Asymmetric mate preference and reproductive interference mediate climate-induced changes in mate availability in a small mammal hybrid zone

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Abstract

Range expansion and contraction are among the most common biotic responses to changing environmental conditions, yet much is to be learned about the mechanisms that underlie range-edge population dynamics, especially when those areas are points of secondary contact between closely related species. Here, we present field-measured parentage data that document the reproductive outcomes of changes in mate availability at a secondary contact zone between two species of woodrat in the genus *Neotoma*. Changes in mate availability resulted from drought-driven differential survival between the species and their hybrids. As the availability of conspecific mates declined, rates of hybridization increased, leading to the accumulation of admixed individuals in the zone of contact. Patterns of reproductive success in the wild appear to be the result of a combination of both pre-mating isolation and post-zygotic selection resulting from genomic incompatibilities between the parental lineages. Evidence of asymmetric mate preference between the parental lineages came from both skewed reproductive output in the field and laboratory preference trials. Moreover, partial genomic incompatibility was evident from the near-zero reproductive success of F1 males and because nearly all surviving hybrids had one pure parent. Nonetheless, high reproductive success of F1 females and backcrossing in both parental directions allow for introgression between the parental species. These findings reveal how climate change may alter evolutionary outcomes for species at the edge of their ranges through an interplay of behavioral, demographic, and genetic mechanisms.

Keywords: reproductive success, secondary contact, mate choice, hybrid incompatibility, Haldane's Rule, *Neotoma*, woodrats

Teaser Text

Species distributional ranges are shifting in response to changing climate conditions, sometimes leading to augmented interspecific mating opportunities. This study presents laboratory behavioral trials and field-measured reproductive success to identify pre- and post-mating isolating mechanisms that determine the genomic and evolutionary consequences of climate-induced changes in mate availability. Our laboratory behavioral trials show strong asymmetry in mate preference with the females of the smaller-bodied species showing stronger preference for conspecific males, while females of the larger-bodied species are more equivocal. Our field-measured patterns of reproductive output are consistent with the asymmetric expectations of the behavioral trials. In addition to mate selection, genomic incompatibilities between the parental lineages also determine reproductive output in the wild because F1 males rarely sire offspring and nearly all surviving hybrids have one pure parent. By combining field and laboratory studies, we show that both pre- and post-mating selection determine the outcomes of climate-driven changes in mate availability in this range-edge hybrid zone.

Introduction

One of the most common ways in which species respond to environmental change is through shifts in geographic distribution (Parmesan 2006). Although these patterns are obvious at latitudinal and elevational scales (Parmesan *et al.* 1999, Moritz *et al.* 2008), we understand little concerning the population-level processes that determine patterns of range-edge expansion and contraction, especially when those dynamics include the potential for hybridization between closely related species (Garroway *et al.* 2010, Chunco 2014). Ecological and genetic interactions between close congeners in range-edge zones of secondary contact can fundamentally change the trajectory of range edge populations, and thus, the ability of species to continue to track shifting niche space (Chunco 2014, Sánchez-Guillén *et al.* 2016, Hunter *et al.* 2017, Driver *et al.* 2022, Brauer *et al.* 2023). Hybridization at species' range edges may partly inhibit range expansion (at a species' leading edge) or exacerbate range contractions (at a species' trailing edge) if mating decisions and selection against hybrids lead to reproductive interference (Barton and Hewitt 1985, Gröening and Hochkirch 2008, Driver *et al.* 2022). Alternatively, introgression at range edges may enhance adaptive evolution by quickly augmenting genetic variation in ecologically relevant loci (Hedrick 2013, Taylor *et al.* 2015, Pfennig *et al.* 2016, Moran *et al.* 2021, Brauer *et al.* 2023).

Initiation or augmentation of secondary contact may itself be a consequence of demographic responses to climate change (Sánchez-Guillén *et al.* 2016). For example, one taxon may be favored by certain climatic conditions and expand into the range previously occupied by a close congener (Bronson *et al.* 2003, Taylor *et al.* 2014, Garroway *et al.* 2010, Hunter *et al.* 2017). Changes in mate availability and interspecific encounter rates can be strong determinants of hybridization rates (Keränen *et al.* 2013, Rohde *et al.* 2017) depending on the pre- and post-

mating isolating mechanisms that characterize the species boundary (Harrison 1986, Abbott *et al.* 2013). If pre-mating isolation is complete and the parental species mate assortatively, there may be minimal reproductive interaction between the species, although other ecological interactions may still influence the evolutionary trajectory of each taxon (Brown and Wilson 1956, Adams and Rohlf 2000, Matocq and Murphy 2005). On the other hand, if pre-mating isolation is incomplete and genomic incompatibilities (Dobzhansky 1936, Muller 1939, Orr 1995) have minimal or moderate effects, interspecific mating could lead to the generation of fertile hybrids. This in turn would further alter the pool of mates in the area of contact, creating an opportunity for introgression between the parental species (Shurtliff *et al.* 2014, Rohde *et al.* 2015, Jahner *et al.* 2021, Klure *et al.* 2023). If genomic incompatibilities are such that there is strong selection against hybrids, the cost of interspecific and admixed mating may result in reproductive interference or decreased fitness within the parental species (Barton and Hewitt 1985, Gröening and Hochkirch 2008, Driver *et al.* 2021). Hence, at range edges, both sexual (*e.g.* mate choice) and natural (*e.g.* intrinsic and extrinsic) selection, and their interplay, are likely to determine rates of hybridization and introgression (Schmidt and Pfennig 2016, Maxwell *et al.* 2021).

When costs to hybridization exist, they may be asymmetric between the lineages and sexes (Wirtz 1999, St. John and Fuller 2021, McFarlane *et al.* 2016). Asymmetric post-zygotic costs depending on which species serves as the mother or father may select for asymmetric female mate choice (Wirtz 1999). Such asymmetry in female mate choice can result from costs associated with interspecific differences in body size (Nagel and Schluter 1998). For example, males of a larger-bodied species may be avoided by females of the smaller-bodied species, perhaps as a response to aggressive encounters (Nagel and Schluter 1998, Shurtliff *et al.* 2013) or the consequences of paternal genomic imprinting (Dawson *et al.* 1993, Zeh and Zeh 2003,

Vrana *et al.* 2007). In addition to mating preferences, reproductive outcomes in hybrid zones will be strongly determined by sex-specific viability and fertility phenotypes of admixed individuals. For example, among hybrids the heterogametic sex often has lower viability or fertility (Haldane 1922, Orr 1997), which can be a strong determinant of the genomic composition of the effective breeding population in a contact zone. The nature of these costs and asymmetries provide insight into the pre- and post-mating isolating mechanisms that characterize a species boundary, while also determining the direction and magnitude of introgression, and the persistence of parental and admixed genotypic classes.

