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A Test of Haldane's Rule in *Neodiprion* Sawflies and Implications for the Evolution of Postzygotic Isolation in Haplodiploids

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ABSTRACT: Haldane's rule—a pattern in which hybrid sterility or inviability is observed in the heterogametic sex of an interspecific cross—is one of the most widely obeyed rules in nature. Because inheritance patterns are similar for sex chromosomes and haplodiploid genomes, Haldane's rule may apply to haplodiploid taxa, predicting that haploid male hybrids will evolve sterility or inviability before diploid female hybrids. However, there are several genetic and evolutionary mechanisms that may reduce the tendency of haplodiploids to obey Haldane's rule. Currently, there are insufficient data from haplodiploids to determine how frequently they adhere to Haldane's rule. To help fill this gap, we crossed a pair of haplodiploid hymenopteran species (*Neodiprion lecontei* and *Neodiprion pinetum*) and evaluated the viability and fertility of female and male hybrids. Despite considerable divergence, we found no evidence of reduced fertility in hybrids of either sex, consistent with the hypothesis that hybrid sterility evolves slowly in haplodiploids. For viability, we found a pattern opposite to that of Haldane's rule: hybrid females, but not males, had reduced viability. This reduction was most pronounced in one direction of the cross, possibly due to a cytoplasmic-nuclear incompatibility. We also found evidence of extrinsic postzygotic isolation in hybrids of both sexes, raising the possibility that this form or reproductive isolation tends to emerge early in speciation in host-specialized insects. Our work emphasizes the need for more studies on reproductive isolation in haplodiploids, which are abundant in nature but underrepresented in the speciation literature.

Keywords: intrinsic postzygotic isolation, arrhenotoky, speciation, haplodiploidy, dominance theory, faster-X theory.

Introduction

Barriers to gene flow enable species to diverge along independent evolutionary trajectories. For this reason, the evolution of reproductive isolation is a central focus of speciation research. Although there are many different types of reproductive barriers (Dobzhansky 1951; Coyne and Orr 2004), the most impermeable and permanent of these is intrinsic postzygotic isolation, which is the inability to produce viable, fertile hybrids. At a genetic level, hybrid inviability and sterility are often caused by the accumulation of incompatible alleles in diverging populations (Bateson 1909; Dobzhansky 1937; Muller 1942). While neutral or beneficial in the parental genomes, negative epistasis among opposite-ancestry alleles in hybrid genomes results in intrinsic postzygotic isolation.

Before the emergence of complete reproductive isolation, sterility or inviability is often restricted to one sex of the hybrid offspring (Coyne and Orr 1989, 1997). When this occurs, it is almost always the heterogametic sex (XY males or ZW females) that is sterile or inviable, a pattern known as Haldane's rule (Haldane 1922; Schilthuizen et al. 2011). To date, multiple non-mutually-exclusive mechanisms have been proposed to explain Haldane's rule. Two explanations that have gained considerable empirical support are dominance theory and faster-X theory (Schilthuizen et al. 2011; Delph and Demuth 2016). Both assume that hybrid incompatibilities are, on average, at least partially recessive in the hybrids.

First, under dominance theory, heterogametic hybrid malfunction is explained by genetic incompatibilities that are located on sex chromosomes (Turelli and Orr 1995). Whereas hybrids of the homogametic sex will express only those X-linked (or Z-linked) incompatibilities that are at least partially dominant, hybrids of the heterogametic sex will express all X-linked (or Z-linked) incompatibilities, regardless of dominance, since they contain only a single X (or Z) chromosome. Second, the faster-X explanation for

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Haldane's rule stems from the observation that the X (or Z) chromosome often has a disproportionate impact on hybrid fitness compared with autosomes, a pattern known as the large X-effect (Charlesworth et al. 1987). One explanation for the large X-effect is that new beneficial mutations that are partially recessive will have a faster substitution rate on the X chromosome compared with the autosomes (Charlesworth et al. 1987). This is because on the X chromosome, new recessive alleles are immediately visible to selection in heterogametic individuals. An increased substitution rate on the X chromosome provides more opportunities for hybrid incompatibilities to arise. Faster-X evolution can lead to Haldane's rule either via exacerbating the effect of dominance or via the fixation of alleles that act in the heterogametic sex only (Coyne and Orr 2004).

A shared feature of dominance and faster-X theories is that the expression of recessive alleles on sex chromosomes in the heterogametic sex results in stronger postzygotic isolation compared with the homogametic sex. All else equal, both mechanisms predict that the rate of evolution of intrinsic postzygotic isolation should correlate positively with the extent of hemizygosity. In support of this prediction, *Drosophila* species that have a larger proportion of their genome on the X chromosome evolve intrinsic postzygotic isolation more rapidly than species with smaller X chromosomes (Turelli and Begun 1997). Additionally, taxa with heteromorphic sex chromosomes evolve intrinsic postzygotic isolation at lower levels of genetic divergence than taxa with homomorphic or no sex chromosomes (Lima 2014).

