSI:}$ 0.72, SD = 0.17, $p = 0.0024$) by diet; by contrast, males were not significantly sexually isolated because of learned mating preferences for parental odors. Although my experimental design did not allow me to determine how dietary information was learned—whether offspring sexually imprinted on diet metabolites from their parents or environmental diet odors in the cage—I was able to conclude that sexual imprinting on diet is possible and likely occurs through olfaction and/or taste. Because *P. leucopus* and *P. gossypinus* are known to eat different diets in nature (Calhoun 1941), diet would likely amplify sexual isolation between the species in sympatry. My results from Chapter 3 support the idea that divergence in diet, when coupled with sexual imprinting, is a plausible mechanism for generating sexual isolation between populations.

Together, my studies have shown that mating preferences create sexual isolation between *P. leucopus* and *P. gossypinus*. While additional barriers likely act in concert with sexual isolation, such as habitat isolation, conspecific sperm precedence, or selection against hybrids (e.g. sexual discrimination or subfertility), the contrast between mating preferences in no-choice and two-way choice trials confirm that mating preferences create a significant amount of reproductive isolation between the species. That this isolation is caused in part by sexual imprinting highlights the relevance of learning in speciation. Sexual imprinting, especially when ecologically divergent traits are learned (e.g. diet or odor), could explain the phenomenon of ecological adaptation and by-product reproductive isolation, one of the major tenets of ecological speciation. My research indicates that sexual imprinting likely plays an important role in the evolution of reproductive isolation and thus has the potential to facilitate speciation.

APPENDIX:

Superovulation in *Peromyscus*

OVERVIEW

In Chapter 2, I found differences in sexual imprinting between *P. leucopus* and *P. gossypinus*. Although *P. leucopus* mating preferences were unaffected by cross-fostering, *P. leucopus* might still learn mating preferences but have a different sensitive period from *P. gossypinus*. If *P. leucopus* learned maternal chemical information before birth, their mating preferences would have been unaffected by my post-natal cross-fostering experiment. An ideal way to test my hypothesis for different sensitive periods between the species would be a 2x2 full factorial experiment with embryo transfers between species such that the effects of learning before and after birth could be quantified. Because superovulation improves embryo yield, and because the ability to manipulate reproductive biology in *Peromyscus* would also be important for making transgenic mice, I was motivated to identify optimal superovulation conditions in *Peromyscus*.

ABSTRACT

We aimed to improve oocyte collection from *Peromyscus* deer mice. Previously published studies concluded that *Peromyscus maniculatus* cannot be superovulated, but did not adequately design experiments to quantify the effects of factors that might influence oocyte production. We designed a 2_V^{5-1} fractional factorial experiment to quantify main effects and interactions for five factors: female age, pregnant mare serum gonadotropin (PMSG) dose, human chorionic gonadotropin (hCG) dose, the length of time between PMSG and hCG injections, and the length of time before harvesting oocytes post-hCG. Using only 17 females, we explored the relevant parameter space and identified three important factors for superovulation: PMSG dose, hCG dose, and the length of time between the administration of the two hormones. We replicated the treatment combination that caused our highest oocyte yield in additional females and found we could reliably collect 29 ± 4.2 oocytes/female (mean ± S.E.M.). We tested these same conditions in two other species within the genus—*P. leucopus* and *P. gossypinus*—but did not achieve the same oocyte yield. We suggest that additional experiments will be necessary to optimize superovulation for other *Peromyscus* species, and recommend using fractional factorial experiments to efficiently test the effects of multiple factors.

INTRODUCTION

The North American genus of *Peromyscus* deer mice contains 55 species representing a diversity of behaviors, mating systems, and adaptations to different habitats (Hooper 1968; Joyner et al. 1997). As QTL mapping and genome-wide association studies identify loci underlying morphological and behavioral adaptations in *Peromyscus* mice (e.g. Linnen et al., 2013; Steiner, Weber, & Hoekstra, 2007; Weber, Peterson, & Hoekstra, 2013), it will become necessary to test the function of these purported causal loci. The genomes of six *Peromyscus* species are being sequenced, assembled, and annotated and will improve identification of specific DNA sequences likely contributing to a phenotype, but the ultimate functional test of specific loci will require transgenic *Peromyscus* mice.