Here, we investigate patterns of behavioral isolation and reproductive success over a five-year period in a zone of secondary contact where the ranges of two woodrat species meet: *Neotoma fuscipes* and *Neotoma macrotis* (Fig. 1A). These species have been diverging from one another for ~2 million years (Matocq 2002a) and although their ranges are largely allopatric, they are known to hybridize where they come into contact (Matocq 2002b, Matocq *et al.* 2012, Coyner *et al.* 2015). During the five years of the current study, drought conditions (dry winters) led to higher survival of *N. macrotis* in comparison to *N. fuscipes*, and greater dispersal of *N. macrotis* into the range previously occupied by *Neotoma fuscipes* (Fig. 1B; Hunter *et al.* 2017). Given this survival advantage and expansion of *N. macrotis* into the range of *N. fuscipes*, hybridization increased and we observed the accumulation of admixed individuals on the site (Fig. 1B). In addition, the center of the hybrid zone shifted over the six-year window in the direction of the area previously occupied by *N. fuscipes* (Fig. 1D, Hunter *et al.* 2017). Here, we sought to identify the patterns of intra- and interspecific reproductive success that accompanied this change in interspecific mate availability and accumulation of admixed genomes. We combined laboratory and field studies to gain insight into the pre- and post-mating factors that

determined reproductive success observed in the wild. We focused on three questions. First, when given a choice between a conspecific and heterospecific male, do purebred females exhibit assortative or non-assortative preferences? Second, are patterns of reproductive success in the wild consistent with expectations of random mating among the genotypic classes or do asymmetries exist? And third, do we find evidence of reproductive interference between the parental lineages? By combining patterns of reproductive success in the wild with measures of affiliative and exploratory behavior in the laboratory, we gained insight into how climatic variation and pre- and post-zygotic isolating mechanisms affect the genomic composition of range-edge populations.

Materials and Methods

Field sampling

The study site occurs in the riparian habitat of the Camp Roberts Military reservation just west of the confluence of the Nacimiento and Salinas Rivers, approximately 6.5 km northeast of San Miguel, CA (Fig. 1). Woodrats are restricted to the narrow strip of riparian habitat along the banks of the Nacimiento River that is dominated by oak (*Quercus* spp.), willow (*Salix* spp.) and elderberry (*Sambucus* spp.) (Fig. 1). We conducted an intensive mark-recapture effort in order to maximize our capture of both adult and juvenile woodrats; the latter emerge from their mother's stick house/nest at approximately one month of age. Adult woodrats maintain conspicuous stick houses/nests that are ~1 meter in height and width, typically located at the base of trees or shrubs. These multi-chambered stick houses are maintained throughout the year and often throughout an individual's lifetime (~2-3 years in the wild, Linsdale and Tevis 1951). These houses are the site of all major activities in a woodrat's life including sleeping, breeding, storing food, and for females raising young. Our trapping efforts focus on houses

because they are the center of activity for these animals, but also allow for a high enough density of traps that the occupants of nearby houses are often trapped at multiple nearby trap sites (*i.e.* near a neighbor's house). Trapping occurred from May-June 2008, March-August 2009, April-August 2010, and April-October 2011 and 2012. At each woodrat house, we set two chipmunk-sized Tomahawk live-traps (Tomahawk Live Trap, Hazelhurst, WI) baited with peanut-butter and oats in the early evening hours and began checking traps approximately 7 hours later. Each woodrat house was trapped for three consecutive nights during a trapping session, and sessions were repeated approximately every 4-6 weeks. Individuals were marked with uniquely numbered metal fingerling ear tags, sexed, aged, weighed, measured (tail length, total length, and foot length), and a small ear biopsy was taken for subsequent genetic analysis. All field activities and behavioral trials (described below) were conducted under a Scientific Collecting Permit from the California Department of Fish and Game (now Wildlife), approved protocols from the University of Nevada Reno's Institutional Animal Care and Use Committee, and in accordance with the standards set by the Animal Care and Use Committee of the American Society of Mammalogists (Sikes *et al.* 2016).

Genotyping and parentage analysis

Genotypes used in this analysis are based on a 15 microsatellite-locus dataset generated using methods described in (Coyner *et al.* 2015) and published therein, and in (Hunter *et al.* 2017). We used this large, available dataset to build on past studies of this system (Coyner *et al.* 2015, Hunter *et al.* 2017) and because studies show high consistency between microsatellites and other datasets (*e.g.* single nucleotide polymorphisms) in estimates of hybrid indices (Jahner *et al.* 2021) and parentage (Kaiser *et al.* 2017). We used STRUCTURE version 2.3.4 (Pritchard *et al.* 2000, Falush *et al.* 2007) to estimate *q*-values and broadly classified individuals as pure *N. macrotis* (proportion of the genome assigned to *N.*

fuscipes = q_{FU} < 0.10), BC-*macrotis* (q_{FU} = 0.10- 0.40), F1 (q_{FU} = 0.40- 0.60), BC-*fuscipes* (q_{FU} = 0.60- 0.90), and pure *N. fuscipes* (q_{FU} > 0.90) as in Coyner *et al.* (2015). Throughout the following analyses we use *q*-values as a continuous variable as well as the basis of classifying individuals into broad genotypic classes as detailed above (*N. macrotis*, BC-*macrotis,* F1, BC-*fuscipes, N. fuscipes*).

We conducted parentage analysis using the likelihood-based approach implemented in CERVUS (Marshall *et al.* 1998). Any adult male or female caught on site during the breeding season (March-August) was considered a potential parent for any juvenile captured that year (Suppl. Table 1). To determine critical Delta values for parent assignments, we ran simulations for 10000 offspring assuming we had captured 90% of potential mothers and fathers with 95% of loci typed and a 1% error rate. We used the same parameters in assigning parents at strict (95% confidence) and relaxed (80% confidence) levels of parentage assignment.

Behavioral trials

To quantify both exploratory behavior and intersexual affiliative preference, we conducted 50 behavioral trials (n = 25 females of each species). Known pure-bred adult females and males of each species were trapped from the hybrid zone and brought briefly into a field laboratory (≤ 3 d) and housed in individual cages (cages ~35 x 35 x 55 cm). We used a standard T-maze design (as in Shurtliff *et al.* 2013) wherein the focal female's cage (home box) was linked to a 61 cm runway tube that ended in two side arms of 61 cm each, that led to the cage of either a conspecific or heterospecific adult male. Males were tethered with a chest harness such that they could only access their own cage, while females could leave their home cage and freely explore the runway, side-arm tubes, and visit both males. To induce mating behavior in these trials, prior to testing, females were injected with 1 mg/ml estradiol benzoate (0.1 ml/ 100 g body weight) 48 hours pre-trial, and 10mg/ml progesterone (1 ml/100 g body weight) 4-6

hours pre-trial (as in Shurtliff *et al.* 2013). Exploratory behavior in female mice is not influenced by oestrous phase (Vošlajerová Bímová *et al.* 2016), so our goal was to induce mating behavior (as in Shurtliff *et al.* 2013) while still providing insight into female exploratory behavior and boldness. Trials were run for 2 hours at night when woodrats would normally be active. Post-trial, we released individuals at their site of capture.

