Sexual imprinting and speciation in two *Peromyscus* species

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ABSTRACT

Sexual isolation, a reproductive barrier, can prevent interbreeding between diverging populations or species. Sexual isolation can have a clear genetic basis; however, it may also result from learned mate preferences that form via sexual imprinting. Here, we demonstrate that two sympatric sister species of mice—the white-footed mouse (*Peromyscus leucopus*) and its closest relative, the cotton mouse (*P. gossypinus*)—hybridize only rarely in the wild despite co-occurring in the same habitat and lack of any measurable intrinsic postzygotic barriers in laboratory crosses. We present evidence that strong conspecific mate preferences in each species form significant sexual isolation. We find that these mating preferences are learned in one species but may be genetic in the other: *P. gossypinus* sexually imprints on its parents, but innate biases or social learning affects mating preferences in *P. leucopus*. Our study demonstrates that sexually imprinting contributes to reproductive isolation that reduces hybridization between otherwise inter-fertile species, supporting a previously underappreciated role for learning in mammalian speciation.

INTRODUCTION

Sexual isolation, when divergent mating preferences limit or prevent interbreeding between individuals from different populations, is a prevalent premating reproductive barrier that may be vital to early stages of speciation. Comparative studies in fruit flies (Coyne and Orr 1989, 1997) and darter fish (Mendelson 2003) have shown that sexual isolation accumulates rapidly among young species relative to postzygotic reproductive barriers (e.g., hybrid sterility and inviability). Sexual isolation also can significantly reduce interbreeding among incipient sympatric species pairs (Coyne and Orr 1997; Noor 1997; Ramsey et al. 2003; Boughman et al. 2005; Nosil 2007; Matsubayashi and Katakura 2009), suggesting that it either allows species to co-exist or is selected via the process of reinforcement. In a few cases, sexual isolation is the sole reproductive barrier preventing hybridization between sympatric species, indicating that sexual isolation alone can be strong enough to inhibit hybridization and maintain genetic differentiation (e.g., Seehausen 1997; Fisher et al. 2006). Yet, despite the important role that sexual isolation can play among incipient species, its mechanistic basis—whether mating preference is genetic or learned—is often unknown.

Sexual isolation forms when mating traits and preferences diverge among populations, suggesting that sexual isolation should evolve when genetic loci for mating traits and preferences become linked (Felsenstein 1981). This scenario is a "two allele mechanism" of reproductive isolation, in which divergent alleles for trait and preference loci must fix between a pair of populations. As this mode of sexual isolation can break down due to recombination, linkage disequilibrium (particularly when caused by physical linkage) can promote sexual reproductive barriers. For example, plumage and plumage preferences are both sex-linked in finches and flycatchers and thus reduce hybridization between alternate color morphs (Pryke 2010) and

congener species (Saether et al. 2007), respectively. Alternatively, the issue of recombination could be circumvented if the loci for mating traits and preferences were under the same genetic control (i.e., pleiotropy). Pleiotropic genes undergoing divergent natural selection can also cause non-random mating and thus behave as "magic traits". Although several examples of magic traits (e.g., Jiggins et al. 2001; Mckinnon et al. 2004; Bradshaw & Schemske 2003) suggest they may not be as rare as previously thought (Servedio et al. 2011), the putative "magic" genes remain elusive. In either of these scenarios, a minimum of two alleles must be present and fixed in each population, requiring selection or drift in opposite directions in each species (Felsenstein 1981).

Sexual imprinting, the process of learning to prefer parental traits at a young age, is an alternate mechanism for establishing sexual isolation that arguably can be more efficient at establishing reproductive isolation than the above-mentioned genetic mechanisms. Sexual imprinting is immune to genetic recombination because learned mating preferences are automatically "inherited" with a given trait locus. Thus, sexual imprinting is considered a "one-allele mechanism" of reproductive isolation because the same "sexual imprinting allele" (e.g., an ability to learn parental traits) could result in assortative mating that reduces interbreeding between divergent populations, thereby reducing the number of steps required to achieve sexual isolation. An example of one-allele assortative mating locus has been localized in the *Drosophila* genome (Ortíz-Barrientos and Noor 2005). In addition to needing only one allele at a learning locus, several theoretical models have shown that learned mating preferences 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). Often, sexual imprinting will maintain reproductive isolation in the face of gene flow because it lowers the amount of divergent natural selection needed to isolate groups (Verzijden et al. 2005). Sexual imprinting may also enhance

61 sexual isolation in sympatry through reinforcement (Servedio et al. 2009). Finally, if sexual
62 imprinting is asymmetric—cases in which offspring prefer even more extreme versions of the
63 traits on which they imprinted—it may even facilitate divergence by creating a bias and thereby
64 selection for more divergent mating traits (ten Cate et al. 2006).

65 While sexual imprinting has long been recognized as a phenomenon that occurs within
66 species, its impact on speciation has become appreciated only recently (Irwin and Price 1999). It
67 is a phenomenon that occurs in species with parental care, and has been documented in over 15
68 orders of birds (ten Cate and Vos 1999) as well as some mammals (Kendrick et al. 1998) and
69 fish (Verzijden and ten Cate 2007; Kozak and Boughman 2009; Verzijden and Rosenthal 2011).
70 A few empirical studies have explicitly tested for a connection between sexual imprinting and
71 sexual isolation between closely related populations or species. For example, benthic and
72 limnetic sticklebacks sexually imprint paternal cues under ecologically divergent selection,
73 which results in significant sexual isolation between the two morphs (Kozak et al. 2011). Other
74 studies in cichlids (Verzijden and ten Cate 2007), great and blue tits (Slagsvold et al. 2002), and
75 Darwin's finches (Grant and Grant 1997) have demonstrated that sexual imprinting can maintain
76 sexual isolation. Therefore, sexual imprinting seems to be a key, but underexplored, avenue to
77 speciation.

78 Here we assess the role of sexual imprinting in generating reproductive isolation
79 between two mammalian species, the white-footed mouse (*Peromyscus leucopus*) and the cotton
80 mouse (*P. gossypinus*), which are thought to have diverged during the Pleistocene (Blair 1950).
81 *P. leucopus* is distributed across the Midwest and eastern United States, whereas *P. gossypinus* is
82 restricted to the Southeast (Figure 1); their ranges overlap in the Gulf Coast states, from Texas to
83 Virginia. These species show some level of sexual isolation: *P. leucopus* and *P. gossypinus*

hybridize and produce viable, fertile offspring in the lab (this study, Dice 1937, 1940); however, when multiple mice of each species are placed in large arenas, both species mate with conspecifics (Bradshaw 1965, 1968). While assortative mating in laboratory studies is potentially strong, there is mixed evidence as to whether it is strong enough to prevent hybridization in wild sympatric populations (Dice 1940, Howell 1921, McCarley 1954).

In this study, we used genomic data to first assess hybridization in the wild and conclusively found that the two species remain genetically distinct in sympatry despite rare hybridization events. We then examined the degree of sexual isolation between *P. leucopus* and *P. gossypinus,* and tested if it had a learned or genetic basis. Our results show that sexual imprinting produces strong sexual isolation between these sister species, and we suggest that learning disproportionately contributes to the total reproductive isolation we have observed in between two inter-fertile, sympatric species.

METHODS

Detection of hybrids in sympatric populations

Wild samples

During April 2008 and January-February of 2010 and 2011, we collected mice from seven allopatric locations and 13 sympatric locations (Figure 1). At each location, we placed up to 300 Sherman traps every 20 feet in transects of 50 traps per line. From each mouse captured, we took liver or tail tissue and stored tissues in 100% ethanol for subsequent DNA extraction. We augmented our own sampling with tissues collected at additional sites from museum specimens at the Harvard Museum of Comparative Zoology, Oklahoma State University Collection of Vertebrates, Sam Noble Museum of Natural History, and the Museum of Texas Tech University.