Superovulation is the first step in making a transgenic mouse, but is thought to be impossible in *Peromyscus* species (Veres et al. 2012; Choi and He 2013). Ideally, a donor female is superovulated with two hormones—for example, pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG)—which stimulate the ovaries to produce oocytes and cause their release, respectively (Gertsenstein et al. 2003). After mating, single-cell fertilized embryos are collected and genetically manipulated (e.g. with lentiviruses, ZFN, CRISPR/Cas, or TALENs), incubated, and then transferred to a pseudo-pregnant recipient female (Gaj et al. 2013). Increasing the number of oocytes released improves embryo yield and the chance for successful implantation in recipient females. In addition, effective superovulation reduces the number of animals needed for a transgenic experiment. Successful superovulation protocols have been developed for a number of rodents, but optimal conditions for hormone injection and egg harvest can vary between strains and species (Popova et al. 2005; Pasco et al. 2012). As of yet,

no published studies have successfully developed a superovulation protocol in *Peromyscus* mice (Veres et al. 2012; Choi and He 2013).

A recent and intriguing study by Choi and He (2013) with *Peromyscus maniculatus* females, however, indicated that superovulation might not be impossible. Although the authors were unable to collect more than ~5 ova per female via oviduct flushing, they were able to collect nearly 21 ova from females if they manually dissected the cumulus oocyte complex (COC). We interpret this to mean that PMSG effectively stimulates oocyte production but their release from the COC is problematic. Although the authors' solution of manual dissection increased oocyte yield four-fold, it is not ideal in that the ova must undergo *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) before genetic manipulation can begin. Adding additional steps lengthens the transgenic protocol and requires more effort, materials, and time from the researcher. Thus, while we were encouraged by Choi & He's results, we questioned the optimality of their published protocol.

We hypothesized that if oocyte release was problematic, as we suspected, allowing more time for oocytes to mature before stimulating their release or waiting longer before harvesting ova would improve yield. Additionally, age might also affect oocyte yield. Previous studies attempted to superovulate 12-14 week-old *P. maniculatus* females (Veres et al. 2012; Choi and He 2013), which are several weeks after females reach sexual maturity at 7-8 weeks (Dewey and Dawson 2001). Because many lab mouse protocols superovulate females before sexual maturity, age might also be an important factor affecting oocyte production.

We designed a fractional factorial experiment to simultaneously quantify main effects and interaction effects among five variables likely to influence superovulation: female age, pregnant mare serum gonadotropin (PMSG) dose, human chorionic gonadotropin (hCG) dose,

the length of time between PMSG and hCG injections, and the length of time before harvesting eggs after hCG injection. We tested these superovulation conditions in *Peromyscus maniculatus*, the same species used in prior studies (Veres et al. 2012; Choi and He 2013). We identified 3 factors that allowed us to consistently superovulate *P. manciulatus* (29 ± 4.2 oocytes/female; mean ± S.E.M.). We the tested whether these conditions are also optimal in two congeneric species, *P. leucopus* and *P. gossypinus*,

MATERIALS & METHODS

Animals

We used virgin females from three species for our experiments: *Peromyscus maniculatus*, *P. leucopus*, and *P. gossypinus*. Both *P. maniculatus* and *P. leucopus* mice were ordered from the Peromyscus Genetic Stock Center (University of South Carolina); *P. gossypinus* are from the Hoekstra lab breeding colony established in 2009 from Washington and Jackson counties in Florida. All females were socially housed in same sex cages prior to our experiment.

Hormones

We diluted each PMSG (EMD Millipore, #367222-5000IU) and hCG (Sigma-Aldrich, #CG10-1VL) to a concentration of 50 I.U.s with sterile-filtered BioXtra water for embryo transfer (Sigma-Aldrich, #w1503). After dilution, we verified that our hormones were functional by testing them on female C57BL/6J or BDF1 lab mice with the assistance of the Harvard University Genome Modification Center.

Superovulation conditions in *Peromyscus maniculatus*

We aimed to test the effects of five factors: (1) female age, (2) PMSG dose, (3) hCG dose, (4) interval between PMSG and hCG injections, and (5) the length of time before harvesting oocytes after hCG injection (Table 1). Because full factorial experiments with two levels would require 2^5 experimental treatments (i.e. combinations of factors), we halved the number of treatments needed by implementing a 2_V^{5-1} fractional factorial experiment design. This design has a resolution of 5 (i.e. V), where no main effects are confounded with any 2- or 3-way interactions. This design required 16 treatments, but we added an extra treatment at intermediate levels for all factors as a center point (column "0" in Table 1).