A female's exploratory behavior and boldness was quantified as the total time spent outside of her own home box interacting with the novel portion of the arena during a trial, *i.e.*, in the runway tube, entry tube to each male's cage, or the cage of either the conspecific or heterospecific male. We compared average total exploratory time by species using log-transformed responses as the data were right-skewed. We quantified intersexual affiliative behaviors using time spent with males (grappling, mutual smelling and grooming) and cage visits. Despite hormonal induction, no copulations were observed. A female's net preference was measured as the difference between her response (times, visits) with the conspecific male and the heterospecific male. We compared the mean net preference by species using *t* tests. Because preference responses were leptokurtic they were squareroot-transformed prior to analysis ($x' = \sqrt{x}$), with negative values indicating preference for the heterospecific male, transformed as follows: $x' = -1\sqrt{-x}$.

Null-model simulations

We simulated expected mate pairings in the wild under a null model of random mating in which the probability of pairing with an opposite-sex adult individual was based solely on distance between individuals (*i.e.*, without reference to genotype, body size, etc.). First, we calculated the empirical distribution function (EDF) of observed distances between known mate pairs from the parentage analysis described above ('edfun' R package, Galili 2016; Fig. S1). For each adult captured each year, we

then sampled (with replacement) multiple (n = 100) potential mate-pairs from the set of all opposite-sex adults captured that year with sampling probabilities set by the mate-distance EDF. We used these simulations to (1) run null-hypothesis tests for assortative mating and/or genetic incompatibility (see below), (2) model the factors driving mate 'selection' for males and females of both pure lineages (*N. macrotis* and *N. fuscipes*), and (3) characterize the change in availability of pure conspecific mates (and the availability of admixed and interspecific mates) across the study period.

Analytical approach

To assess the consistency of observed mating patterns with the null model (pairings based solely on distance), we compared sampling distributions for test statistics (see below) generated under the null model with the equivalent statistics computed from the observed data. In each case, bootstrapped p-values were computed as the fraction of simulated test statistics that provided at least as much evidence in favor of rejecting the null model as the observed test statistic.

It should be noted that our field measure of reproductive output (test data for these analyses) is taken at the stage of juvenile emergence from the nest (~4 weeks); as such, our measure of reproductive success is a composite of mate choice, fertilization efficiency, and early-stage (*i.e.* embryonic or first weeks postpartum) survival. Thus, although we test for common patterns associated with assortative mating and common genomic incompatibilities (*e.g*. greater male infertility consistent with Haldane's Rule; Haldane 1922), we cannot definitively distinguish between assortative mating and early-stage genomic incompatibility.

To confirm that individuals of similar genetic lineage were more likely to successfully interbreed than random mate-pairings sampled under the null hypothesis, we compared the following test statistics with their corresponding null sampling distributions: (1) the fraction of successful mate pairings

with both pure parents of the same type (*N. macrotis*-*N. macrotis* or *N. fuscipes*-*N. fuscipes*; fraction should be higher under assortative mating), (2) the fraction of successful mate pairings with both pure parents of different types (*N. fuscipes*-*N. macrotis* or vice versa; fraction should be lower under assortative mating), and (3) the mean absolute q_{FU} difference between each pair of mates. We also performed separate tests for females of each genotype class using mean q_{FU} (fraction *N. fuscipes* genotype) across all successful pairings as the primary test statistic.

To assess whether two hybrid parents (F1, BC-*macrotis*, BC-*fuscipes*) were less likely to produce viable offspring , and whether hybrid males would be less likely to produce viable offspring than hybrid females (consistent with Haldane's rule), we compared the following test statistics with their corresponding null sampling distributions: (1) the fraction of all successful mate-pairings with at least one pure parent (*N. fuscipes* or *N. macrotis*), (2) the fraction of all admixed mate pairings (pairings comprising at least one hybrid or backcross individual) in which the other parent was pure (*N. fuscipes* or *N. macrotis*), (3) the global fraction of successful F1 hybrid-hybrid pairings, (4) the global fraction of pairings that included an admixed male vs. admixed female parent, and (5) the fraction of admixed females and males (assessed separately) that were observed to mate with a pure individual.

We ran a proportion test (Fisher's exact test) to assess whether the strength of apparent outbreeding avoidance differed for *N. macrotis* versus *N. fuscipes* females. Specifically, we tested whether the proportion of successful cross-species pairings (*N. macrotis* x *N. fuscipes*) in which the female partner was *N. macrotis* (versus cross-species pairings in which the female partner was *N. fuscipes*) was consistent with the null model in which all females mated randomly with nearby males. We hypothesized that the smaller-bodied *N. macrotis* females would be less likely to mate with larger-bodied (and possibly more aggressive) *N. fuscipes* males than the reverse scenario (female *N. fuscipes* x male *N. macrotis*) based on similar findings in mate-choice trials between size differentiated, closely related species of woodrats, *Neotoma lepida* and *N. bryanti* (Shurtliff *et al.* 2013).

To assess the factors driving reproductive success in our study system, we modeled probability of reproduction (defined as the probability of any captured adult being assigned as a parent of one or more offspring each year) as a function of genotype (pure *N. fuscipes*, pure *N. macrotis*, and admixed (F1, BC-*macrotis*, BC-*fuscipes*), body size, and local availability of admixed mates (to test for reproductive interference as a decline in reproductive output with an increase in admixed mate pairings). To do this, we ran logistic regression models in which the observations comprised all adults captured each year and reproductive success was defined based on whether ≥ 1 offspring was assigned to that individual (see Parentage Analysis, above). We ran separate reproductive success models for each sex.

To better understand the factors differentiating observed from potential mate pairings in this system, we modeled the probability of females of each pure parental genotype (*N. fuscipes* or *N. macrotis*) successfully pairing with available males as a function of their genotype and body size (only females observed to successfully mate with a known male were included in this analysis). We used a conditional logistic regression framework (with strata defined by the set of available mate-pairs), implemented in the 'glmmTMB' package in R (Brooks *et al.* 2017, R Core Team 2021). We ran separate models for each genotype class (only female-focused models are presented here). All analyses and null model simulations were performed in R v4.1; code for performing analyses and visualizations are available on GitHub: https://github.com/kevintshoemaker/hybridzone_repro.

Results

Composition of the contact zone population

We trapped and genotyped a total of 1,651 individuals between 2008 and 2012, 959 of which were known to be born on site. At the start of the study, the proportion of individuals in each genotypic class were as follows: *N. macrotis* ($q_{FU} < 0.10$) = 0.50, BC-*macrotis* (q_{FU} = 0.11-

0.40) = 0.04, F1 (q_{FU} = 0.40- 0.60) = 0.03, BC-*fuscipes* (q_{FU} = 0.60- 0.90) = 0.03, and pure *N. fuscipes* (q_{FU} > 0.90) = 0.40, for a total of 10% of the population being admixed (Table S1). The admixed proportion of the population remained stable during 2009 and 2010 but then increased to 21% in 2011 and 25% in 2012 (Fig. 1; Table S1).

On average, both *N. fuscipes* males (355g ± SD = 57g) and females (295g ± 38g) are larger than *N. macrotis* males (296g ± 42g) and females (265g ± 35g), but F1 hybrid males (380g ± 66g) and females (305g ± 34g) are, on average, larger than pure-bred individuals (Fig. S2). BC-*fuscipes* males (320g ± 62g) and females (283g ± 36g), as well as BC-*macrotis* males (309g ± 52g) and females (272g ± 36g) are intermediate to the pure parentals (Fig. S2). In terms of interspecific mate-pair body size disparities, on average, *N. fuscipes* females and *N. macrotis* males do not differ in size, while *N. fuscipes* males are, on average, 34% larger than *N. macrotis* females.