Although Haldane's rule has primarily been studied in diploid taxa with sex chromosomes, it has been argued that this rule should also apply to haplodiploids (Haldane 1922; Koevoets and Beukeboom 2009; but see Kulathinal and Singh 2008). Haplodiploidy (arrhenotoky) is a sex determination mechanism in which males develop from unfertilized eggs and are haploid and females develop from fertilized eggs and are diploid (Normark 2003; Bachtrog et al. 2014). The primary rationale for expanding Haldane's rule to include haplodiploids is that the pattern of inheritance for a haplodiploid genome is like that of an X chromosome; therefore, both dominance and faster-X mechanisms are applicable to haplodiploids (Koevoets and Beukeboom 2009). Moreover, because the proportion of the genome that is hemizygous in males is maximized under haplodiploidy, these mechanisms predict that Haldane's rule will evolve more rapidly in haplodiploid taxa than in diploid taxa.

Haldane's rule is a composite phenomenon, however, and the relative importance of dominance, faster-X, and other causal mechanisms for this pattern likely varies across taxa (Wu and Davis 1993; Coyne and Orr 2004; Kulathinal and Singh 2008; Koevoets and Beukeboom 2009; Schilthuizen et al. 2011; Delph and Demuth 2016). Despite some similarities, haploid males differ from heterogametic males in several respects that may reduce adherence to Haldane's rule in haplodiploids relative to diploids (table 1). For example, because haploid males produce sperm via mitosis, they may be less likely than diploid males to evolve hybrid sterility via antagonistic coevolution of meiotic drivers and suppressors (conflict theory; Hurst and Pomiankowski 1991; Phadnis and Orr 2009; Meiklejohn and Tao 2010) or via disruption of spermatogenesis (faster-male theory; Wu and Davis 1993; Clark et al. 2010). Another important difference between diploids and haplodiploids is that when haplodiploids hybridize, the F_1 generation consists of hybrid females (diploid) and pure-species males (haploids). Hybrid males are not formed until after hybrid females themselves reproduce, which provides opportunities for (1) selection to eliminate alleles that reduce hybrid viability and fertility (i.e., only viable, fertile hybrid females can give rise to hybrid males) and (2) recombination to reconstitute viable parental allele combinations in haploid hybrid males (fig. 1).

Taken together, Haldane's rule mechanisms and haplodiploid transmission genetics suggest that comparative patterns of hybrid inviability and sterility—including the tendency to obey Haldane's rule—may differ between haplodiploid and diploid taxa. However, in contrast to the abundance of data for evaluating Haldane's rule in diploid species (Schilthuizen et al. 2011; Matute and Cooper 2021), we know almost nothing about patterns of hybrid sterility and inviability in haplodiploids. Evidence to date suggests that at least some haplodiploid species pairs do obey Haldane's rule and that intrinsic postzygotic isolation—especially hybrid sterility—may evolve more slowly in haplodiploids than in diploids (Breeuwer and Werren 1995; Bordenstein et al. 2001; Koevoets and Beukeboom 2009; Clark et al. 2010; Kulmuni et al. 2010; Koevoets et al. 2012; Kulmuni and Pamilo 2014; Beukeboom et al. 2015; Cordonnier et al. 2020; Zhang et al. 2021). To address the generality of these patterns, however, data from many more haplodiploid taxa are needed. Except for experimental work in *Nasonia* wasps (Breeuwer and Werren 1995; Koevoets and Beukeboom 2009; Clark et al. 2010; Koevoets et al. 2012) and *Tetranychus* spider mites (Knegt et al. 2017; Villacis-Perez et al. 2021), controlled laboratory crosses between haplodiploid species that evaluate the fitness of both male and female hybrids are rare. To start filling this data gap, we investigate hybrid viability and fertility in crosses between *Neodiprion lecontei* and *Neodiprion pinetum*, a pair of haplodiploid pine sawfly species (Order: Hymenoptera; Family: Diprionidae).

Neodiprion pinetum and *N. lecontei* are sister species (Linnen and Farrell 2008) that diverged an estimated 1.5 million generations ago (Bendall et al. 2022). Although their ranges overlap (Linnen and Farrell 2010) and they hybridize in the wild (Linnen and Farrell 2007; Bendall et al. 2022),

Table 1: Evolutionary and genetic mechanisms that may increase or decrease haplodiploid adherence to Haldane's rule relative to diploids