Precise collecting locations and sample sizes for all animals included in this study are provided in Supplementary Table 1.

Lab strains

We obtained *P. leucopus* animals from the *Peromyscus* Genetic Stock Center (University of South Carolina). The line was originally established with 38 founders brought into the lab during 1982-1985. In 2009, we established a breeding colony of *P. gossypinus* animals with 18 founders caught in Jackson and Washington counties in Florida. In captivity, both breeding colonies have been have been deliberately outbred to preserve genetic diversity.

All animals were housed in standard mouse cages in either mate pairs (one female and one male) or in same sex cages with a maximum of five adults. Offspring were weaned into same sex cages 23 days after birth. We set the light cycle to 14 hours of light and 10 hours of dark and maintained a room temperature between 70 and 77 degrees Fahrenheit. All mice were fed a regular Purina diet (Purina Iso Pro 5P76) *ad libitum*.

In addition to maintaining these two species strains, we also bred hybrids in the laboratory. First generation hybrids (F1) were generated from both *P. gossypinus* female x *P. leucopus* male matings as well as the reciprocal cross. Next, these F1 hybrids were backcrossed to either *P. gossypinus* or *P. leucopus*.

ddRADseq library construction and genotyping

We extracted genomic DNA from 374 wild-caught individuals and two lab-raised hybrids using an Autogen kit and AutoGenprep 965 instrument. We prepared double digest restriction-associated DNA tag (ddRAD) libraries from each individual following the protocol described in

Peterson et al. (2012). Briefly, we digested 100-200 ng of DNA from every individual with two restriction enzymes, EcoRI-HF and MspI (New England Biolabs) and purified the reactions with AMPure XP beads (Beckman Coulter Genomics). After quantifying the cleaned and digested product on a spectrophotometer plate reader (SpectraMax Gemini XS Plate Reader), we ligated approximately 50 ng of digested DNA to uniquely barcoded EcoRI adapters and MspI adapters in a 40 µl reaction volume with T4 DNA ligase (New England Biolabs). We pooled equal amounts of 48 ligated samples and used two rounds of AMPure XP bead purification to reduce the total pooled volume of up to 30 µl. We loaded each ligation pool onto a 2% agarose Pippin Prep cassette (Sage Science) and selected fragments with a size of 300 ± 35 bp. We ran five replicate Phusion PCRs according to the Finnzyme kit directions (Thermo Scientific) for 12 cycles with 5 µl of eluted Pippin Prep product as template. 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 purified them with AMPure XP beads 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 reads and aligned them 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 with default parameters (Lunter and Goodson 2011). We identified and removed adapter sequences with Picard-tools 1.100 (http://picard.sourceforge.net). We realigned potential indels with the Genome Analysis Tool Kit v. 3.2-2 IndelRealigner (GATK; McKenna et al. 2010) and performed SNP discovery across all samples simultaneously using the GATK UnifiedGenotyper (DePristo et al. 2011). We filtered alignments, keeping regions with 100 or more total reads and an average base quality greater than 20. We retained bi-allelic SNPs with a

minimum mapping quality of 30 that were present in at least 90% of our individuals at a depth of 10 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 and we randomly selected one SNP per cluster. Our final dataset contained 3,707 SNPs and 321 mice that had over 90% of genotypes present at these SNPs. Of these mice, 21 *P. leucopus* were caught at allopatric sites or lab-raised, 54 *P. gossypinus* were caught in allopatric sites or lab-raised, two individuals were hybrids from our colonies, and 244 individuals were of unknown ancestry from the predicted sympatric species range. Short read data were deposited in GenBank (accession number: SRPXXXXXX).

Identification of hybrids

We used genetic principal component analysis (PCA) to evaluate admixture between *P. leucopus* and *P. gossypinus*. We implemented genetic PCA using smartpca from the Eigensoft 6.0.1 package (Patterson et al. 2006) and output the first ten eigenvectors. After excluding outlier individuals and SNPs, our final dataset contained 288 individuals and 2,528 SNPs. We expected the first principal component to separate genetic differences between species and included known individuals from each species (allopatric and identified museum specimens) to identify PC1 values corresponding to each species. We assessed the eigenvector significance using Tracy-Widom statistics (Patterson et al. 2006) implemented using twstats in Eigensoft 6.0.1.

Measurement of sexual isolation between species

Using lab-based assays, we first tested for intrinsic postzygotic isolation and then estimated sexual isolation without mate choice. We could then compare our sexual isolation

176 estimate from no-choice assays to those with mate choice to quantify the contribution of mating
177 preferences to reproductive isolation between *P. leucopus* and *P. gossypinus*.
178
179 *Intrinsic postzygotic isolation and sexual isolation without choice*
180 We tested for intrinsic postzygotic sexual isolation and sexual isolation between lab-
181 raised *P. leucopus* and *P. gossypinus* using no-choice trials. We set up 20 crosses for each
182 conspecific and heterospecific pairing: L♀ x L♂, G♀ x G♂, L♀ x G♂, and G♀ x L♂ (in which
183 "L" represents *P. leucopus* and "G" represents *P. gossypinus*). When F1 offspring were
184 produced, we used these mice as F1 hybrids in additional no-choice trials in backcross mating
185 pairs: F1♀ x L♂, F1♀ x G♂, L♀ x F1♂, and G♀ x F1♂. We avoided any sib-sib or sib-parent
186 pairings.
187 We set up mating pairs by adding a sexually receptive virgin female to the cage of a
188 virgin, sexually mature male. We determined female sexual receptivity through vaginal lavage
189 and considered a female to be receptive between proestrus and estrus stages. We gave pairs 60
190 days to produce a litter, which is approximately 12 estrous cycles (mean estrous cycle length for
191 both species is 5-6 days; Dewsbury et al. 1977) or opportunities for successful reproduction. We
192 considered the production of offspring as a successful mating event and inferred the latency to
193 the first successful mating by subtracting the average gestation period—23 days in both species
194 (Pournelle 1952; Wolfe and Linzey 1977; Lackey et al. 1985)—from the total number of days
195 until a litter was born. Although our metric for mating success is conservative because it is
196 confounded with any fertility differences that might exist among individuals or between the
197 species, our assay nonetheless captures hybridization between these species.

We first used the no-choice assays to test hybrid viability and fertility in our laboratory strains of *P. leucopus* and *P. gossypinus*. We scored offspring survival to reproductive age in heterospecific crosses (L♀ x G♂, G♀ x L♂), and then used these F1 hybrids in backcrosses to look for evidence of reduced fertility relative to conspecific crosses. To compare the proportion of successful mating events between conspecific and heterospecific crosses, we used a logistic regression to quantify the effects of the female species, male species, or the interaction between female and male species. We then selected the best-fit model based on the lowest Akaike Information Criterion (AIC). We compared the 95% confidence intervals for the mean mating success among backcross pairs (F1♀ x L♂, F1♀ x G♂, L♀ x F1♂, G♀ x F1♂) to those of conspecific crosses. Together, these no-choice data provide an estimate of hybrid viability and relative fertility.