Laboratory behavioral trials

Our behavioral trials provided insight into female exploratory behavior and boldness, as well as preference for conspecific or heterospecific males. We found that *N. macrotis* females emerged from their home box for much more time on average (20.8 min. [12.2, 34.9]; back-transformed means [95% CI]) than did *N. fuscipes* females (3.1 min. [1.5, 5.6]; t_{49} = 5.0, P < 0.001). A majority (22 of 26) of the *N. macrotis* females spent at least 5 minutes in the novel portion of the test arena, while only 10 of 25 *N. fuscipes* females did so (Fig. 2A).

Preference for conspecific versus heterospecific males differed between *N. macrotis* and *N. fuscipes* females. Compared to *N. fuscipes*, *N. macrotis* females interacted more with conspecific over heterospecific males (two-sample t_{df}) in terms of visits (t_{49} = 4.0, P < 0.001) and

time spent (t_{49} = 3.4, P = 0.002). Considering each species separately, mate preferences were significantly asymmetric (Fig. 2B & C). *Neotoma macrotis* females, the smaller-bodied species, preferred males of their own species (one-sample t_{df}) in terms of net visits (t_{25} = 3.5, P = 0.002) and net time (t_{25} = 2.3, P = 0.028). Conversely, *N. fuscipes* females, the larger-bodied species, preferred heterospecific males in terms of net visits (t_{24} = 2.1, P = 0.047) and net time (t_{24} = 3.0, P = 0.006).

Reproductive success in the field

Of 959 juveniles included in the parentage analysis, 794 were assigned both parents at the 80% confidence level or higher. The parental classes (particularly *N. fuscipes*) tended to have higher per-capita reproductive success than admixed individuals for both sexes, with the notable exception of F1 hybrid females (Fig. 3; Table S2). F1 females produced an average of 1.45 offspring per year versus 1.31 and 0.88 for pure *N. fuscipes* and *N. macrotis* females, respectively (Fig. 3, Table S2). Likewise, F1 females had the highest apparent fraction of known-reproductive individuals with approximately 70% of F1 females being assigned at least one offspring versus 61% and 52% for pure *N. fuscipes* and *N. macrotis* females, respectively (Table S2). In contrast to the high reproductive success of F1 females, F1 males produced a mean of 0.13 offspring in comparison to the 1.6 and 1.03 for pure *N. fuscipes* and *N. macrotis* males, respectively (Fig. 3, Table S2). Finally, only 13% of F1 males (n = 4 individuals) were identified as fathers versus 61% and 44% for pure *N. fuscipes* and *N. macrotis* males, respectively (Table S2). Finally, for individuals from which we recovered two or more offspring within a single year (N = 178 males; N = 220 females) and thus had the opportunity to identify multiple partners, we

found that males had an average of 2.5 partners and females had 1.8. Further, for females that had more than one offspring, 53% of subsequent offspring had a different father than the first.

Reproductive success of pair types in comparison to null expectations and mate availability

We recovered significantly fewer offspring from interspecific mate pairs than expected under a null model in which pairings occur in proportion to mate availability (Table S3). We found that 82.7% of successful pairings comprised two conspecific pure parentals (*N. macrotis-N. macrotis* or *N. fuscipes-N.fuscipes*), versus 60.1% expected under the null model ($P < 0.001$). Further, only 2.6% of successful pairings were between heterospecific pure parentals, versus 15.3% expected under the null model ($P < 0.001$). Of the successful heterospecific pairings, though, there was significant asymmetry. For direct interspecific crosses, we were much more likely to observe offspring from a *Neotoma fuscipes* female coupled with a *N. macrotis* male than the reciprocal cross (Table S3). Further, observed mate pairings were more likely to include at least one pure parent (98.6%), which exceeded the null model (95.5%; $P = 0.002$). Only 1.6% of successful pairings included two hybrid parents, versus 4.2% expected under the null model ($P = 0.002$). Admixed males rarely were observed to successfully produce offspring; 6.2% of all successful pairings included an admixed male, versus 15.3% expected under the null model ($P < 0.001$). This is in contrast to females, where hybrids were identified as successful parents at a rate consistent with the null model (10.5% observed vs 12.9% expected; $P = 0.11$). Finally, admixed females (backcross and F1) successfully mated with pure males more often than expected under the null model (87% observed vs 68% expected; $P = 0.009$), whereas admixed males produced young with pure females in accordance with their availability (75% observed vs. 72.5% expected; $P = 0.88$).

We compared reproductive output (field-measured observation of successful reproduction) with random pairings generated from the null model (based only on male availability) for females of each genotypic class (Fig. 4). *Neotoma fuscipes* and *N. macrotis* females have mate choice opportunities primarily at their respective conspecific ends of the genomic continuum (grey histograms), and yet, field-measured reproductive output is even more extreme towards each conspecific end of the spectrum (red line; Fig. 4). While both F1 and BC-*fuscipes* females successfully reproduced with males across the genotypic spectrum, both of these types of females were somewhat more likely to reproduce with more *N. macrotis*-like males (Fig. 4). BC-*macrotis* females had a very strong tendency to reproduce with *N. macrotis*-like males (Fig. 4).

We found that the probability of reproduction in females is positively related to body size (Fig. 5A, see below), but the probability of reproduction was similar between females of the parental classes, and more variable in admixed females (Fig. 5B). Overall, though, as the proportion of admixed mates increased on the site, the probability of reproduction declined for *N. fuscipes* females while remaining comparatively stable for *N. macrotis* females (Fig. 5C).

Our conditional logistic regression models, which model the probability of successful mate pairing (for known-reproductive individuals) conditional on mate availability, show that reproductive *N. fuscipes* females had a greater chance of successfully pairing with larger males (Fig. 6A). Reproductive *N. macrotis* females were more likely to successfully pair with males in the range of 300 g and had less success with both smaller and larger males (Fig. 6C). Further, both *N. fuscipes* and *N. macrotis* females were more likely to successfully pair with males towards the conspecific end of their respective genotypic spectrum (Fig. 6B & D), although reproductive *N. fuscipes* females successfully paired with males across the genotypic spectrum.

Discussion

Species' distributional limits are shifting in response to changing climate conditions. When changes in range edges create an opportunity for interspecific mating, the nature of the species boundary between contacting lineages will partly determine the evolutionary consequences of range-edge shifts. At the start of this study, approximately 10% of the individuals occupying the area of secondary contact between *N. macrotis* and *N. fuscipes* had admixed ancestry. During the subsequent five years, drought-like conditions gave *N. macrotis* a survival advantage over *N. fuscipes* (Hunter *et al.* 2017), which, coupled with movement of *N. macrotis* into the range of *N. fuscipes*, ultimately led to a doubling of mixed ancestry in the population (24%). We sought to identify the patterns of reproduction that accompanied the change in interspecific mate availability and led to the accumulation of admixed genomes in the area of secondary contact.