Mechanism	Male inviability	Male sterilty	Explanation	Reference(s)
Dominance	+	+	All recessive alleles—and therefore all sterility- or inviability-causing incompatibilities—will be expressed in haploid male hybrids	Turelli and Orr 1995; Koevoets and Beukeboom 2009[a]
Faster-X evolution	+	+	All beneficial recessive mutations will be expressed in haploid males, increasing the probability that they will fix via natural selection and providing more opportunities for incompatibilities to arise	Charlesworth et al. 1987; Koevoets and Beukeboom 2009[a]
Faster-male evolution	NA	+/−	Lack of meiosis and sex chromosomes may make haploid spermatogenesis insensitive to faster-male evolution; within-ejaculate sperm competition is absent; haplodiploid inheritance impedes sexual selection via Fisherian runaway selection (because fathers do not produce sons) but promotes it via the handicap principle	Wu and Davis 1993; Koevoets and Beukeboom 2009;[a] de La Filia et al. 2015[a]
Improper segregation of sex chromosomes	NA	−	Because haplodiploid males do not undergo meiosis, missegregation of nonhomologous chromosomes cannot cause sterility	Clark et al. 2010[a]
Conflict (meiotic drive)	−	−	Because haplodiploid males do not undergo meiosis, there is no opportunity for antagonistic coevolution of meiotic drivers and suppressors to produce hybrid male sterility; if this mechanism also gives rise to male inviability, that too will be absent in haplodiploids	Hurst and Pomiankowski 1991; Phadnis and Orr 2009; Meiklejohn and Tao 2010[a]
Purifying selection	−	−	Efficient purging of recessive deleterious alleles in haplodiploid populations reduces one potential source of incompatibilities; also, recessive alleles with female-limited expression will not be purged as easily, giving rise to female-limited incompatibilities	Crozier 1976;[a] Avery 1984;[a] Werren 1993;[a] Hedrick and Parker 1997[a]
Haplodiploid transmission genetics	−	−	Alleles that reduce viability and fertility in hybrid females can be purged by selection before the formation of hybrid males; also, recombination in hybrid females will reconstitute viable and fertile parental genotypes in hybrid males	Figure 1

Note: Plus sign indicates that the mechanism is predicted to increase adherence to Haldane's rule in haplodiploids relative to diploids. NA indicates that the mechanism is not relevant to that form of postzygotic isolation. Minus sign indicates that the mechanism is predicted to reduce adherence to Haldane's rule in haplodiploids relative to diploids. Plus sign/minus sign indicates that the mechanism could either promote or reduce adherence to Haldane's rule in haplodiploids, depending on the specific scenario. References are provided for each mechanism, regardless of whether haplodiploids were discussed explicitly.

[a] Explicitly discusses implications of mechanisms for haplodiploids.

N. lecontei and *N. pinetum* are genetically and phenotypically distinct in sympatry ($F_{ST} = 0.63$; Nei's $D = 0.36$). This distinctness is maintained in part by extrinsic postzygotic isolation stemming from female adaptation to different pine hosts (Bendall et al. 2017). Whereas *N. pinetum* females embed their eggs within the needles of a thin-needled pine species (*Pinus strobus*), *N. lecontei* females deposit their eggs in thicker, more resinous needles of multiple other pine species. Hybrid females have maladaptive combinations of egg-laying traits that lead to oviposition failure: they prefer the thin-needled host but have an ovipositor morphology and egg pattern better suited to thicker, more resinous needles (Bendall et al. 2017). Nothing is currently known about the viability and fertility of hybrid males. If these species obey Haldane's rule, hybrid males should have reduced fertility and/or viability relative to hybrid females. To evaluate Haldane's rule, we used multigeneration laboratory crosses between *N. lecontei* and *N. pinetum*. Our data revealed a hybrid viability pattern opposite to that expected under Haldane's rule. In