We randomly assigned 16 females to the treatments plus two additional females to the center point treatment (Supplemental Table 1) and administered the appropriate hormone doses by intraperitoneal injection with 0.3 cc insulin syringes (BD Medical) at the designated time (Supplemental Table 2). At harvest time, we euthanized females and dissected oviducts into 300 µl room temperature M2 medium (Sigma-Aldrich, # M7167-100ML). We flushed oviducts with M2 medium into 24 well plates and recorded the number and quality of ova ("good" oocytes typically have granulated ooplasm and an incomplete and/or expanded cumulus; "bad" oocytes typically have homogeneous ooplasm with a compact and complete cumulus). We replicated the treatment producing the maximum yield of good oocytes yield in 4 additional *P. maniculatus* females to estimate the average number of oocytes collected (Supplemental Table 2).

We analyzed our data with linear models in R. We used the coded.data() function in the rsm package (Lenth 2009) to code our treatments and the cubePlot() function in the FrF2 package (Gromping 2014) to visualize our results. We constructed with a full model of all main

effects and pairwise interactions among the factors (y=lm(oocyte_number~.*.), and used the step() function to implement backward stepwise AIC model selection.

Table 1. Superovulation factors and levels tested in *P. maniculatus*. "-" represents the low setting of a factor, "+" represents the high setting of a factor, and "0" represents the intermediate setting of a factor.

Factor	**-**	**0**	+
Female age (weeks)	4	8	12
PMSG dose (I.U.s)	2.5	5	7.5
hCG dose (I.U.s)	5	10	15
Interval between hormones (hrs)	48	56	64
Harvest time (hrs)	18	21	24

Superovulation conditions in *Peromyscus leucopus & P. gossypinus*

We also tested superovulation conditions for two species from the sister group to *P. maniculatus*: *P. leucopus* and *P. gossypinus*. We performed a two-level 2^3 full factorial experiment for the three factors we identified as important for superovulation in *P. maniculatus*: PMSG dose, hCG dose, and the interval between the two hormones. We set the optimal *P. maniculatus* doses of PMSG and hCG as the lower and higher levels in our experiments with *P. leucopus* and *P. gossypinus*, respectively (Table 2; Supplemental Table 3). We held age and harvest oocyte time constant, using 4-week-old females and harvesting oocytes 18 hours post-hCG. This design had 8 treatments per species. We randomly assigned 8 females of each species to the 8 treatments and administered the appropriate hormone doses at the designated time intervals following Supplemental Tables 4 & 5.

Table 2. Superovulation factors and levels tested in *P. leucopus and P. gossypinus*. "-" represents the low setting of a factor and "+" represents the high setting of a factor.

Factor	**-**	+
PMSG dose (I.U.s)	7.5	12.5
hCG dose (I.U.s)	2.5	5
Interval between hormones (hrs)	56	64

RESULTS

Superovulation conditions in *Peromyscus maniculatus*

In analyzing our data, we realized that we had accidentally aliased harvest time with a two-way interaction effect of female age*PMSG dose when designing our experiment. This mistake was caused in part by the United States government shutdown in October of 2013, which prevented us from accessing the tables for a 2_V^{5-1} fractional factorial design from the National Institute of Standards and Technology (NIST/SEMATCH e-Handbook of Statistical Methods, http://www.itl.nist.gov/div898/handbook/, October 2013); this led us to construct these tables by hand, but we made an error in aliasing harvest time. However, when we selected a minimal model from our full regression model for the number of good oocytes collected using a backward stepwise algorithm and the Akaike Information Criterion (AIC), harvest time was dropped. If we assume that harvest time—the number of hours post-hCG before collection—had little significant effect on oocyte yield, which is reasonable because we set the levels so that oocytes should still be within the oviduct, then we have a fully replicated 4-factor factorial experiment. This is one of the advantages of factorial and fractional factorial designs—if factors are unimportant in a regression model, then collapsing data at those factors increases replication.

We thus analyzed a full regression model with four factors (excluding harvest time) with number of good oocytes collected as our response variable. When we minimized AIC through a backward stepwise algorithm from our full model, we found PMSG dose significantly affected oocyte yield and hCG dose and the interaction between hCG dose*Interval to be nearly significant (Table 3). Although we replicated this experiment once, we interpret the near significance of hCG dose and its interaction with the interval between hormone injections as evidence that these factors also influence oocyte yield. Surprisingly, female age did not appear to

affect oocyte yield; we did not detect a significant difference between oocyte yield from 4-week-old and 12-week-old females when grouping treatments by female age only (two-sided t-test: t = 0.6747, df = 12.432, p = 0.5122).