We next tested for differences in mating latency between conspecific, heterospecific, and backcross mating pairs using a non-parametric Kruskal-Wallis rank sum test followed by pairwise Wilcoxon tests with adjusted Bonferroni-corrected p-values. To quantify sexual isolation, we counted the number of successful mating events to estimate a joint isolation index, I_{PSI} (Rolán-Alvarez and Caballero 2000), which compares observed to expected mating events (assuming random mating among individuals) among conspecific and heterospecific pairs. This index ranges from -1 (all mating occurred between species) to +1 (all mating occurred within species), with a value of 0 indicating equal mating among pair types. We used the number of conspecific and heterospecific pairs that produced litters to estimate I_{PSI} in JMATING v. 1.0.8 (Carvajal-Rodriguez and Rolán-Alvarez 2006). We used 10,000 bootstrap replicates to estimate the isolation indices, their standard deviation, and to test the hypothesis that our estimates of the joint isolation deviated significantly from a null hypothesis of random mating.

221

222 *Sexual isolation with choice*

223 We contrasted our estimate of the sexual isolation index (I_{PSI}) from no-choice assays to
224 the sexual isolation index estimated from two-way choice assays. We measured conspecific
225 mating preferences in a two-way electronically-controlled gated mate choice apparatus that
226 consisted of three collinear rat cages, with each pair of cages separated by two RFID antennae
227 and gates (FBI Science Gmbh; Figure 3A). Each pair of gates was programed to allow passage
228 depending on the identity of the mouse. Specifically, for each trial we implanted three mice with
229 small transponders (1.4 mm x 9 mm, ISO FDX-B, Planet ID Gmbh) in the interscapular region
230 using a sterile hypodermic implanter and programmed the gates to allow the designated
231 "chooser" mouse (i.e. the individual whose preference we tested) to pass freely through all cages
232 while constraining each "stimulus" mouse to the left or right cage, respectively.

233 With this apparatus, we tested mate preferences of males and females of each species for
234 conspecific and heterospecific stimuli of the opposite sex. We allowed the chooser mouse—
235 either a sexually receptive virgin female (in proestrus or estrus as determined by vaginal lavage)
236 or a sexually mature virgin male—to acclimate to the apparatus for one day, adding food, water,
237 used nesting material, and a hut from each stimulus mouse's colony housing cage to the flanking
238 cages of the apparatus. Approximately 24 hours later, we returned the chooser mouse to the
239 center cage if it had not already nested there, closed all gates, and added stimulus mice to the two
240 flanking cages to allow them two to four hours to acclimate to their new environment. At lights
241 out (4:00 pm; 14:10 hour light:dark cycle), we re-opened the gates and recorded RFID readings
242 at all antennae as well as webcam video streams from each flanking cage for two nights (~44
243 hours; camera model: DLINK DCS-942L). Each chooser mouse was tested once.

244 At the end of each trial, we parsed a log file of RFID readings and calculated chooser
245 preference for a stimulus as the proportion of time spent with that stimulus divided by the time
246 spent with both stimuli (i.e. a ratio of association time between the stimuli). We analyzed only
247 trials in which the chooser mouse investigated both cages during the acclimation, spent at least
248 10 minutes investigating at least one stimulus during the trial, and the stimuli mice were in their
249 cages at least 75% of the trial period (we discarded 15% of trials that did not meet these criteria).
250 We compared the preferences of 8-11 adults (at 9-14 weeks of age) of each species and
251 sex for conspecific and heterospecific stimuli of the opposite sex. For female-choice trials, we
252 tested virgin female preferences for either: (1) pairs of sexually experienced males that had
253 successfully sired offspring with a conspecific female prior to use in the two-way choice trials
254 (*P. leucopus*, $N = 5$ trials; *P. gossypinus*, $N = 7$ trials), or (2) pairs of virgin males as stimuli (*P.*
255 *leucopus*, $N = 6$ trials; *P. gossypinus*, $N = 4$ trials). Because we did not detect a significant
256 difference in female preference based on male stimulus sexual experience (two-sided Wilcoxon
257 rank sum test, *P. gossypinus* females: $W = 9$, $p = 0.41$; *P. leucopus* females: $W = 15$, $p = 1$), we
258 combined female preference data from trials with sexually experienced and virgin male stimuli.
259 For male-choice trials, we used only virgin females as stimuli.
260 We estimated I_{PSI} for each sex separately in JMATING v. 1.0.8 (Carvajal-Rodriguez and
261 Rolán-Alvarez 2006) because behavior of the stimuli may not be similar across male- and
262 female-choice trials. We estimated I_{PSI} by considering the chooser and its most preferred
263 stimulus as a "mated" pair; when we observed no mating, we replaced zero values with a 1 to
264 allow for bootstrapping with resampling. We used 10,000 bootstrap replicates to estimate the
265 isolation indices and test for deviation from random mating ($I_{PSI} = 0$).
266

267 **Testing for sexual imprinting**

268 To determine whether conspecific mating preferences are learned in the nest, we
269 measured the preferences of mice from each species after they had been cross-fostered—raised
270 from birth until weaning—by parents of the opposite species. We swapped whole litters at birth
271 between breeding pairs of *P. leucopus* and *P. gossypinus*, reducing litters to the same number of
272 offspring if litters differed in number of pups. All cross-fostering attempts were successful,
273 indicating that parents readily attended to unrelated offspring. We allowed cross-fostered
274 offspring to remain with their foster parents until weaning (23 days after birth), when we
275 separated offspring into same sex cages; this matches the life cycle of all other mice in our study.
276 As a control, we also cross-fostered offspring within species (i.e. swapped litters between
277 conspecific families) to partition the effects of litter transfer and foster parent species on mating
278 preference. Although there is mixed (or incomplete) information for whether fathers contribute
279 parental care in *P. leucopus* and *P. gossypinus* (Hartung and Dewsbury 1979; Schug et al. 1992),
280 we cross-fostered offspring to both parents because we maintain male-female breeding pairs in
281 our laboratory colonies of *P. leucopus* and *P. gossypinus* and aimed to compare preferences of
282 mice from cross-fostered and non-cross-fostered trials.

283 We tested the mating preferences of all cross-fostered mice in the two-way gated choice
284 assay described above. We predicted that if young mice sexually imprint on their parents, cross-
285 fostered mice raised with the opposite species should prefer heterospecific stimuli and exhibit a
286 weaker preference for conspecifics compared to individuals raised by their biological parents or
287 other unrelated conspecific parents. We evaluated the effects of chooser sex and cross-fostering
288 treatment on preferences for *P. leucopus* in each species, using linear modeling after applying an
289 arcsin transformation to the proportion of time spent with *P. leucopus*. To test for the possibility

that the sexes within each species might react differently to cross-fostering, we considered models with and without an interaction between chooser sex and cross-fostering treatment and selected the best-fit models based on the lowest AIC.

Assessment of two-way choice assay

We confirmed that our two-way mate choice assay accurately predicts mating preference by measuring whether the most preferred stimulus corresponded to mating events in a subset of trials in which mating occurred. We identified trials with successful mating events by either 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 the male stimuli and the pups at two to three microsatellite markers (loci 14, 35, and 80 from Weber et al. 2010) following the protocol described in Weber et al. 2010 (N = 15 trials) or screening video data for copulation events (N = 5 trials). We tested whether the most preferred individual (as determined by the greatest proportion of association time) predicted mating success using a linear regression. We applied an arcsin transformation to association time proportions. This analysis allowed us to determine that association time is an accurate predictor of mating, and thus reflects mating preference.