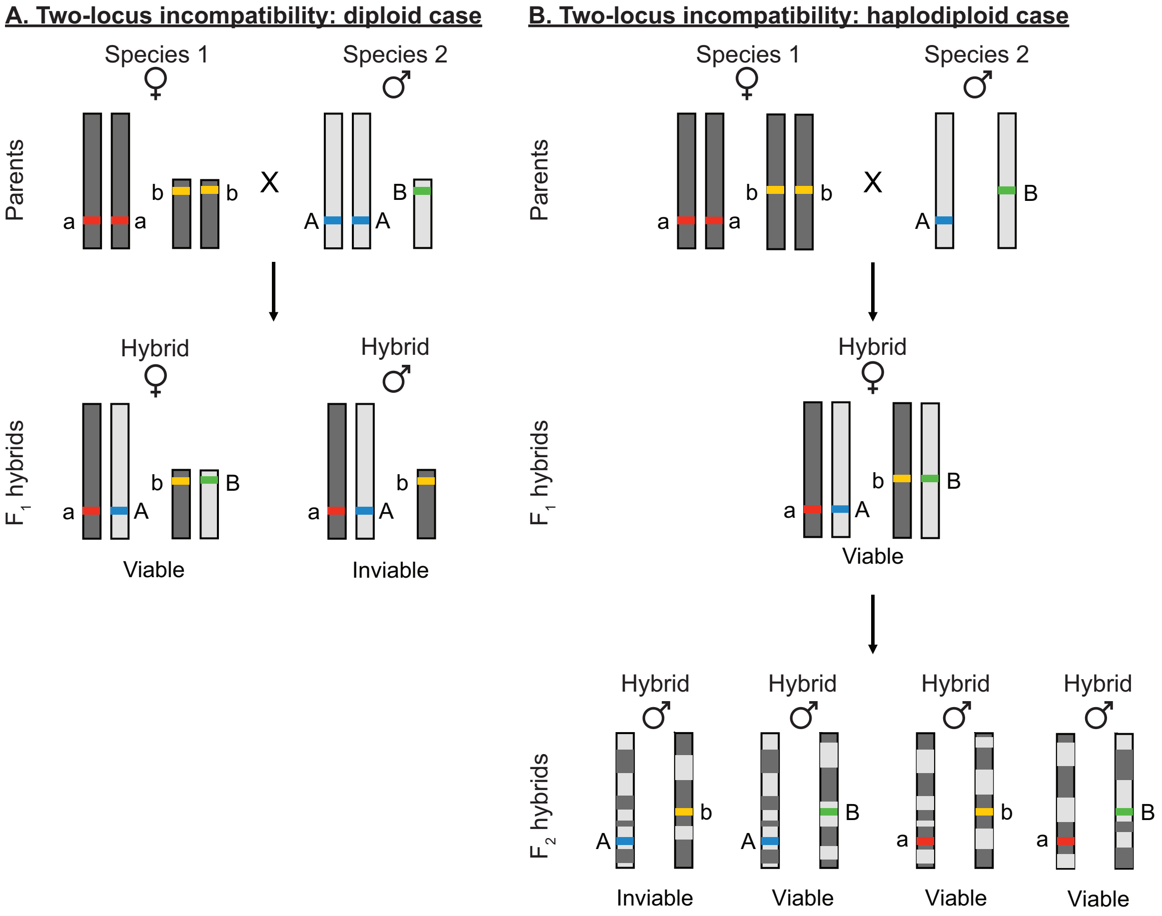

Figure 1: Two-locus incompatibility in diploids (*A*) and haplodiploids (*B*). In diploids, the A locus is on an autosome (long bars), and the B locus is on the X chromosome (short bars; Y chromosome not shown). In haplodiploids, all loci are on autosomes (long bars), and males are haploid. An inviable phenotype is produced when an individual with at least one A allele (dominant) is homozygous or hemizygous for the b allele (recessive). All hybrid diploid males (*A*) have an inviable genotype. In haplodiploids (*B*), hybrid males are formed in the second generation. Because of recombination, only 25% of the haploid hybrid males will have the incompatible allele combination. Note that while this model will produce a viability pattern consistent with Haldane's rule in both cases, viability differences between hybrid females and hybrid males are less extreme in the haplodiploid case (*B*) relative to the diploid case (*A*). Also note that any incompatibility loci that are polymorphic in the parental species and expressed in hybrid females will be eliminated by selection before the production of hybrid males only in the haplodiploid case (*B*).

"Discussion," we consider implications of these findings for patterns of postzygotic isolation and expression of Haldane's rule in haplodiploid taxa.

Methods

Lab Populations and Crosses

The *Neodiprion pinetum* and *Neodiprion lecontei* lab lines that were used in this study were derived from larvae collected from multiple field sites between 2013 and 2017 (table S1) and propagated in the lab for one to four generations following standard lab protocols (Harper et al. 2016; Bendall et al. 2017). Briefly, because females of both species tend to lay their entire egg complement within the needles of a single pine branch and colonies of larval siblings tend to remain intact throughout development (Coppel and Benjamin 1965), eggs and larvae were always reared together with their siblings. After emerging from cocoons, adult females were either mated to nonsibling males (to produce mixed-sex colonies) or left as virgins (to produce male-only colonies) and released into a mesh cage with one or more seedlings of a preferred *Pinus* host (*P. strobus* for *N. pinetum*; *P. banksiana* for *N. lecontei*). Egg-bearing seedlings were watered as needed until hatching, and once larvae had defoliated the seedlings, they were transferred to a plastic rearing box with a mesh lid and fed pine clippings from a preferred host as needed until they spun cocoons. Cocoons were stored individually in gelatin capsules and checked daily for adult emergence. To generate hybrid females and males, we used the crossing scheme illustrated in figure 2. Pure-species lines were always propagated alongside the interspecific crosses. Because *Neodiprion* are haplodiploid, interspecific crosses produced a combination of diploid hybrid females (F_1) and haploid pure-species males. To obtain haploid hybrid males (F_2), we allowed both mated and unmated F_1 hybrid females to reproduce (fig. 2).

Viability of Hybrid Females and Males

We evaluated hybrid viability in two ways: adult sex ratios for both directions of the cross and embryo viability for one direction of the cross (fig. 2). First, to evaluate adult sex ratios for each family type, we used rearing logs from intraspecific and interspecific crosses that occurred between 2013 and 2017. In these logs, we recorded the date and sex of adults that emerged from each of our laboratory colonies. Each colony consisted of the male and female progeny of a single mated pair. After compiling data from rearing logs, our total sample sizes (number of adults and families) for adult emergence data for the six family types (fig. 2) were 485 adults from 27 *N. pinetum* crosses (each family was a mix of *N. pinetum* females and males), 453 adults from 18 *N. lecontei* crosses (each family was a mix of *N. lecontei* females and males), 899 adults from 23 $F_{1(L\times P)}$ crosses (each family was a mix of $F_{1(L\times P)}$ hybrid females and *N. lecontei* males), 208 adults from 15 $F_{1(P\times L)}$ crosses (each family was a mix of $F_{1(P\times L)}$ hybrid females and *N. pinetum* males), 666 adults from 32 $F_{2(L\times P)}$ crosses (each family was a mix of backcross females and hybrid males), and 104 adults from 9 $F_{2(P\times L)}$ crosses (each family was a mix of backcross females and $F_{2(P\times L)}$ hybrid males). To ensure comparable data for families produced by intraspecific and interspecific crosses, we used data from only nonhybrid families that were present in the lab at the same time and derived from the same source populations as our hybrid families.