Asymmetry in female mate preference and exploratory behavior

Our behavioral trials provided insight into two female behavioral traits pertinent to range edge dynamics: exploratory behavior (boldness), and affiliative behavior. First, we measured an index of exploratory behavior by assessing how much time *N. macrotis* and *N. fuscipes* females spent outside their home box in the novel test apparatus. We found that nearly all *N. macrotis* females emerged from the home box, exhibiting a least a minimal level of exploratory behavior and boldness, while less than half the *N. fuscipes* females did so. While we cannot decipher the cause of *N. macrotis'* apparent greater exploration and boldness, nor the persistence of this behavior under natural field conditions, exploratory behavior in artificial test arenas has been

shown to be positively correlated with greater dispersal in the wild (Fraser *et al.* 2001, Dingemanse *et al.* 2003, Hoset *et al.* 2011, reviewed in Cote *et al.* 2010). Further, immigrants exhibit greater exploratory behavior (Dingemanse *et al.* 2003, Quinn *et al.* 2011), which leads to the theoretical expectation that an expanding population will be composed of more bold and exploratory individuals (Fraser *et al.* 2001). If *N. macrotis'* greater boldness and exploration leads to higher rates of dispersal in the field, these behavioral traits could have contributed to the observed expansion of this species into the range of *N. fuscipes*, thus changing interspecific mating opportunities during the study period.

Our behavioral trials also provided insight into intersexual affiliative behavior, a proxy of mate preference (Grize *et al.* 2019, Dougherty and Shuker 2015). We found that *N. macrotis* females, the smaller-bodied species, significantly preferred males of their own species. In contrast, *N. fuscipes* females, the larger-bodied species, significantly preferred heterospecific males. As such, *N. macrotis* males were preferred by both types of females. These results are consistent with laboratory mate choice trials between a pair of closely related woodrat species that show similar size disparity, *N. lepida* and *N. bryanti*. The relatively small-bodied *N. lepida* females very rarely preferred/chose the relatively large-bodied *N. bryanti* males, while *N. bryanti* females often preferred/chose *N. lepida* males (Shurtliff *et al.* 2013). Importantly, the directionality of mate choice suggested by these behavioral trials are consistent with the field-measured reproductive success discussed below.

Asymmetric reproductive success

The parental classes (particularly *N. fuscipes*) tended to have higher per-capita reproductive success than admixed individuals for both sexes, with the notable exception of F1 hybrid females. The high reproductive success of F1 females suggests that these individuals

represent a key gateway for genomic introgression between the two parental species. In contrast, F1 males had particularly low reproductive success, consistent with Haldane's Rule (Haldane 1922) wherein the heterogametic sex is more likely to exhibit the consequences of genomic incompatibility between the parental species through sterility or inviability (Davis 1993). Among the backcross classes, BC-*fuscipes* males had particularly low reproductive output, while BC-*macrotis* males appear to regain function and achieve levels of productivity comparable to backcross females, albeit still lower than the pure-bred classes.

As in previous studies of *N. macrotis* (Matocq 2004), *N. fuscipes* (McEachern *et al.* 2009) and other species of woodrats [*N. cinerea* (Topping and Millar 1998) and *N. micropus* (Baxter *et al.* 2009)], we found that both males and females mate with multiple partners and that the mating system is best described as promiscuous. However, despite access to heterospecific mates and a promiscuous mating system, at least according to offspring production, mating seemed to rarely cross the species boundary. We recovered significantly fewer offspring from interspecific mate pairs than expected under a null model in which pairings occur in proportion to mate availability. Overall, the vast majority of successful pairings in this hybrid zone included at least one pure parent, suggesting that genomic stability requires at least one full complement of a parental genome. Genomic-based estimates of interparental ancestry between two closely related species of woodrats, *N. lepida* and *N. bryanti*, suggested the same pattern (Jahner *et al.* 2021), but the current study is the first to confirm these mating patterns in *Neotoma* using field-collected parentage data.

The lowered reproductive success of F1 males and other admixed genotypes (with the exception of F1 females) and the fact that nearly all surviving hybrids had one pure parent are patterns consistent with genomic incompatibility between the parental lineages. The lowered

fertility or sterility of F1 males is expected to be one of the first consequences to arise during the process of speciation (Orr 1997). Potential underlying causes of lowered male F1 fertility and lowered fertility of the backcross classes include X or Y-linked hybrid male sterility and sex chromosome-autosome interactions (Good *et al.* 2008, Campbell *et al.* 2012). Mismatched cytonuclear interactions may also partly underlie genomic incompatibilities between the species (Edmands and Burton 1999, Ellison and Burton 2006). However, at this hybrid zone, mtDNA of both species is found on the alternate genetic background. Yet, consistent with the directionality of reproductive output we observed in the field, an *N. fuscipes* mtDNA on a *N. macrotis* nuclear background was more common than the reverse pattern (Coyner *et al.* 2015). Identifying possible negative sex chromosome-autosome-mtDNA interactions in this system will require laboratory experimental crosses (Britton-Davidian *et al.* 2005, Good *et al.* 2008, Campbell *et al.* 2012, Bize *et al.* 2018).

Chromosomal differences between the species can also result in lowered fertility through meiotic imbalances (Grize *et al.* 2019), but early inspection of the first available genome sequences of *N. fuscipes* and *N. macrotis* show that the species have the same number of chromosomes ($2n = 56$, as determined for *N. macrotis* by Mascarello and Hsu 1976) and do not exhibit large inversions (NCBI PRJNA951688; SAMN34055418 and SAMN34051485). Nonetheless, woodrats differ greatly from one another and other rodents in the expansion of certain gene families involved in detoxification (Greenhalgh *et al.* 2022). Whole genome sequencing of additional individuals will be needed to identify any structural or sequence variation that may be involved in genomic incompatibilities between the species.

Potential role of body size in reproductive output

Our conditional logistic regression models indicated that pure females of both species tended to successfully breed with larger conspecific males. The apparent selection for large body size was especially strong in *N. fuscipes*, where the set of 'selected' males was heavily skewed toward large males. In contrast, *N. macrotis* females tended to successfully pair with males of body size around 300 g and appeared to avoid pairing with very large males and very small males after accounting for genotype effects, thus only rarely crossing the species boundary to mate with the larger *N. fuscipes* males. As such, *N. fuscipes* males appear to be largely restricted to females on the *fuscipes*-end of the genotypic spectrum. In contrast, *N. macrotis* males, especially larger-bodied individuals, appear to have a wider genotypic spectrum of mates spanning from *N. macrotis* to *N. fuscipes* females. In fact, the large-bodied *N. macrotis* males that may be most likely to mate with *N. fuscipes* females may be less favored by females of their own species due to their preference for males of an intermediate body size. Thus, behavioral mechanisms may favor pairings between *N. fuscipes* females and larger-bodied *N. macrotis* males. Our behavioral trials are consistent with these patterns wherein *N. fuscipes* females will affiliate with *N. macrotis* males when given the choice in an arena, while *N. macrotis* females avoid *N. fuscipes* males. Indeed, the behavioral trials demonstrate that both *N. fuscipes* and *N. macrotis* females readily affiliate with *N. macrotis* males, a pattern reflected in field-measured reproductive success. Finally, while we only tested pure females in our behavioral trials, it appears from the field-reproductive data that backcross females may be making similar mate choices to pure parental females. That is, BC-*macrotis* females produced more offspring with *N. macrotis* males than *N. fuscipes* males, while BC-*fuscipes* females successfully reproduced with both *N. fuscipes* and *N. macrotis* male. Comparison of observed successful pairings with random

pairings generated from the null model (pairing based only on male availability) showed that BC-*macrotis* females had a very strong tendency to reproduce with *N. macrotis*-like males (males with low q_{FU}), whereas BC-*fuscipes* females had a much weaker tendency to reproduce with *N. fuscipes*-like males and were somewhat more likely to reproduce with more *N. macrotis*-like males.