RESULTS

Hybridization is rare in sympatric populations

Using thousands of markers across the genome summarized in a genetic PCA, we tested for evidence of hybridization between *P. leucopus* and *P. gossypinus* in sympatric populations. We identified six significant principal components by Tracy-Widom statistics with the following

313 eigenvalues: (1) 37.855, (2) 4.352, (3) 3.627, (4) 3.161, (5) 3.054, and (6) 2.941. Based on
314 clustering with known allopatric and previously identified *P. leucopus* and *P. gossypinus*
315 individuals, the first eigenvector clearly separates *P. leucopus* (negative values) and *P.*
316 *gossypinus* (positive values) (Figure 1A). As expected, a control lab-generated F1 hybrid falls at
317 the midpoint along the first eigenvector and a lab backcross mouse (F1 x *P. gossypinus*) falls half
318 way between the F1 hybrid and the mean value of *P. gossypinus* values (Figure 1A). Of the 244
319 sympatric mice we collected, all could be easily assigned to either the *P. leucopus* or *P.*
320 *gossypinus* species, with only two exceptions: two mice from Big Lake Wildlife Management
321 Area, Louisiana had intermediate values along eigenvector 1 (Figure 1A). These admixed
322 individuals showed greater *P. leucopus* ancestry, similar to a F1 backcross or advanced
323 backcross to *P. leucopus*, indicating that there may be biased gene flow from *P. gossypinus* into
324 *P. leucopus*.

325 The second eigenvector revealed two genetically distinct *P. gossypinus* subgroups. These
326 likely reflect genetic differences between *P. gossypinus* subspecies, *P. gossypinus gossypinus*
327 and *P. gossypinus megacephalus*. Specifically, higher PC2 values corresponded to mice caught
328 east of the Mississippi River—which are more likely to be *P. g. gossypinus*—whereas lower PC2
329 values corresponded to mice caught west of the river—which are more likely to be *P. g.*
330 *megacephalus* (Wolfe and Linzey 1977). The Mississippi river is a known biogeographic barrier
331 for many species (Soltis et al. 2006), and our data suggest that this may also be the case for *P.*
332 *gossypinus*. Only one individual from the Tunica Hills wildlife management area population in
333 Louisiana failed to fit this pattern (Figure 1A): this individual occurred to the east of the
334 Mississippi River but it clustered with individuals from the western group. We did not find any
335 evidence to suggest a similar barrier to gene flow in *P. leucopus*, but we also did not have the

equivalent population-level sampling on both sides of the river. The remaining four eigenvectors (3, 4, 5, and 6) identified population structure within *P. leucopus* (Supplemental Figure 1).

P. leucopus and _P. gossypinus_ co-occur in mosaic sympatry

Using cluster assignments based on the genetic PCA, we identified six of 13 sympatric sites that contained both species (Figure 1B). However, the other seven sites contained only a single species, highlighting the patchy distribution of both species within their broadly sympatric range from Texas and Virginia. Thus, our data confirm that the two species occur in mosaic sympatry.

No evidence for intrinsic postzygotic isolation

Previous studies have suggested that there is no measurable intrinsic postzygotic isolation in laboratory crosses of *P. leucopus* and *P. gossypinus* (Dice 1937). We confirmed this result in our independent lines (i.e., different spatial and temporal origin) of these two species. We first measured reproductive success within and between species in no-choice assays. The proportion of crosses that produced offspring was determined largely by the female (logistic regression: β = 1.25, SE = 0.47, p = 0.008), with *P. leucopus* females showing greater mean mating success than *P. gossypinus* (Supplemental Figure 2). Importantly, this means that *P. leucopus* females had greater reproductive success with *P. gossypinus* males (12/20 pairs had offspring) than the reciprocal cross between *P. gossypinus* females and *P. leucopus* males (6/20 pairs had offspring), indicating some asymmetry in either mate preferences, copulation attempts, or female fertility. Successful heterospecific crosses confirmed that we can produce viable F1 hybrids, which survive until reproductive age. In addition, we compared the mating successes of backcrosses to

conspecific and heterospecific crosses. We found that these F1 hybrids are as fertile in backcrosses (i.e., had similar frequency of litter production) as either conspecific or heterospecific crosses, and that all backcross offspring are also viable (Supplemental Figure 2).

Mate choice causes significant sexual isolation

We examined whether mating preferences lead to significant sexual isolation between the species in a laboratory environment (Supplemental Table 2). In no-choice assays, heterospecific pairs hybridized and produced viable offspring, indicating no measurable sexual isolation exists in the absence of mate choice (I_{PSI} = 0.00, *SD* = 0.19, p = 0.960; Figure 2). However, conspecific, heterospecific, and backcross mating pairs had significantly different latencies to produce offspring (Figure 2; Kruskal-Wallis: χ^2 = 6.7626, df = 2, p = 0.034). Pairwise comparisons between mating pairs revealed significance differences in latency to mating only between conspecific and heterospecific mating pairs (W = 69, $p_{Bonferroni}$ = 0.010). Heterospecific pairs took an average of 5.4 days longer to produce litters than conspecific pairs, indicative of either delayed heterospecific mating or longer hybrid gestation times. This delay is roughly equivalent to one estrus cycle in *Peromyscus* (Dewsbury et al. 1977).

By contrast, we detected significant sexual isolation between the species in two-way choice assays (Supplemental Table 2). Sexual isolation estimates were similar in female- and male-choice trials: *P. leucopus* and *P. gossypinus* females strongly preferred conspecific mates (Figure 3B; I_{PSI} = 0.75, *SD* = 0.14, $p < 0.01$) as did *P. leucopus* and *P. gossypinus* males (Figure 3B; I_{PSI} = 0.75, *SD*= 0.15, $p < 0.01$). More generally, there were strong preferences for conspecific mates in both species, regardless of sex.

382 **Sexual imprinting contributes to sexual isolation in at least one species**

383 We next investigated whether mating preferences in these species had a learned or
384 genetic basis using a series of cross-fostering experiments and found that cross-fostering had
385 different effects on mating preference in the two focal species. In *P. leucopus*, the AIC selected a
386 model in which cross-fostering, sex, and their interaction was significant ($F = 5.09$ on 3 and 25
387 df, $p = 0.007$). When raised with their own parents, *P. leucopus* of both sexes preferred *P.*
388 *leucopus* stimuli (Figure 3B; female *P. leucopus* preference $\hat{p} = 0.689$; male *P. leucopus*
389 preference $\hat{p} = 0.959$). *P. leucopus* males that were cross-fostered significantly changed their
390 preference (Figure 3C; *P. leucopus* preference $\hat{p} = 0.184$; t = 3.853, $p_{Bonferroni} = 0.001$), whereas
391 cross-fostering did not significantly change female preferences (Figure 3; *P. leucopus* preference
392 $\hat{p} = 0.764$; t = -0.390, $p_{\text{Bonferroni}} = 1$). Thus, *P. leucopus* females always preferred *P. leucopus* to
393 *P. gossypinus* mates, whereas males spent more time with the species by whom it was raised
394 In *P. gossypinus*, the model selected by AIC showed a strong cross-fostering effect but no
395 significant sex effects or interactions between cross-fostering and sex ($F = 51.31$ on 1 and 33 df,
396 $p < 0.001$). When raised with their own parents, *P. gossypinus* of both sexes preferred *P.*
397 *gossypinus* stimuli (Figure 3B; *P. leucopus* preference $\hat{p} = 0.069$), whereas *P. gossypinus* raised
398 with *P. leucopus* preferred *P. leucopus* stimuli (Figure 3C; *P. leucopus* preference $\hat{p} = 0.781$).
399 To confirm that the preference reversal in *P. gossypinus* was not an effect of transferring
400 litters but rather an effect of the foster parent species, we collected an additional control dataset
401 for *P. gossypinus.* We cross-fostered *P. gossypinus* to unrelated *P. gossypinus* foster parents
402 (females: $N = 4$, males: $N = 7$) and found that foster species, and not the litter transfer itself,
403 affected *P. gossypinus* preferences (Supplemental Figure 3). Pairwise t-tests on arcsin-
404 transformed proportion of time spent with *P. leucopus* revealed no significant differences

between *P. gossypinus* raised with their own parents or unrelated conspecific parents (t = -0.72, df = 15.38, $p_{Bonferroni}$ = 1). Thus, *P. gossypinus* males and females preferred *P. gossypinus* to *P. leucopus* mates, independent of if they were related to their conspecific parents.