In *Neodiprion*, sex ratios tend to be female biased (Craig and Mopper 1993; Harper et al. 2016). For an individual family, adult sex ratios are the product of the proportion of eggs that the mother fertilized and egg-to-adult survival rates for each sex. If hybrid females have reduced viability, families produced by interspecific (F_1) crosses should have more male-biased sex ratios than those produced by intraspecific crosses; if hybrid males have reduced viability, families produced by interspecific (F_2) crosses should have more female-biased sex ratios. To determine whether sex ratio varied among the six different family types, we used the glmer function in R package lmerTest version 3.1-3 to fit a mixed effects logistic regression model to the sex ratio data (proportion of adults that were female), with family type and individual family as fixed and random effects, respectively. We then used a type II ANOVA to evaluate significance of family type (Anova function from car ver. 3.0-10), followed by the emmeans function (emmeans ver. 1.5.2-1) for post hoc comparisons among family types, with the Benjamini-Hochberg method (adjust = "fdr") for adjusting *P* values. These and all other statistical analyses were performed in R (ver. 4.1.2; R Core Team 2020). Because sample sizes (total number of adults) were highly variable across families, we repeated these analyses for four different sample size minimums for individual families to be included in the analysis: $N \geq 1$ adult, $N \geq 5$ adults, $N \geq 10$ adults, and $N \geq 15$ adults.

Second, for one direction of the cross (*N. lecontei* female × *N. pinetum* male), we were also able to evaluate embryo viability using data from an oviposition performance assay. In this experiment females were mated to a conspecific male, heterospecific male, or no male, yielding four different types of families (embryos): (1) nonhybrid families containing a mix of *N. lecontei* males and females, (2) nonhybrid families containing a mix of *N. pinetum* males and females, (3) families consisting of a mix of $F_{1(L\times P)}$ hybrid females and nonhybrid *N. lecontei* males, and (4) families consisting of $F_{2(L\times P)}$ hybrid males only (progeny of virgin $F_{1(L\times P)}$ hybrid females).

We released individual females (mated or virgin) into mesh cages with *P. banksiana* and *P. strobus* seedlings, as described in Bendall et al. (2017). For each female, we then recorded whether eggs were laid. If eggs were laid, we recorded the host selected (eggs were always laid on a single host species) and the number of eggs laid. Each egg-bearing pine seedling was checked daily for hatching. Once hatchlings were observed on a seedling, we gave the family an additional 48 h to allow sufficient time for all viable eggs to hatch. We then counted the newly hatched larvae by hand with a fine paintbrush. For each family, embryo viability was calculated as the number of hatchlings divided by the total egg number. For eight families, poor seedling health (e.g., completely dried out needles) led to complete hatching failure. These families, which were observed in all four family types and on both hosts, were excluded from further analysis. In total we scored embryo viability in 18 clutches (families) of *N. lecontei* eggs, all laid on *P. banksiana* ($N = 2{,}081$ embryos); 10 clutches of *N. pinetum* eggs, all laid on *P. strobus* ($N = 857$ embryos); 13 clutches containing a mix of male *N. lecontei* and hybrid female $F_{1(L\times P)}$ eggs, all laid on *P. banksiana* by *N. lecontei* females ($N = 1{,}870$ embryos); and 13 clutches of hybrid male $F_{2(L\times P)}$ eggs laid by $F_{1(L\times P)}$ hybrid females (4 clutches with $N = 401$ embryos on *P. banksiana*, 9 clutches with $N = 768$ embryos on *P. strobus*).

The four types of families we examined had two potential sources of variation in hatching rates: maternal