We combined data from female age and harvest time and plotted the average number of good oocytes as a cube plot (Figure A.1). The top front right corner, treatment 11, represents the highest average oocyte yield: 7.5 I.U.s of PMSG, 5 I.U.s of hCG, and 64 hours between the two hormones. We administered these hormones doses with a 64-hour interval to four additional 4-week-old females and harvested oocytes after 18 hours. The mean number of good oocytes ranged from 17 to 42, with a mean of 29 ± 4.2 S.E.M oocytes/female. If one imagines an axis from the bottom back left corner of the cube to the front top right corner, it is clear that the optimal superovulation conditions might be further maximized with a higher dose of PMSG, a lower dose of hCG, and a longer inter-dose interval.

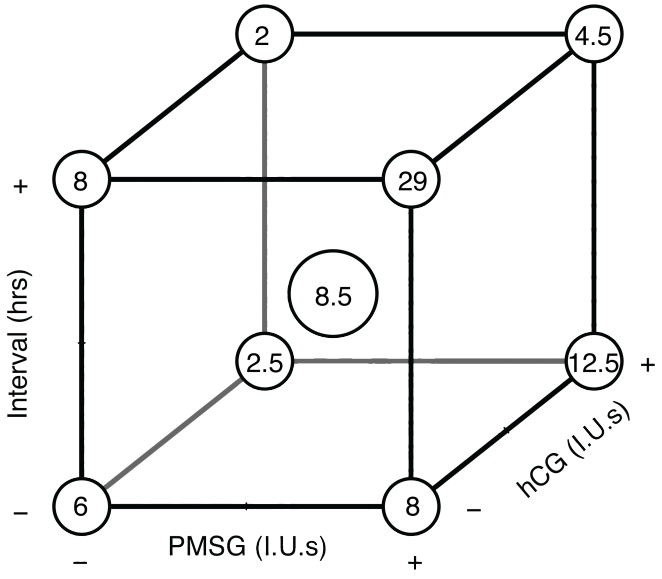

Figure A.1. Cube plot representing the average number of good oocytes collected at low and high levels for PMSG dose, hCG dose, and the interval between the two hormone injections. We collapsed data from runs at different ages and harvest times.

Table 3. Results from *P. maniculatus* regression model. Significance: *** p < 0.001, ** p < 0.01, * p < 0.05, . p < 0.1.

	Estimate	Std Error	t	Pr (>|t|)	Significance
(Intercept)	8.765	1.768	4.957	0.000333	***
PMSG	4.438	1.823	2.435	0.031462	*
hCG	-3.687	1.823	-2.023	0.065914	.
Interval	1.812	1.823	0.994	0.339634	
hCG*Interval	-3.937	1.823	-2.16	0.051678	.

Superovulation conditions in *Peromyscus leucopus & P. gossypinus*

We tested whether the conditions optimal for superovulation in *P. maniculatus* also worked in two other congeneric species. We tested the effects of PMSG dose, hCG dose, and the interval between the two hormones in a full factorial experiment. Cube plots for these experiments revealed that the best treatment for *P. maniculatus* (7.5 I.Us. PMSG, 5 IUs hCG, and 64 hours between injections—which corresponds to the top back left corner in Figure A.2) was ineffective for both *P. leucopus* and *P. gossypinus* (0 oocytes/female; top back left corner in each panel of Figure A.2). None of the factors tested significantly increased the number of good oocytes collected in a full linear regression model. After backward stepwise model selection with AIC, the intercept for good oocytes in each species was 1.4 for *P. leucopus* and 4.6 for *P. gossypinus*. If one were to collapse interval information for *P. gossypinus*, it would appear that higher doses of hCG may produce greater numbers of oocytes although hCG dose was not significant in our regression model.