To examine the effects of sexual imprinting on sexual isolation, we calculated the sexual isolation index assuming the most preferred stimulus from each heterospecifically cross-fostered choice trial (Figure 3C) as a "successful mating". Cross-fostering eliminated sexual isolation in female-choice trials (I_{PSI} = 0.25, SD = 0.34, p = 0.57) and male-choice trials (I_{PSI} = -0.29, SD = 0.42, p = 0.32). Thus, our cross-fostering results confirm that sexual isolation between *P. leucopus* and *P. gossypinus* is the result of sexual imprinting.

Two-way choice test accurately measures preference

To confirm that the time spent with either stimulus mouse was an accurate predictor of mate preference and hence mate choice, we recorded 20 mating events in our two-way choice assays: 12 mating events occurred in trials where choosers were raised with their own parents and 8 trials where choosers were raised with heterospecific foster parents. In 19 out of 20 trials, choosers mated with the stimulus individual with which they spent the most time (Figure 4). Mating outcome (with conspecific or heterospecific stimulus) was predicted by proportion of time spent with the conspecific stimulus (logistic regression: β = 10.06, SE = 4.86, p = 0.04), indicating that our two-way choice assay accurately detects mating preferences.

DISCUSSION

Sexual imprinting could be a powerful generator of sexual isolation because it quickly and effectively associates preferences with traits in populations. Furthermore, sexual imprinting

has been documented in a diversity of taxa—e.g. birds, fish, mammals, amphibians, and insects—suggesting it could be a broadly important driver of speciation (Immelmann 1975). Our study shows that sexually imprinted mate-choice has maintained and contributed to strong sexual reproductive isolation in a classic mammalian system.

Rare hybridization in sympatry indicates a high degree of reproductive isolation

To test the strength of reproductive isolation between *P. leucopus* and *P. gossypinus* in nature, we collected 374 mice from across their range overlap and generated genomic data to measure the frequency of hybridization between these species in sympatry. Classic studies by mammalogists in the mid 1900's reported mixed evidence as to the extent of interspecific hybridization in sympatric populations. In Louisiana, Alabama, and southern Illinois, Howell (1921) and later McCarley (1954) identified a few intermediate individuals resembling hybrids based on morphology and allozyme genotypes. By contrast, Dice (1940) found no evidence of morphological intermediates in his studies in Virginia. Thus, it was unclear how frequently these species hybridize in the wild.

In our study, we found less than 1% of individuals collected from sites in which both species co-occurred were admixed. Our genomic analysis thus supports the conclusions of previous studies that suggested *P. leucopus* and *P. gossypinus* may remain genetically distinct in nature (Dice 1940; Price and Kennedy 1980; Robbins et al. 1985) in spite of rare hybrids (Howell 1921; McCarley 1954b; Barko and Feldhamer 2002). The two hybrids that we found were both from Big Lake Wildlife Management Area (Louisiana), and they had greater proportions of *P. leucopus* ancestry. At this site, *P. gossypinus* were less common than *P. leucopus*, potentially accounting for the biased gene flow into *P. leucopus*. Previous behavioral

451 data also support the possibility of biased gene flow from *P. gossypinus* into *P. leucopus*. For
452 example, using population cages to test interactions among pairs of male and female *P. leucopus*
453 and *P. gossypinus*, Bradshaw (1965) found that *P. leucopus* females were far more tolerant of *P.*
454 *gossypinus* males than vice versa. Although no hybrid offspring were produced in this study, if
455 heterospecific matings were to occur, one would predict they would be in the direction of a *P.*
456 *leucopus* female with a *P. gosyspinus* male.
457 Although we detected some admixture and possible biased gene flow from *P. gossypinus*
458 into *P. leucopus*, the overall lack of pervasive hybridization between these otherwise inter-fertile
459 species suggests that they are strongly reproductively isolated in nature. Our genomic data
460 revealed that *P. leucopus* and *P. gosssypinus* are distributed in a mosaic sympatry, with many
461 sites containing only one species (seven of thirteen sampling sites). This patchiness could be
462 driven by differences in microhabitat use: *P. leucopus* often occupy upland habitat and use more
463 arboreal nest sites while *P. gossypinus* often occupy swamps and bottomland habitat and use
464 more ground nest sites when they co-occur (McCarley 1954c, 1963; Taylor and Mccarley 1963).
465 However, these habitat differences are not enough to exclude contact in sympatry because both
466 species can be trapped in the same patch of forest, especially where these habitat types abut
467 (Dice 1940; Calhoun 1941; Price and Kennedy 1980; Roehrs et al. 2012). In fact, we often
468 caught both species in the same trap line. Similarly, there do not appear to be any significant
469 differences in breeding seasons: the two species have overlapping peak reproductive activities in
470 the winter months, but adults from both species can also be caught in reproductive condition
471 throughout the year in Texas, Louisiana, and Alabama (Pournelle 1952; McCarley 1954a; Wolfe
472 and Linzey 1977). Thus, the distributions, habitat preferences, and breeding seasons do not

appear to form complete or even strong reproductive barriers, suggesting that behavioral differences may be the most important cause of reproductive isolation.

P. leucopus and _P. gossypinus_ remain genetically distinct due to learned sexual isolation

We tested for evidence of sexual isolation, as previous studies suggested that mating preferences might explain the lack of hybridization in the wild. Using no-choice and choice trials to examine *P. leucopus* and *P. gossypinus* mating preferences, we found that conspecific preferences form a significant sexual reproductive barrier between the two species. Without a choice of mates, *P. leucopus* and *P. gossypinus* did not show significant sexual isolation, although there was an increase in latency to mate in heterospecific relative to conspecific pairs. However, when given a choice of mates, the species mated assortatively, and we estimated the average joint sexual isolation index (I_{PSI}) between the species to be 0.651. While sexual isolation is not yet complete ($I_{PSI} < 1$) between these species, the amount of sexual isolation we have observed is far greater than what has been detected among cactophilic ($I_{PSI} = 0.12$; Etges and Tripodi 2008) or Caribbean *Drosophila* ($I_{PSI} = 0.159$-0.282; Yukilevich and True 2008), walking stick insect populations ($I_{PSI} = 0.24$-0.53; Nosil et al. 2013), or gold and normal Nicaraguan cichlid color morphs ($I_{PSI} = 0.39$ and 0.86; Elmer et al. 2009), placing *P. leucopus* and *P. gossypinus* quite far along a speciation continuum.

We cannot determine if sexual isolation resulted because of strong female preferences and weak male preferences (where males select mates based on female acceptance or rejection) or the reverse, weak female preferences and strong male preferences (where females select mates based on male courtship). However, because our test apparatus provides the opportunity for

choosers to avoid either stimulus, we interpret a pattern of sexual isolation in female- and male-choice trials to be the result of active conspecific preferences in both sexes.