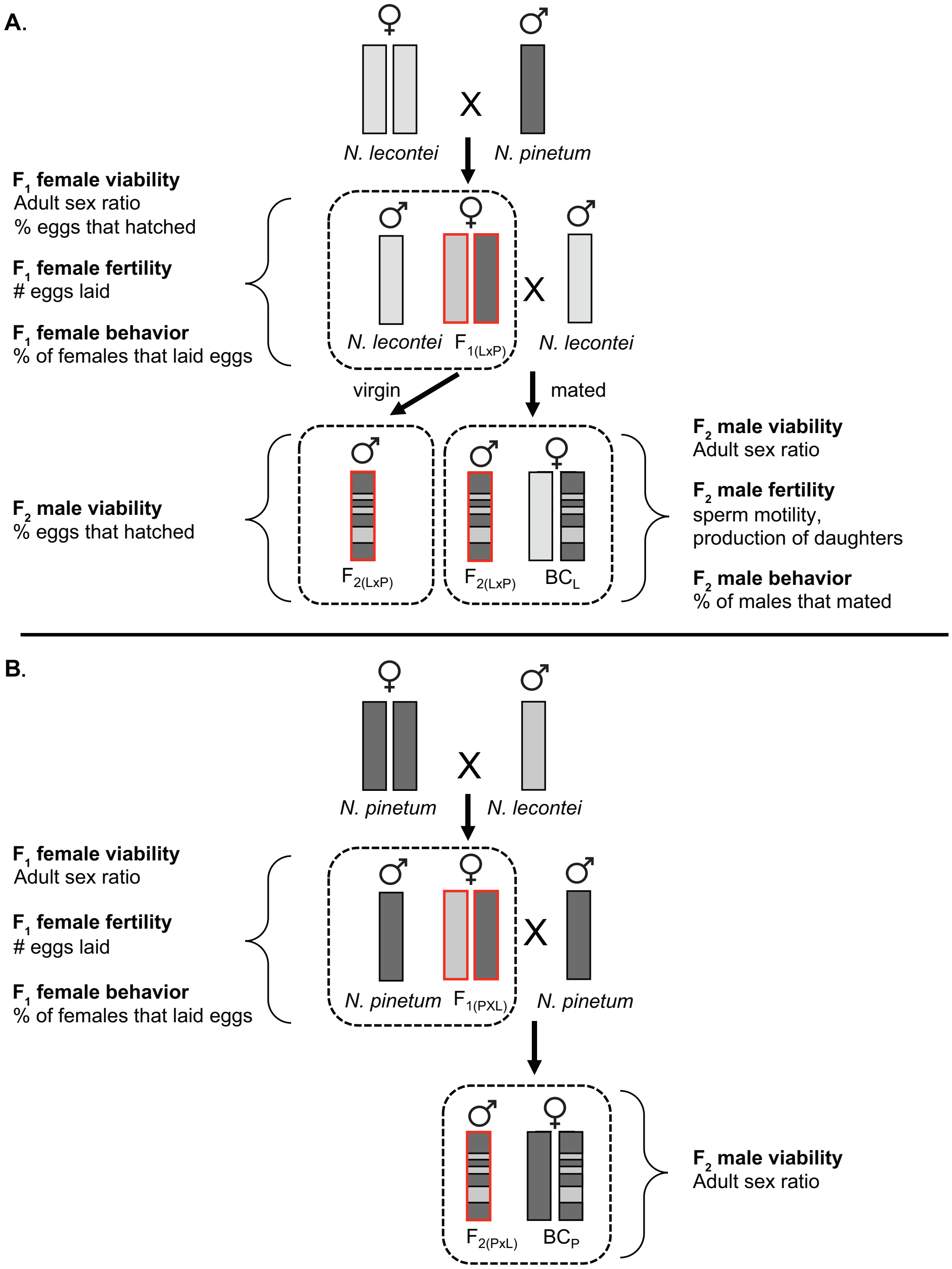

Figure 2: Crossing scheme for measuring hybrid female and hybrid male viability and fertility. Rectangles depict ploidy (one indicates haploid, two indicates diploid), with coloring indicating genomic ancestry (light gray indicates *Neodiprion lecontei*, dark gray indicates *Neodiprion pinetum*). Dashed boxes enclose different family types produced by mated or virgin females, and red outlines indicate focal hybrid individuals within families. Note that within hybrid families, males and females have different genomic ancestry proportions (see text for how this could bias some hybrid viability estimates). Both directions of the reciprocal cross are shown: *N. lecontei* females × *N. pinetum* males (*A*) and *N. pinetum* males × *N. lecontei* females (*B*). Note that viability and fertility data were more limited for one direction of the cross (*B*).

oviposition success on different species of pines (due to egg-laying traits of the mother) and embryo viability. However, with our experimental design, we cannot statistically disentangle host effects, family-type effects, and host-by-family-type interactions because only F_1 hybrid females varied in host choice. Instead, we fitted three separate logistic regression models to the data to explore the effects of family type and host plant in different contexts. First, to determine whether the probability of hatching on *P. banksiana* differed depending on the genomic composition of the embryos, we used the glmer function (lmerTest ver. 3.1-1) to fit a mixed effects logistic regression model to the hatching data, with family type (*N. lecontei*, $F_{1(L\times P)}$, or $F_{2(L\times P)}$, all on *P. banksiana*) as a fixed effect and family as a random effect to account for hatching differences among families that were unrelated to hybrid viability. To evaluate significance of model terms and conduct post hoc tests, we used a type II ANOVA and the emmeans function as described above. Haldane's rule predicts reduced viability of F_2 males relative to other embryo types.