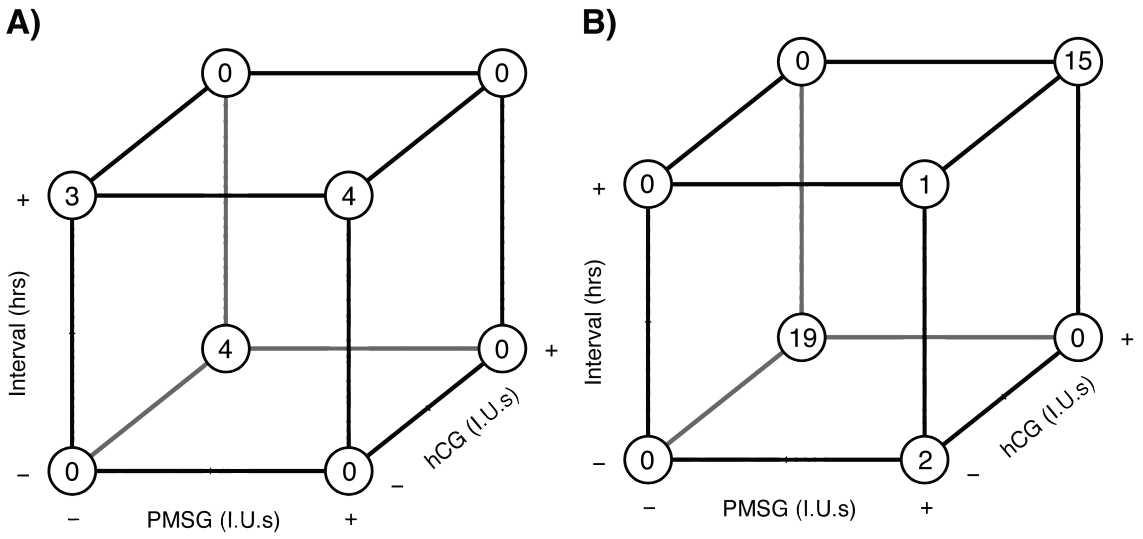

Figure A.2. Cube plots with number of good oocytes collected at low and high levels for PMSG dose, hCG dose, and the interval between hormone injections. (A) *Peromyscus leuocpus* results. (B) *Peromyscus gossypinus* results.

DISCUSSION

Prior to our experiment, no superovulation protocols could produce more than approximately 8-10 oocytes per female in *Peromyscus maniculatus*. While it was shown that the COC could be manually dissected to retrieve an average of 21 oocytes per female (Choi and He 2013), this procedure required two additional steps: IVM and IVF. With the superovulation protocol we have designed here, we can reliably collect nearly 30 oocytes per female and avoid manual dissection and IVM altogether. Our protocol also permits fertilized embryos to be created through natural mating instead of IVF, although IVF is possible if desired. We tested natural mating with three females by pairing each with a singly housed male (adding the female into the male's cage) immediately following hCG injection. With natural mating, we have been able to collect fertilized embryos that continue to grow and divide in M2 media for at least 4 hours after oviduct flushing (unpublished data). In addition to avoiding IVM and IVF, our superovulation protocol is also time efficient. Because we show that female age had no significant effect on

oocyte yield, we use 4-week-old females and save up to 8-10 weeks of time compared to other published protocols that used older mice (Veres et al. 2012; Choi and He 2013).

Now that we have optimized superovulation in *P. maniculatus*, the next steps will be to optimize natural mating and cryopreservation. The current practice for obtaining fertilized embryos is to add a superovulated female to a male's cage after hCG injection (Gertsenstein et al. 2003). Depending on how long a stud male takes to copulate upon pairing, the timing of when to establish pairs may need tuning. Cryopreservation may also need to be optimized, as it has been shown to vary depending on the strain of lab mouse (Byers et al. 2006). If optimization appears necessary, tweaking existing protocols would be a worthwhile venture because being able to freeze and store fertilized single-cell embryos would greatly enhance the ease with which transgenic experiments can be performed.

Despite our advances in developing a successful superovulation protocol for *P. maniculatus*, we were surprised to find that the same conditions did not produce *any* oocytes in two closely related species, *P. leucopus* and *P. gossypinus*. We tested just 3 factors in *P. leucopus* and *P. gossypinus* – PMSG dose, hCG dose, and interval between PMSG and hCG – holding female age and harvest time constant (4-weeks and 18 hours, respectively). Although we did not detect significant age effects in *P. maniculatus*, it is possible that age matters in *P. maniculatus* as well as *P. leucopus* and *P. gossypinus*. Moving forward, we recommend testing superovulation at a range of ages and hormone doses (possibly higher doses of hCG) in *P. leucopus* and *P. gossypinus*. Ideally, values of each hormone would be chosen to test whether the amount of hormones vs. the ratio between them is more important. In addition, other hormones may be worth testing (e.g. equine chorionic gonadotropin or follicle stimulating hormone instead of PMSG, or luteinizing hormone instead of hCG) (Martín-Coello et al. 2008; Pasco et al. 2012).