Our two-way choice results, compared to our no-choice results, indicate that mating preferences increase sexual isolation (Coyne et al. 2005). Testing the mating preferences of heterospecifically cross-fostered mice revealed that both species are affected by sexual imprinting, but that the degree of imprinting differed by species and sex. For one, both male and female *P. gossypinus* strongly sexually imprinted on their foster parent species, indicating that mating preferences in *P. gossypinus* are entirely learned. By contrast, we found that *P. leucopus* also sexually imprint on their parents, though weakly. Some *P. leucopus* males had a reduced preference for conspecifics when raised with heterospecific parents, whereas all *P. leucopus* females appeared unaffected by cross-fostering. In other words, *P. leucopus* females showed an own species bias, suggesting that their mate preferences could be genetic. *P. leucopus* showed a similar sexual difference in imprinting in a study that examined *P. leucopus* preferences for soiled bedding after cross-fostering to grasshopper mice, *Onychomys torridus* (McCarty and Southwick 1977a): male and female *P. leucopus* raised with *O. torridus* parents had decreased preference for conspecific soiled bedding, but the effect was more dramatic in males than females. Thus, both *P. leucopus* and *P. gossypinus* appear to learn mating preferences, but the degree of sexual imprinting varies between the two species, particularly in cross-fostered *P. leucopus* females which show a bias towards mates of their own species.

Genetic preference or preference reversal after socialization may account for the lack of sexual imprinting observed in *P. leucopus* females

517 Sex differences in sexual imprinting are most likely caused by sex-specific genetic (or
518 epigenetic) differences in learning, or learning in both sexes followed by preference reversal in
519 one sex after social interaction. Sex-specific genetic differences that affect the length of the
520 sensitive learning period or how the learned preference is internalized could create a sex bias in
521 learning. In some species such as sticklebacks and cichlids, only one sex (females) sexually
522 imprints (Verzijden and ten Cate 2007; Verzijden et al. 2009; Kozak et al. 2011); in other species
523 such as zebra finches, both sexes sexually imprint but on different parental cues (Vos 1995).
524 Learning in these species may be affected by sex-specific differences, but genetic loci that cause
525 these differences have yet to be identified. Alternatively, conspecific mating preferences could
526 be epigenetically-determined by parental behaviors or experiences. Some behavioral traits such
527 as pup licking in rats (Francis et al. 1999), fear of odors in mice (Dias and Ressler 2013), anxiety
528 in stickleback fish (McGhee and Bell 2014), have been linked to epigenetic inheritance. We
529 cannot rule out that conspecific preferences in female *P. leucopus* were determined
530 epigenetically, but suspect that social interactions most likely explain the own species bias in *P.*
531 *leucopus* females.

532 Social interactions can also influence sexual imprinting, and may be more likely to
533 account for sex differences. Irwin & Price (1999) describe a learning model in which offspring
534 might initially develop a generalized behavioral response to a range of traits resembling their
535 parents that later contracts after experience with heterospecifics. In cross-fostering studies, this
536 model would be supported by instances in which individuals sexually imprint on their foster
537 parents but subsequently alter their preferences in the presence of conspecifics. For example, a
538 longitudinal study of the effects of cross-fostering between sheep and goats found that females
539 initially preferred males of their foster species but later preferred conspecifics after a year of

socialization with conspecifics (Kendrick et al. 1998). Such preference reversals might be driven because of courtship behavior.

Species in which females show an own species bias after cross-fostering treatments with heterospecific parents have often been shown to be affected by differences in the courtship activity of male stimuli. For example, female zebra finches raised with heterospecific Bengalese foster parents spent more time with Bengalese males but directed more sexually receptive tail quivering behavior to conspecific males (ten Cate and Mug 1984); this own species bias was shown to be the result of greater conspecific male courtship activity. Male zebra finches sang more vigorously and frequently than Bengalese males, biasing female sexual behavior toward conspecifics (ten Cate and Mug 1984). Male courtship activity has also been shown to modify sexually imprinted preferences in female mallards (Bossema and Kruijt 1982; Kruijt et al. 1982). In our trials, male *P. leucopus* stimuli could have directed more copulatory behavior toward *P. leucopus* females, whereas male *P. gossypinus* stimuli may have been antagonistic, causing *P. leucopus* females to develop stronger preferences for conspecific males. Females could have been responsive to differences in ultrasonic vocalizations which help attract mates (Pomerantz et al. 1983; Musolf et al. 2010), male mounting attempts, or aggression. Because our choice assay permits physical interaction between the stimuli and the chooser mice, we cannot rule out the very likely possibility that *P. leucopus* females did sexually imprint but are affected by the behavior of stimuli in our two-way choice assay.

Further studies designed to test for sexual imprinting as a function of species-directed male courtship may be able to determine whether *P. leucopus* females sexually imprint but alter their preferences based on conspecific courtship activity, or if they truly show an innate preference for males of their own species. Whether *P. leucopus* females show biased conspecific

563 preferences because of genetic differences or subsequence social interactions, the asymmetry in
564 *P. leucopus* female and *P. gossypinus* female responses suggest that the species differ
565 meaningfully in either female learning or male courtship behaviors.
566
567 **Sexual imprinting cues**
568 Although we do not know the precise imprinting cues (e.g., odors, vocalizations) that *P.*
569 *leucopus* and *P. gossypinus* learn in the nest, they most likely learn olfactory cues as other
570 rodents are known to imprint on nest odors (Mainardi et al. 1965; Marr and Gardner 1965; Carter
571 and Marr 1970; Quadagno and Banks 1970; McCarty and Southwick 1977a; Porter et al. 1983).
572 If *P. leucopus* and *P. gossypinus* diverged in odor sources containing species, sex, or individual
573 information such as saliva (Gray et al. 1984; Smith and Block 1991; Talley et al. 2001), urine
574 (Doty 1973; Smadja and Ganem 1998; Pillay 2000; Hurst et al. 2001), scent marks (Johnston and
575 Brenner 1982; Becker et al. 2012), or major histocompatibility complex alleles (Yamazaki et al.
576 1979; Brown et al. 1989), these could serve as imprinting cues (e.g. Penn & Potts 1998). *P.*
577 *leucopus* and *P. gossypinus* have diverged in small urinary proteins (Cain et al. 1992) and *P.*
578 *leucopus* have been shown to sexually imprint on olfactory information (McCarty and Southwick
579 1977b), suggesting that sexual imprinting could be olfactory-based in this species pair.
580 Natural olfactory signals, which are affected by diet, could have been obscured by the
581 common Purina laboratory diet that we fed our *P. leucopus* and *P. gossypinus* stocks. Diet
582 influences odors in guinea pigs (Beauchamp 1976), mice (Schellinck et al. 1992), and voles
583 (Ferkin et al. 1997), and *P. gossypinus* and *P. leucopus* consume different diets in nature. *P.*
584 *gossypinus* is mainly carnivorous, with over two thirds its stomach contents containing animal
585 matter (insects and gastropods) compared to *P. leucopus*, which is primarily herbivorous, with

586 greater than two thirds of its stomach contents containing plant matter (Calhoun 1941). If
587 species-specific olfactory signals (e.g., urine, feces, scent marks) were affected by different diets,
588 imprinting cues and thus sexual isolation may be much more pronounced between natural
589 populations of *P. leucopus* and *P. gossypinus* than what we have detected in the laboratory.
590
591 **Sexual imprinting may limit the effects of rare hybridization**
592 When gene flow occurs, theoretical models have shown that sexual imprinting can create
593 substantial reproductive isolation and facilitate sympatric speciation more often than genetic
594 preferences (Verzijden et al. 2005). When hybrid fitness is high, as is the case for *P. leucopus*
595 and *P. gossypinus*, mating preferences formed by sexual imprinting may be more effective at
596 producing reproductive isolation than if the preferences were genetically controlled on
597 autosomes or sex chromosomes (Servedio et al. 2009).
598 We suspect that occasional hybridization between *P. leucopus* and *P. gossypinus* might
599 be tolerated because sexual imprinting would prevent the formation of a hybrid swarm. The two
600 hybrids that we found showed greater *P. leucopus* ancestry, indicating asymmetrical gene flow
601 from *P. gossypinus* to *P. leucopus*. While we did not have mitochondrial or Y chromosome
602 markers in our dataset that would have allowed us to identify which type of heterospecific
603 mating pair produced these hybrids, our no-choice data indicate that *P. leucopus* females have
604 greater reproductive success with *P. gossypinus* males than *P. gossypinus* females have with *P.*
605 *leucopus* males. We therefore predict that the few hybrids we found are likely offspring from an
606 initial cross between a *P. leucopus* mother and a *P. gossypinus* father. Futhermore, *P. leucopus*
607 females are far more tolerant of *P. gossypinus* males than the reciprocal direction (Bradshaw
608 1965). If *Peromyscus* offspring sexually imprint on their mothers as they do in other mammals,