While the directionality of reproductive output for interspecies crosses is consistent with our behavioral expectations, it is also possible that *N. macrotis* females often mate with *N. fuscipes* males, but that male F1 embryos are genetically inviable, or that *N. macrotis* females are unable to carry these young through the gestation process and abort early. The latter case would be evidence of genomic imprinting, a form of genomic incompatibility that may influence reproductive output in hybrid zones such as this wherein hybridization occurs between differentially-sized species. Because embryonic nutrient acquisition and fetal growth is largely determined by paternally expressed (imprinted) alleles (*e.g.* IGF-II, DeChiara et. al. 1991) it is possible that small-bodied *N. macrotis* females carrying large *N. fuscipes*-fathered embryos may fail to properly gestate these young (Dawson *et al.* 1993, Vrana *et al.* 2007). This phenomenon has been well-documented in crosses between the relatively large *Peromyscus maniculatus* and the smaller *Peromyscus polionotis* wherein female *P. maniculatus* crossed with male *P. polionotus* lead to viable and fertile F1 offspring, yet few offspring survive from the reciprocal cross (small-bodied *P. polionotus* females crossed to large-bodied *P. maniculatus* males) at least in part due to fetal overgrowth (Dawson *et al.* 1993).

While our sample size of F1 juveniles is limited, the sex ratios we observed suggest possible asymmetric parent-of-origin effects. That is, all 5 F1 juveniles assigned to *N. macrotis* mothers (n = 3) were female, whereas approximately half (7) of the 16 F1 juveniles assigned to

N. fuscipes mothers (n = 12) were female (Table S3). These data suggest that F1 male embryos with *N. macrotis* mothers are inviable due to intrinsic genetic effects, or because their relatively small-bodied mothers can carry F1 hybrid female fetuses to term, but large F1 male fetuses are aborted early. We do not know the size of developing fetuses in woodrats, but F1 adult males are, on average, 25% larger than adult F1 females and 29% larger than *N. macrotis* males. In *P. maniculatus* x *P. polionotus* F1 offspring, adult size is reflective of fetal size (Dawson *et al.* 1993, Vrana pers. comm.) so it is likely that F1 *Neotoma* males (especially *N. fuscipes*-fathered males) are large at the fetal stage and may be difficult for *N. macrotis* females to properly gestate.

Reproductive interference

As the availability of admixed mates increased during the time of our study, average reproductive success of *N. fuscipes* females declined, which suggests reproductive interference (Gröening and Hochkirch 2008). We did not detect such a decline in males or in *N. macrotis* females. The fact that *N. macrotis* females appear to have stronger assortative affiliative preferences suggests that pre-mating barriers may be better developed in these females, reducing their participation in cross-species matings and exposure to the reproductive costs associated with those matings. *Neotoma macrotis* females at this site may have already evolved pre-mating barriers through the process of reinforcement (Rundle and Schluter 1998) or they may come into this area of contact with assortative preferences that, on average, reduce their exposure to post-zygotic selection associated with hybridization.

The fine-scale, but stark, parapatry in zones of secondary contact between woodrats has most often been explained by competition for optimal nest sites (Dial 1988) and food resources

(Cameron 1971, Shurtliff *et al.* 2014, Matocq and Murphy 2007, Matocq *et al.* 2020, Nielsen and Matocq 2021). However, our study suggests that, perhaps in addition to classic forms of resource competition, the reproductive "mistakes" (Gröening and Hochkirch 2008) made in sympatry may generate a fitness cost that limits the spatial overlap of closely related woodrat species that have not achieved full reproductive isolation (Vallin *et al.* 2012). The promiscuous mating system of woodrats (Topping and Millar 1998, Matocq 2004, McEachern *et al.* 2009, Baxter *et al.* 2009) where individuals of both sexes have multiple mates, may minimize the risk or cost of hybridization when in sympatry (Zeh and Zeh 1996, 1997, and 2001, Knief et al. 2020, Villa et al. 2021). Yet, despite the potential buffer of promiscuity and multiple paternity known in these species, *N. fuscipes* females experienced a decline in reproductive success. This suggests that the cost of hybridization could be a factor contributing to the decline of *N. fuscipes* and continued retraction of their range at the trailing edge of their distribution in this region.

Conclusions

During the time of our study, dry winters facilitated the survival of *N. macrotis*, and this, potentially coupled with the exploratory tendencies we observed in female behavioral trials, contributed to movement of *N. macrotis* into the range of *N. fuscipes*. From the behavioral and parentage data presented here, we suspect that the admixture process was largely instigated by *N. fuscipes* female x *N. macrotis* male crosses, with the reciprocal cross happening much more rarely. As such, while *N. macrotis* female mate choice or incompatibility with *N. fuscipes* males may limit hybridization at the expansion front, the mating success of *N. macrotis* males across the genomic spectrum of females contributes most heavily to the initial accumulation of admixed genomes. The high fertility of F1 females generates backcrosses in both parental directions.

Although their BC-*fuscipes* sons show lowered reproductive success, all backcross-sex combinations bred with both parental classes, creating the potential for interspecific gene flow and introgression. However, females of all admixed classes (who should be predominantly carrying *N. fuscipes* mtDNA) showed a greater tendency to successfully backcross towards *N. macrotis*. This provides the reproductive link that underlies the previous observation of *N. fuscipes* mtDNA introgression towards *N. macrotis* at this site (Coyner et al. 2015) and the expectation that introgression should occur in the direction of the expanding/invading taxon (Wirtz 1999, Currat et al. 2008; but see Zhang 2014). Given the overall preference for *N. macrotis* males, the cost of hybridization to *N. fuscipes* females, and *N. macrotis'* survival advantage under winter drought conditions, we would expect that continued drought would lead to *N. macrotis* replacing *N. fuscipes* in this region, and movement of the hybrid zone in a northeast direction.