Unfortunately, though, our results suggest that *Peromyscus* species will require different superovulation protocols.

In summary, we have shown that fractional-factorial and factorial experiments can efficiently identify which factors and levels produce high number and quality of oocytes in *Peromyscus* species. Contrary to previously published results, we show that *P. maniculatus* can be reliably superovulated. However, further optimization is still needed for *P. leucopus* and *P. gossypinus*, two species from the sister group to *P. maniculatus*. Although unsuccessful in finding the perfect superovulation conditions for these other congeneric species, our results suggest where to put more effort. With several more iterations of experiments testing different hormones and for possible age effects, we are confident that superovulation will be possible in these species too. We recommend the use of factorial and fractional factorial experiments in developing superovulation protocols so researchers can minimize the number of animals needed per experiment, test multiple factors simultaneously, and detect interaction effects if they exist.

Supplemental Table 1. Fractional factorial design tables used for *P. maniculatus* superovulation experiment. Values highlighted in yellow indicate deviation in our experimental design from a 2_V^{5-1} fractional factorial experiment. These values should have used the opposite level.

Run	Age (weeks)	PMSG (I.Us.)	hCG (I.U.s)	Interval (hrs)	Harvest (hrs)
1	-	-	-	-	+
2	+	-	-	-	-
3	-	+	-	-	-
4	+	+	-	-	+
5	-	-	+	-	+
6	+	-	+	-	-
7	-	+	+	-	-
8	+	+	+	-	+
9	-	-	-	+	+
10	+	-	-	+	-
11	-	+	-	+	-
12	+	+	-	+	+
13	-	-	+	+	+
14	+	-	+	+	-
15	-	+	+	+	-
16	+	+	+	+	+
17	0	0	0	0	0

	-	0	+
Age	4	8	12
PMSG	2.5	5	7.5
hCG	5	10	15
Interval	48	56	64
Harvest	18	21	24

Run	Age (weeks)	PMSG (I.Us.)	hCG (I.U.s)	Interval (hrs)	Harvest (hrs)
1	4	2.5	5	48	24
2	12	2.5	5	48	18
3	4	7.5	5	48	18
4	12	7.5	5	48	24
5	4	2.5	15	48	24
6	12	2.5	15	48	18
7	4	7.5	15	48	18
8	12	7.5	15	48	24
9	4	2.5	5	64	24
10	12	2.5	5	64	18
11	4	7.5	5	64	18
12	12	7.5	5	64	24
13	4	2.5	15	64	24
14	12	2.5	15	64	18
15	4	7.5	15	64	18
16	12	7.5	15	64	24
17	8	5	10	56	21

Supplemental Table 2. *P. maniculatus* superovulation treatments and results.

Female ID	Treatment	DOB	Age (weeks)	PMSG (I.U.s)	hCG (I.U.s)	Interval (hrs)	Harvest (hrs)	# good oocytes	# bad oocytes
BW4808	1	9/30/13	4	2.5	5	48	24	0	3
BW3675	2	7/28/13	12	2.5	5	48	18	12	0
BW4163	3	9/15/13	4	7.5	5	48	18	8	0
BW3674	4	7/28/13	12	7.5	5	48	24	8	0
BW4189	5	9/20/13	4	2.5	15	48	24	5	0
BW3604	6	7/20/13	12	2.5	15	48	18	0	0
BW4812	7	10/1/13	4	7.5	15	48	18	22	0
BW3676	8	7/28/13	12	7.5	15	48	24	3	0
BW4810	9	9/30/13	4	2.5	5	64	24	14	0
BW3603	10	7/20/13	12	2.5	5	64	18	2	0
BW4169	11	9/16/13	4	7.5	5	64	18	33	4
BW3655	12	7/24/13	12	7.5	5	64	24	25	0
BW4807	13	9/30/13	4	2.5	15	64	24	0	5
BW3656	14	7/24/13	12	2.5	15	64	18	4	0
BW4809	15	9/30/13	4	7.5	15	64	18	4	1
BW3785	16	8/12/13	12	7.5	15	64	24	5	0
BW4058	17	9/4/13	8	5	10	56	21	4	0
BW4167	extra (3)	9/16/13	4	7.5	5	48	18	19	0
BW4170	extra (11)	9/16/13	4	7.5	5	64	18	42	0
BW4769	extra (11)	9/26/13	4	7.5	5	64	18	17	1
BW4770	extra (11)	9/26/13	4	7.5	5	64	18	29	0
BW4771	extra (11)	9/26/13	4	7.5	5	64	18	24	0
BW4059	extra (17)	9/4/13	8	5	10	56	21	13	0

Supplemental Table 3. Full factorial design tables used for *P. leucopus* and *P. gossypinus* superovulation experiment.