609 we would expect F1 hybrids to preferentially backcross with *P. leucopus* mates, facilitating
610 asymmetrical gene flow while simultaneously preventing rampant hybridization. This type of
611 sexual imprinting analysis in F1 hybrids is seldom done (but see Albert 2005), but could reveal
612 how sexual imprinting might bias gene flow. In a limited number of unpublished trials, we found
613 evidence that *Peromyscus* mice sexually imprint on mothers (i.e., sexual imprinting still occurs
614 when no fathers are present). Although more rigorous testing is necessary to determine if
615 *Peromyscus* imprint on one or both parents, we predict that maternal imprinting leads to
616 introgression but prevents complete admixture, allowing the species to remain genetically
617 distinct in sympatry even with a small amount of hybridization.
618 Sexual imprinting might also prevent rampant gene flow between inter-fertile sympatric
619 species if it becomes reinforced (Irwin and Price 1999; Servedio et al. 2009). There is some
620 evidence for reinforcement between *P. leuocopus* and *P. gossypinus*. In a nesting assay,
621 sympatric *P. gossypinus* males and females and *P. leucopus* females preferred to spend more
622 time near conspecific individuals, while allopatric mice from both species showed no significant
623 preference for conspecifics (McCarley 1964). Similar patterns of increased species recognition in
624 sympatry have also been observed between *P. eremicus* and *P. californicus* (Smith 1965; Carter
625 and Brand 1986). However, for reinforcement to occur, hybrids must have reduced fitness. We
626 did not find evidence of hybrid inviability or sterility in our laboratory study, but we did not
627 quantify the degree of hybrid fertility which can vary in severity in hybrid zones (e.g. Turner et
628 al. 2011). In contrast to fertility-related traits, there is some evidence of postzygotic behavioral
629 sterility. A previous study found that *P. leucopus* and *P. gossypinus* reciprocal hybrids have
630 copulatory behaviors like each parental species but that they initiate copulation less frequently
631 than either *P. leucopus* or *P. gossypinus* (Lovecky et al. 1979). Similarly, both reciprocal hybrids

have similar exploratory behavior to both parental species but spent more time freezing in open-field exploratory behavior tests (Wilson et al. 1976). In nature, any reduced mating success or exploratory behavior would reduce hybrid fitness relative to their parents. Finally, hybrids might also be behaviorally sterile if they have intermediate mating traits. For example, hybrids between *M. m. musculus* and *M. m. domesticus* have intermediate urinary signals that are sexually selected against by each subspecies (Latour et al. 2014). The potential for hybrid behavioral sterility, coupled with the fact that moderate sexual imprinting induces sexual isolation in our allopatric lab stocks, suggests that it may be possible for reinforcement to boost reproductive isolation in sympatry, helping explain the paucity of hybrids we have observed in our study.

CONCLUSION

Our study supports an emerging view that sexual imprinting could be vital to the generation and maintenance of sexual reproductive barriers. Pending divergent natural selection on an imprintable trait, a species that learns mating preferences may develop significant sexual isolation that might mitigate the effects of hybridization. Our demonstration of sexual imprinting in *Peromyscus leucopus* and *P. gossypinus*, sympatric sister species that have few other reproductive barriers between them, indicates that sexual imprinting may disproportionately contribute to their total reproductive isolation. Sexual imprinting may sculpt reproductive isolation in subspecies (e.g. benthic and limnetic sticklebacks) undergoing initial morphological and behavioral divergence, or help preserve reproductive isolation between already divergent species, as we have shown to be the case in *P. leucopus* and *P. gossypinus*. Examining the role of sexual imprinting in similar cases of speciation driven by sexual reproductive barriers will continue to expand our understanding of the role of behavior in speciation.

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Figure 1

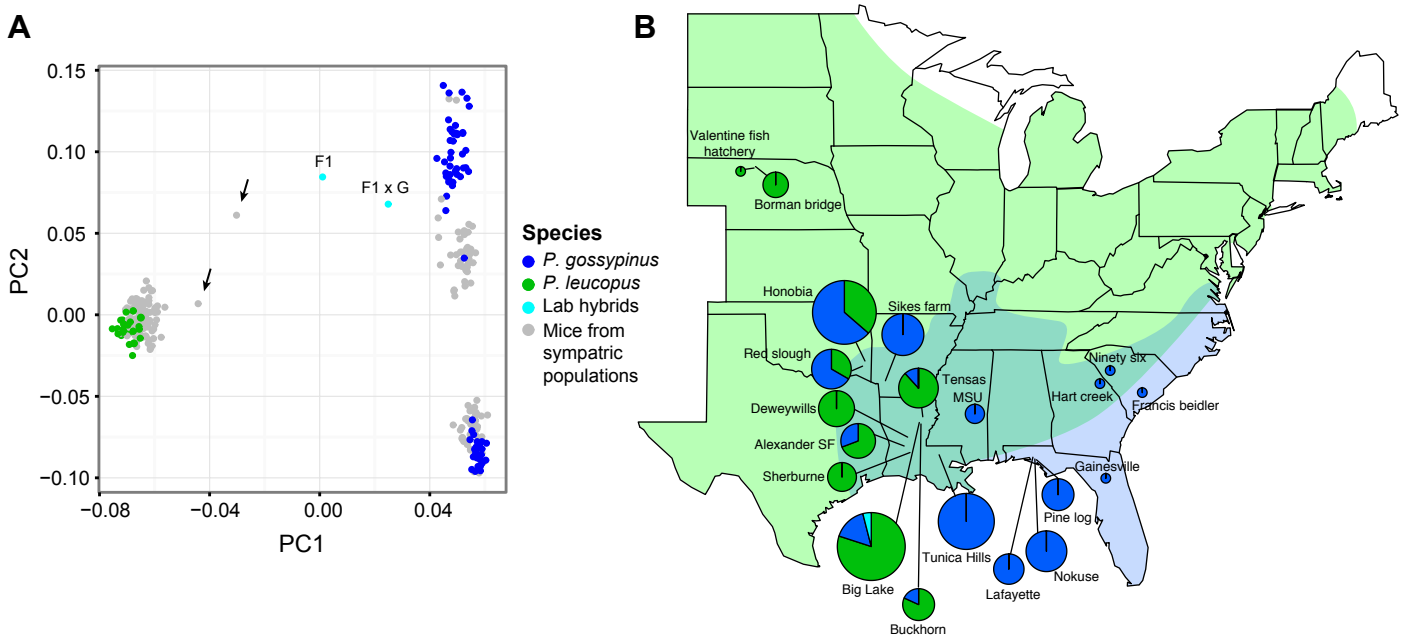

Figure 1. Hybridization is extremely rare between sympatric *P. leucopus* and *P. gossypinus* mice. **(A)** Genetic PCA discriminates between species. The first eigenvector strongly separates species based on the values of known allopatric *P. leucopus* (green dots) and *P. gossypinus* (blue dots) mice. The second eigenvector detects population structure among *P. gossypinus* populations that could correspond to mice collected east (higher values) and west (lower values) of the Mississippi river. Known lab-generated F1 and backcross (F1 x *P. gossypinus*) hybrids (cyan dots) fall intermediate along the first eigenvetor. Mice collected from the sympatric range overlap (grey dots) cluster discretely with *P. leucopus* or *P. gossypinus* with the exception of two mice that may be hybrids (arrows), but showing greater *P. leucopus* ancestry. **(B)** Range map of the two species: *P. leucopus* (green) and *P. gossypinus* (blue) adapted from (Hall and Kelson 1959; Hall 1981), showing areas of allopatry and sympatry. Pie diagrams show collecting locations and frequencies of each species scaled in size to represent the number of mice sampled at each site. Mice were classified as *P. leucopus* (green dots), *P. gossypinus* (blue dots), or potential hybrids (cyan dots) based on the genetic PCA (shown in **A**). The two possible hybrids were collected at “Big Lake” wildlife management area in Louisiana.