Given that the reproductive success of F1 females was high and that hybrids on this site had high survival in some years (Hunter *et al.* 2017), the cost to outbreeding may change across years and may be balanced, especially at the expansion front, by the risk of not breeding if the acceptance threshold of non-like mates is too restrictive (Reeve 1989, Shizuka and Hudson 2020). In fact, for range-edge individuals that may find themselves surrounded by non-like or heterospecific mates, it may be beneficial to have a more permissive threshold, and thus, the partial reproductive isolation of this system could be viewed as adaptive (Servedio and Hermisson 2020). Although our study only captured a narrow window of the breadth of environmental conditions that characterize this system over time, the conditions we observed provide unique insight into the mechanisms underlying the potential for introgression between these species. The weather conditions, survival patterns, and mating opportunities our sampling

captured may be transient, but even ephemeral bouts of hybridization and introgression can have profound evolutionary consequences. Because areas of secondary contact and hybridization are often at the respective edges of species' ranges, these areas provide an opportunity not only to discover mechanisms of reproductive isolation, but also how the nature of species boundaries determine population trajectories at leading and trailing edges. In this and other systems, predicting response to environmental change will require continued tracking of population and genomic dynamics through a greater range of environmental fluctuation.

Data availability

Data and code are available at: https://github.com/kevintshoemaker/hybridzone_repro.

Author contributions

MM designed the study, obtained funding, conducted parentage analysis and wrote the first draft of the manuscript; PM conducted and analyzed the behavioral trials; CA conducted spatial data analyses; EH and KS conducted simulations and associated analyses. All authors contributed to the final version of the manuscript.

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Conflict of Interest

The authors declare no conflicts of interest.

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Figure Legends

Figure 1. (A) Location of secondary contact (red star) between *Neotoma fuscipes* (full distribution in blue) and *N. macrotis* (full distribution in yellow) on the western coast of North America from Oregon, USA at the northern edge of the *N. fuscipes* range to Baja California, Mexico at the southern edge of the *N. macrotis* range, with the study area indicated by the red star. (B) Change in relative abundance and distribution of *N. fuscipes* (blue squares), *N. macrotis* (yellow circles) and admixed (green triangles) individuals from 2008- 2013; symbols represent individual woodrats. (C) Inset showing the riparian corridor along the Nacimiento River of California, USA occupied by woodrats as shown in panel B. (D) Movement of center of hybrid zone from 2008-2013 as a result of expansion of *N. macrotis* and augmented hybridization (reproduced from Hunter et al. 2017). Conspecific mating opportunities declined for each parental species (E, F) from 2008-2012 as the fraction of admixed mates available increased (G).

Figure 2. Activity and preference of individual *Neotoma. fuscipes* (blue) and *N. macrotis* (yellow) females in behavioral trials run in a T-maze for 120 minutes. (A) Total time spent outside starting home box interacting with novel portion of test arena by *N. fuscipes* and *N. macrotis* females during behavioral trials. Net preference of a female for the conspecific male minus the heterospecific male for time in (B) and visits to (C) the male's home cage. Dark plusses are averages, back-transformed from squareroot-transformed values, with the 95% confidence interval represented as the vertical crossbar.

Figure 3. Average reproductive success of male and female woodrats (*Neotoma fuscipes* and *N. macrotis*) across parental and admixed classes from 2008-2012. Points are means and error bars are standard errors. BC = backcross; F1 = first generation hybrid.

Figure 4. Bootstrapped tests for deviation from random mating in a hybrid zone between *Neotoma fuscipes* and *N. macrotis*. In each panel, histograms represent the distribution of the mean mate genotype (n=100; fraction of genome derived from *Neotoma fuscipes* [$q_{fuscipes}$], averaged across all known-reproductive individuals) under a null hypothesis in which mate pairing is solely a function of availability (and not phenotype or genotype). Red vertical lines indicate the observed mean mate genotype. BC = backcross; F1 = first generation hybrid.

Figure 5. (A) Female reproductive probability (annual probability of successfully producing one or more offspring), modeled as a function of body size, genotype (classified as *Neotoma fuscipes*, *N. macrotis*, and admixed), and the fraction of admixed mates within the pool of available mates. Panels B and C represent partial-dependence plots depicting the probability of reproduction (y axis) as a function of a single covariate (x axis), with all other covariates held at their mean or most frequent value. Closed circles and solid lines represent mean predicted reproductive probabilities, and dashed lines/error bars/shaded regions represent two standard errors from the mean.

Figure 6. Female mate-pairing success (relative probability of successfully pairing and producing offspring with available males) modeled separately for *Neotoma fuscipes* (top row) and *N. macrotis* (bottom row) as a function of mate body size (left panels) and genotype (fraction

of mate genotype derived from *N. fuscipes*). Each panel represents a partial-dependence plot depicting mate-pairing success (y-axis) as a function of a single covariate (x-axis), with other covariates held at their mean or most frequent value. Solid lines represent mean predicted reproductive probabilities, and dashed lines represent two standard errors from the mean.