Second, to evaluate the effect of host choice on hatching success while controlling for the genetic composition of embryos, we fitted a mixed effects logistic regression model to hatching data for $F_{2(L\times P)}$ eggs laid by $F_{1(L\times P)}$ hybrid females, with host as a fixed effect and family as a random effect. A significant host effect would be indicative of extrinsic (host-dependent) postzygotic isolation stemming from some mixture of F_1 female egg-laying traits and F_2 male hatchling-host interactions. Third, because extrinsic postzygotic isolation also predicts that hatching rates of eggs laid by hybrid females will be reduced relative to pure-species females, we also fitted a mixed effects logistic regression model to hatching data for $F_{2(L\times P)}$ eggs and *N. pinetum* eggs on *P. strobus*, with cross type as a fixed effect and family as a random effect. We did not directly compare the hatching rates of *N. pinetum* on *P. strobus* with the other cross types on *P. banksiana* because host and family type were confounded for these comparisons.

Fertility and Behavior of Hybrid Females and Males

To evaluate hybrid female fertility, we used fecundity data from a host preference experiment in which we released nonhybrid and hybrid females from both cross directions (fig. 2) individually into cages with *P. banksiana* and *P. strobus* seedlings, as described above. After oviposition or female death, we counted the total number of eggs laid by each female. Because *N. lecontei* and *N. pinetum* females emerge from cocoons with a full complement of eggs, tend to lay all or most of this complement in a single clutch, and die shortly after laying eggs, the total number of eggs laid in our experiment is a good approximation of lifetime fecundity. Females for this assay included all of the females from the embryo viability assay and additional females for which we recorded egg number but did not count hatchlings. Our sample size for the four types of mated female were $N = 124$ *N. lecontei* females, $N = 108$ *N. pinetum* females, $N = 32$ $F_{1(L\times P)}$ hybrid females, and $N = 41$ $F_{1(P\times L)}$ hybrid females.

Many females did not lay any eggs at all, despite having distended abdomens that were full of eggs (i.e., they looked like typical females). To determine whether different types of females differed in their willingness to lay eggs in our host choice assays, we used the glm function to model binary oviposition outcome (laid or did not lay) as a function of female type. If there is behavioral sterility of hybrid females (e.g., as in Linn et al. 2004), hybrid females should exhibit a reduced willingness to lay eggs relative to nonhybrid females. For those females that did lay eggs ($N = 87$ *N. lecontei* females, $N = 43$ *N. pinetum* females, $N = 15$ $F_{1(L\times P)}$ hybrid females, and $N = 25$ $F_{1(P\times L)}$ hybrid females), we next used linear regression (lm function in R) to determine whether different types of females differed in the number of eggs they laid. For this analysis, we excluded females that did not lay eggs because we obtained egg counts from trees rather than via dissection. We therefore did not have any information on the number of eggs produced (but not laid) by nonlaying females. Nevertheless, if hybrid females have reduced fecundity, they should lay fewer eggs than nonhybrid females. For both analyses, we used type II ANOVAs to evaluate significance of model terms and emmeans for post hoc tests with false discovery rate correction, as described above.

To determine whether there was evidence of behavioral sterility in hybrid males, we used no-choice mating assays. We placed a single *N. lecontei* female in a clear 3.25-oz container with either a *N. lecontei* male ($N = 36$) or a $F_{2(L\times P)}$ hybrid male ($N = 37$; *N. pinetum* males and females and $F_{2(P\times L)}$ hybrid males were not available at the time). We observed each pair for 2 h and recorded whether they mated during that time. To evaluate whether mating success differed between *N. lecontei* and hybrid males, we performed a logistic regression.

To evaluate hybrid male fertility, we examined sperm motility in *N. pinetum* ($N = 20$), *N. lecontei* ($N = 47$), and $F_{2(L\times P)}$ males ($N = 39$). We were able to quantify hybrid male fertility in only one direction of the cross because of a lack of $F_{2(P\times L)}$ hybrid males at the time of our male fertility experiments (fig. 2). Upon eclosion from cocoons, adult males were stored at 4°C until use to prolong life. In some cases males were used in mating assays (see above) before testes dissection and then returned to 4°C for a minimum of 24 h until further use. Males were warmed to room temperature for a minimum of 1 h before dissection. From each male, we removed both testes and placed each testis on a siliconized slide in 50 μL of testes buffer (183 mM KCl, 47 mM NaCl, 10 mM Tris-HCl, pH 6.8). After piercing a testis, we imaged the sperm at ×40 with a Nikon E800 DIC. In doing so, we discovered that *Neodiprion* males have sperm that form bundles. We scored the approximate percentage (to the nearest 5%) of bundles that were moving in each testis in each male and then averaged the two scores to obtain a single motility score per male. We then used the lm function in R to fit a linear model to the motility data, with male type (*lecontei*, *pinetum*, or $F_{2(L \times P)}$ hybrid), mating status (mated or unmated), and male age as predictors. On the basis of regression diagnostics, we applied a normal quantile transformation to the motility data before fitting the regression model. To evaluate the significance of model terms, we used a type II ANOVA. Because having motile sperm and copulating with a female do not necessarily mean that a hybrid male is fertile, we took additional steps to confirm hybrid male fertility. To do so, we placed each female that mated with a $F_{2(L \times P)}$ hybrid male in a cage with a *P. banksiana* seedling and reared resulting offspring as described above. For all adult-producing colonies, we scored the presence/absence of adult females, a clear indicator of successful fertilization.