Figure 2

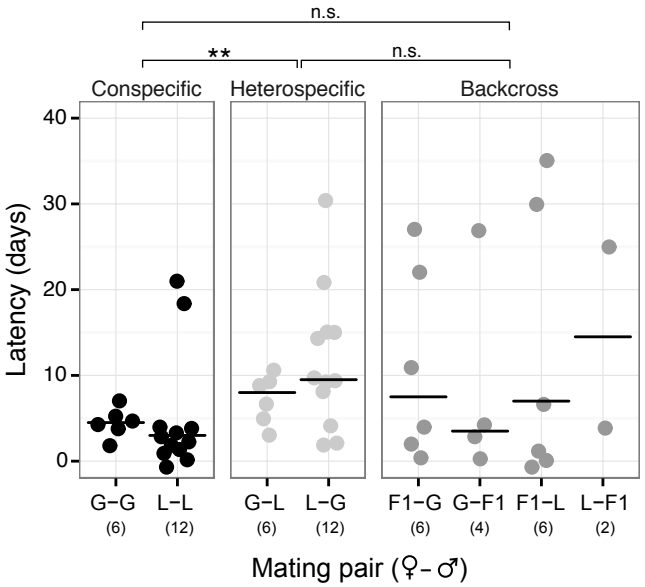

Figure 2. Latency to mating between *P. leucopus* (L), *P. gossypinus* (G) and their hybrids (F1). Estimated days since copulation are shown for conspecific, heterospecific, and backcross mating pairs that produced offspring (sample size in parentheses) in no-choice assays. F1 hybrids were generated with both LxG and GxL crosses. In all pairs, female individual is listed first. ** p = 0.01.

Figure 3

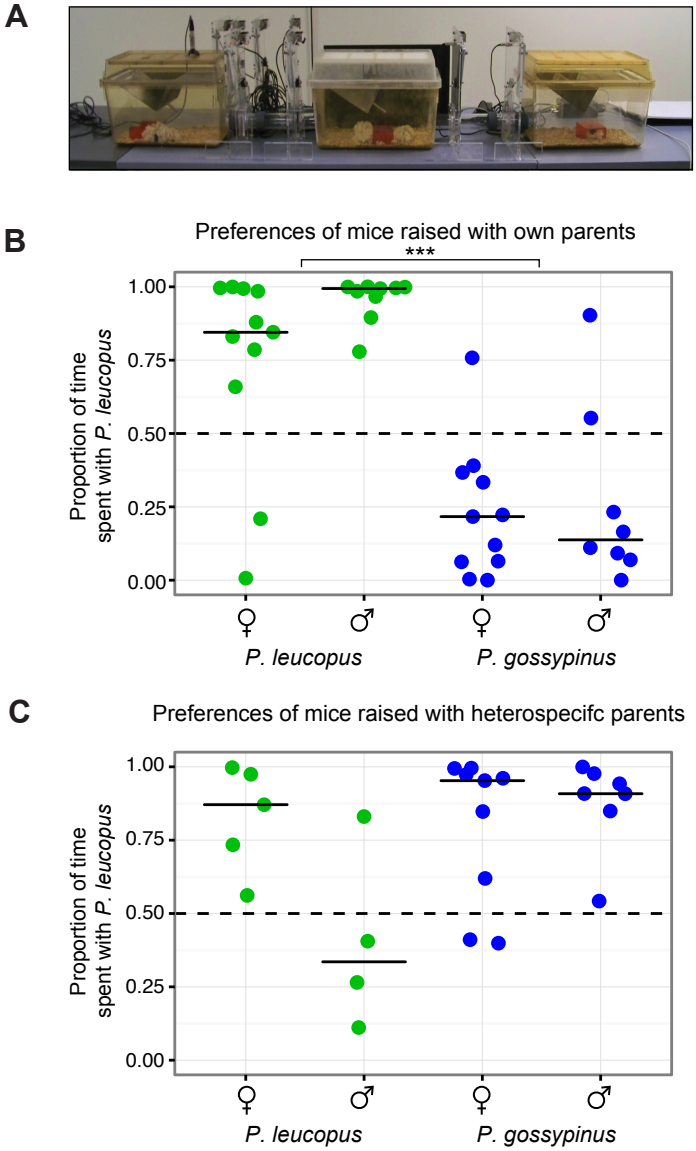

Figure 3. Mating preferences in two-way choice trials. **(A)** Photograph of the mate-choice apparatus. Center chamber is connected to two test chambers, each housing a "stimulus" animal, separated by gated doors activated by only the "chooser" animal. **(B)** Mating preferences for mice raised by their own parents. *P. leucopus* spent greater time with P. leucopus stimuli than both *P. gossypinus* sexes. **(C)** Mating preferences for mice raised by heterospecific foster parents. *P. leucopus* males were strongly affected cross-fostering ($p = 0.004$), whereas *P. leucopus* females were not (asterisks). Both *P. gossypinus* sexes spent significantly more time with the heterospecific stimulus than when raised by their own parents ($p < 0.001$).

Figure 4

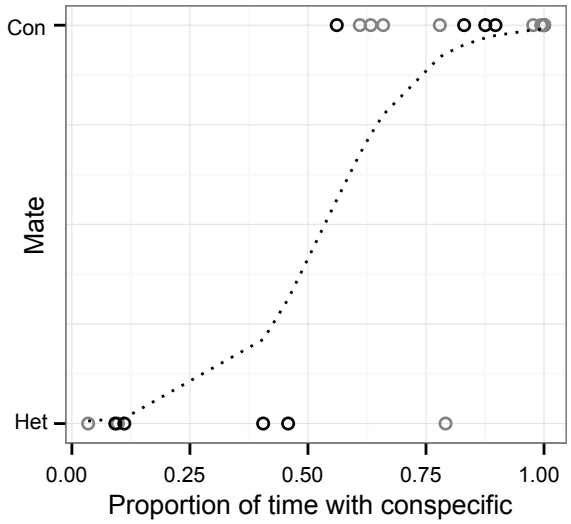

Figure 4. The proportion of time spent with a stimulus predicts mating outcome in trials when mating occurred. Mating occurred in 12 trials where choosers were raised with their own parents (gray dots) and 8 trials in where choosers were raised with heterospecific parents (black dots). Dotted line indicates the predicted probability for mate choice (conspecific versus heterspecific) given the proportion of time a chooser spent with a conspecific individual. The proportion of time a chooser individual spent with the conspecific stimulus strongly predicts the mating partner ($p = 0.038$). With the exception of one *P. leucopus* female raised with her own parents, all mice spent more time with their preferred mate.