Run	PMSG (I.U.s)	hCG (I.U.s)	Interval (hrs)
1	-	-	-
2	+	-	-
3	-	+	-
4	+	+	-
5	-	-	+
6	+	-	+
7	-	+	+
8	+	+	+

	-	+
PMSG	7.5	12.5
hCG	2.5	5
Interval	56	64

Run	PMSG (I.U.s)	hCG (I.U.s)	Interval (hrs)
1	7.5	2.5	56
2	12.5	2.5	56
3	7.5	5	56
4	12.5	5	56
5	7.5	2.5	64
6	12.5	2.5	64
7	7.5	5	64
8	12.5	5	64

Supplemental Table 4. *P. leucopus* superovulation hormone injection schedule and results. The hormone injection schedule was arranged such that all 8 females would be ready for oviduct harvest and flushing at (9 am on a Wednesday).

Female ID	Run	DOB	PMSG (I.U.s)	Time PMSG	hCG (I.U.s)	Time hCG	Interval (hrs)	Time Harvest	# good oocytes	# bad oocytes
LLF591	1	12/9/13	7.5	Sat @ 7 AM	2.5	Tues @ 3 PM	56	Wed @ 9 AM	0	4
LLF592	2	12/9/13	12.5	Sat @ 7 AM	2.5	Tues @ 3 PM	56	Wed @ 9 AM	0	0
LLF565	3	11/5/13	7.5	Sat @ 7 AM	5	Tues @ 3 PM	56	Wed @ 9 AM	4	0
LLF568	4	11/12/13	12.5	Sat @ 7 AM	5	Tues @ 3 PM	56	Wed @ 9 AM	0	0
LLF566	5	11/12/13	7.5	Fri @ 11 PM	2.5	Tues @ 3 PM	64	Wed @ 9 AM	3	1
LLF562	6	11/5/13	12.5	Fri @ 11 PM	2.5	Tues @ 3 PM	64	Wed @ 9 AM	4	0
LLF590	7	12/9/13	7.5	Fri @ 11 PM	5	Tues @ 3 PM	64	Wed @ 9 AM	0	0
LLF569	8	11/12/13	12.5	Fri @ 11 PM	5	Tues @ 3 PM	64	Wed @ 9 AM	0	3

Supplemental Table 5. *P. gossypinus* superovulation hormone injection schedule and results. The hormone injection schedule was arranged such that all 8 females would be ready for oviduct harvest and flushing at (9 am on a Wednesday).

Female ID	Run	DOB	PMSG (IU)	Time PMSG	hCG (IU)	Time HCG	Interval (hrs)	Time Harvest	# good oocytes	# bad oocytes
GOF663	1	11/25/13	7.5	Sat @ 7 AM	2.5	Tues @ 3 PM	56	Wed @ 9 AM	0	0
GOF662	2	11/25/13	12.5	Sat @ 7 AM	2.5	Tues @ 3 PM	56	Wed @ 9 AM	2	0
GOF660	3	11/25/13	7.5	Sat @ 7 AM	5	Tues @ 3 PM	56	Wed @ 9 AM	19	11
GOF664	4	11/26/13	12.5	Sat @ 7 AM	5	Tues @ 3 PM	56	Wed @ 9 AM	0	0
GOF668/7	5	11/27/13	7.5	Fri @ 11 PM	2.5	Tues @ 3 PM	64	Wed @ 9 AM	0	1
GOF670	6	11/27/13	12.5	Fri @ 11 PM	2.5	Tues @ 3 PM	64	Wed @ 9 AM	1	0
GOF669	7	11/27/13	7.5	Fri @ 11 PM	5	Tues @ 3 PM	64	Wed @ 9 AM	0	3
GOF661	8	11/25/13	12.5	Fri @ 11 PM	5	Tues @ 3 PM	64	Wed @ 9 AM	15	2

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