Figure 1

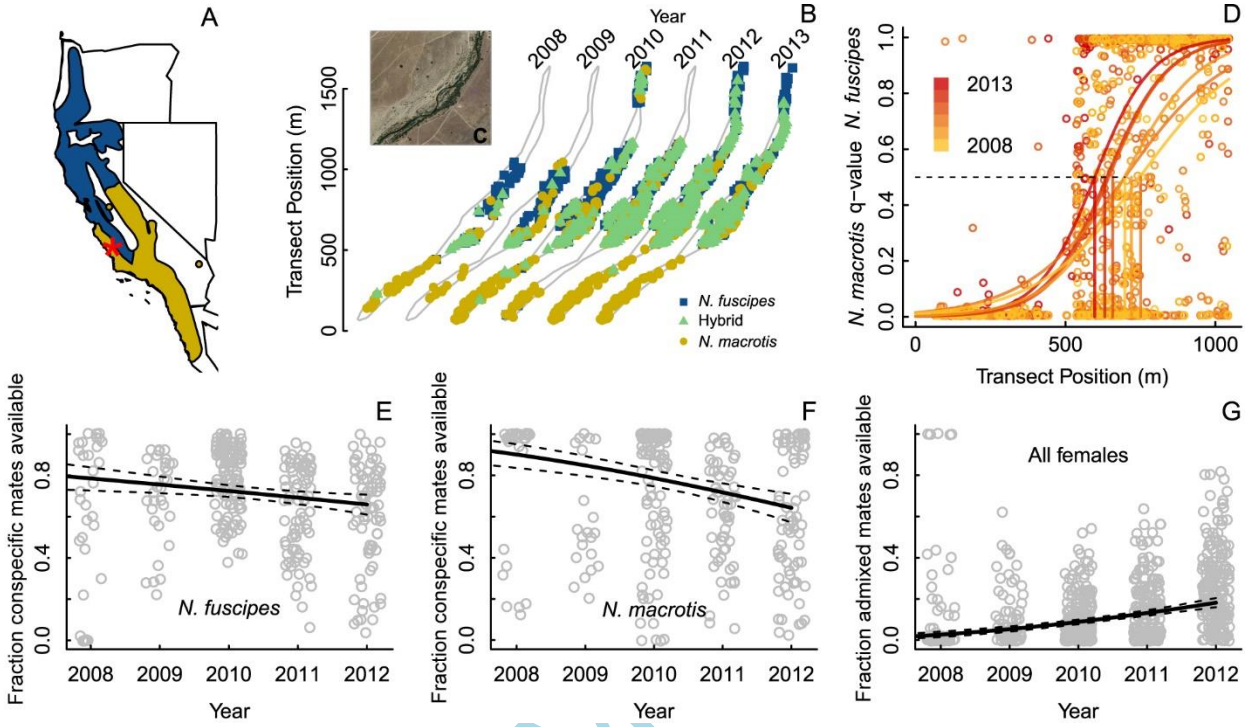

Figure 2

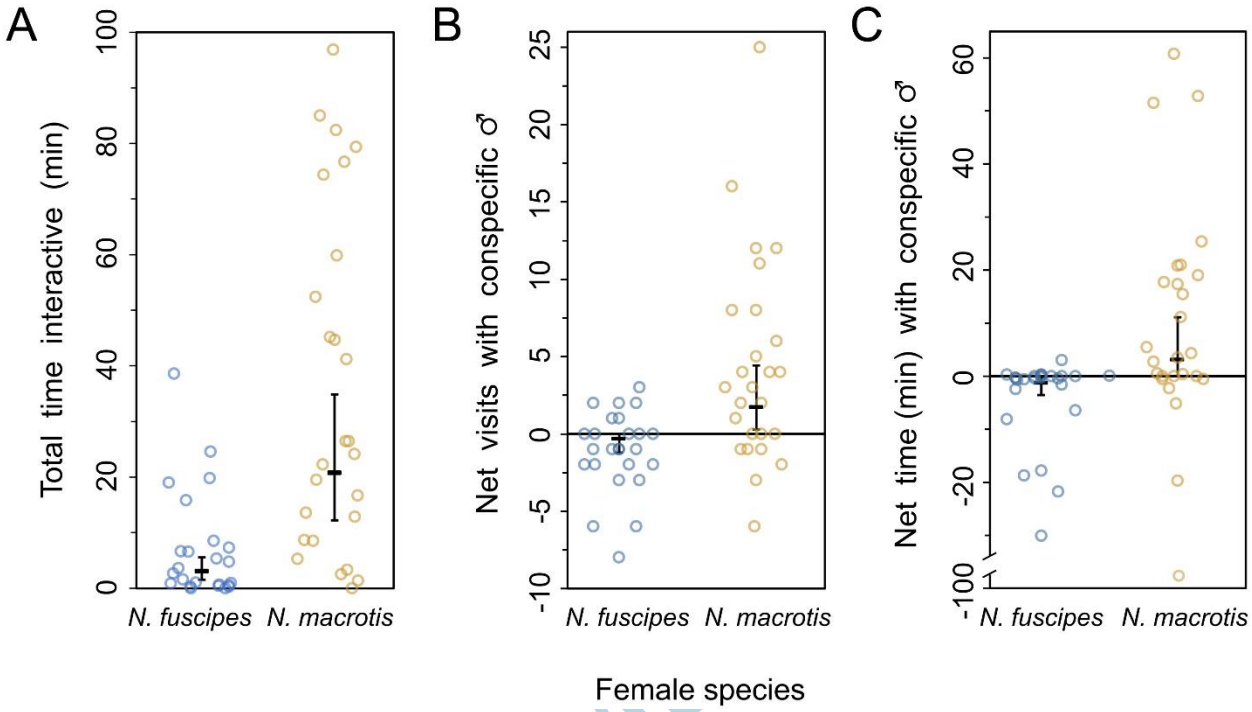

Figure 3

Figure 4

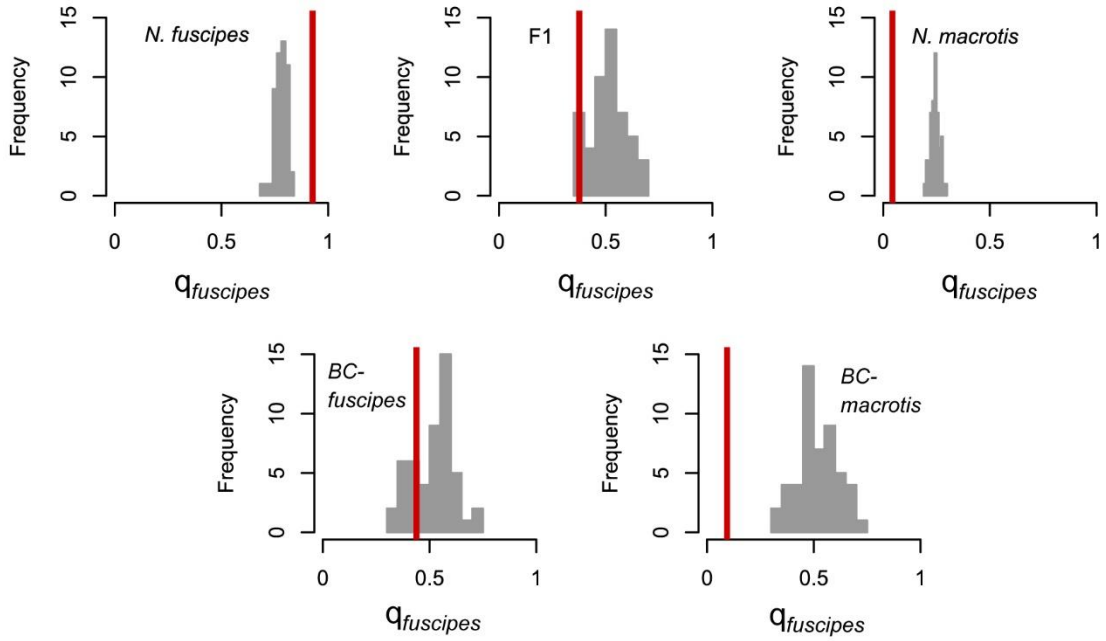

Figure 5

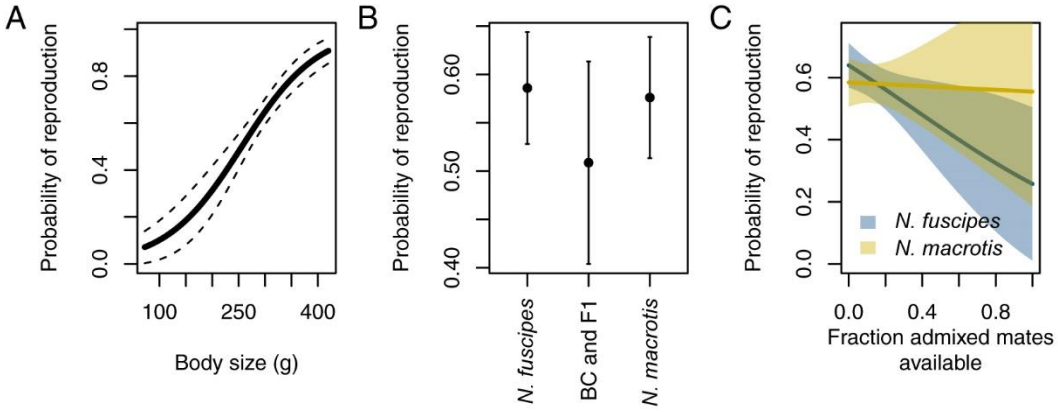

Figure 6