Results

Viability of Hybrid Females and Males

Regardless of sample size thresholds (minimum number of adults per family), there was a significant effect of family type on adult sex ratio ($P < 1 \times 10^{-7}$ for $N \geq 1$, $N \geq 5$, $N \geq 10$, and $N \geq 15$ adults; tables S2–S5). In general, families with F_1 hybrid females tended to have lower proportions of females than nonhybrid families, whereas families with F_2 hybrid males tended to have slightly higher proportions of females than nonhybrid families (fig. 3*A*). In post hoc tests $F_{1(L \times P)}$ families had significantly different sex ratios from all other family types, regardless of sample size minimums per family (tables S2–S5). Additionally, $F_{2(L \times P)}$ families differed significantly from one or both parental species in some post hoc tests; however, these results were not robust to sample size cutoffs (table S2–S5). Overall, these results are consistent with reduced hybrid viability in one direction of the cross (*Neodiprion lecontei* female × *Neodiprion pinetum* male), with hybrid females experiencing a more pronounced reduction in viability than hybrid males.

Differences in adult sex ratio (fig. 3*A*) were partially attributable to differential survival of embryos (fig. 3*B*). Family type had a significant effect on the proportion of eggs that hatched on *Pinus banksiana* ($\chi^2 = 8.47$, df = 1, $P = .014$), with reduced hatching success of families with $F_{1(L \times P)}$ females compared with *N. lecontei* and $F_{2(L \times P)}$ families (fig. 3*B*). However, while the hatching success of families with $F_{1(L \times P)}$ females differed significantly from *N. lecontei* families in post hoc tests ($Z = -2.67$, $P = .023$), a comparison between $F_{1(L \times P)}$ families and $F_{2(L \times P)}$ families did not quite reach statistical significance ($Z = -2.07$, $P = .057$), possibly because of the small number of $F_{2(L \times P)}$ families on *P. banksiana*. The viability of $F_{2(L \times P)}$ embryos was not reduced relative to *N. lecontei* embryos (fig. 3*B*; $Z = 0.29$, $P = .70$). Embryo viability data also revealed evidence of extrinsic (host-dependent) postzygotic isolation. $F_{2(L \times P)}$ families that were laid on *Pinus strobus* had reduced hatching success compared with both the same genotype laid on *P. banksiana* ($\chi^2 = 89.79$, df = 1, $P < 2.2 \times 10^{-16}$) and *N. pinetum* families laid on *P. strobus* ($\chi^2 = 22.26$, df = 1, $P = 2.4 \times 10^{-6}$; fig. 3*B*).

Fertility of Hybrid Females and Males

Females of four different types (parental species and reciprocal hybrids) differed in their willingness to lay eggs ($\chi^2 = 11.42$, df = 3, $P = .0097$). In general, a higher proportion of *N. lecontei* females laid than all other female types (fig. 4*A*, 4*B*). *Neodiprion lecontei* females differed significantly from both *N. pinetum* and $F_{1(L \times P)}$ females in willingness to lay eggs (table S6). However, the proportion of $F_{1(P \times L)}$ females that laid eggs did not differ significantly from any of the other female types (table S6). Of those females that laid eggs, there was also a significant difference in the number of eggs laid among female types ($F = 8.98$, df = 3, $P = 1.51 \times 10^{-5}$). On average, *N. pinetum* females laid fewer eggs than any other female type (fig. 4*C*, 4*D*; table S7). These results suggest that while hybrid females tend to resemble *N. pinetum* in their willingness to lay in choice arenas (fig. 4*A*, 4*B*), they have fecundity comparable to *N. lecontei* females (fig. 4*C*, 4*D*). Overall, we did not see any evidence of reduced fertility or behavioral sterility in hybrid females relative to both parental species.

Mating outcomes for pairs consisting of *N. lecontei* females and hybrid ($F_{2(L \times P)}$) males differed significantly from outcomes for *N. lecontei* pairs ($\chi^2 = 3.99$, df = 1, $P = .046$): pairs containing hybrid males mated less often than those containing *N. lecontei* males (fig. 4*E*, 4*F*). This constitutes a potential source of extrinsic postzygotic isolation in at least one direction of the cross. We also found that sperm motility was unaffected by male mating status ($F = 0.068$, df = 1, $P = .80$) but declined with male age ($F = 5.09$, df = 1, $P = .026$). We did not find differences in sperm motility between $F_{2(L \times P)}$ hybrid males and *N. lecontei* and *N. pinetum* males ($F = 0.80$, df = 2, $P = .45$; fig. 4*G*, 4*H*). Moreover, of the 10 hybrid male–fathered colonies that

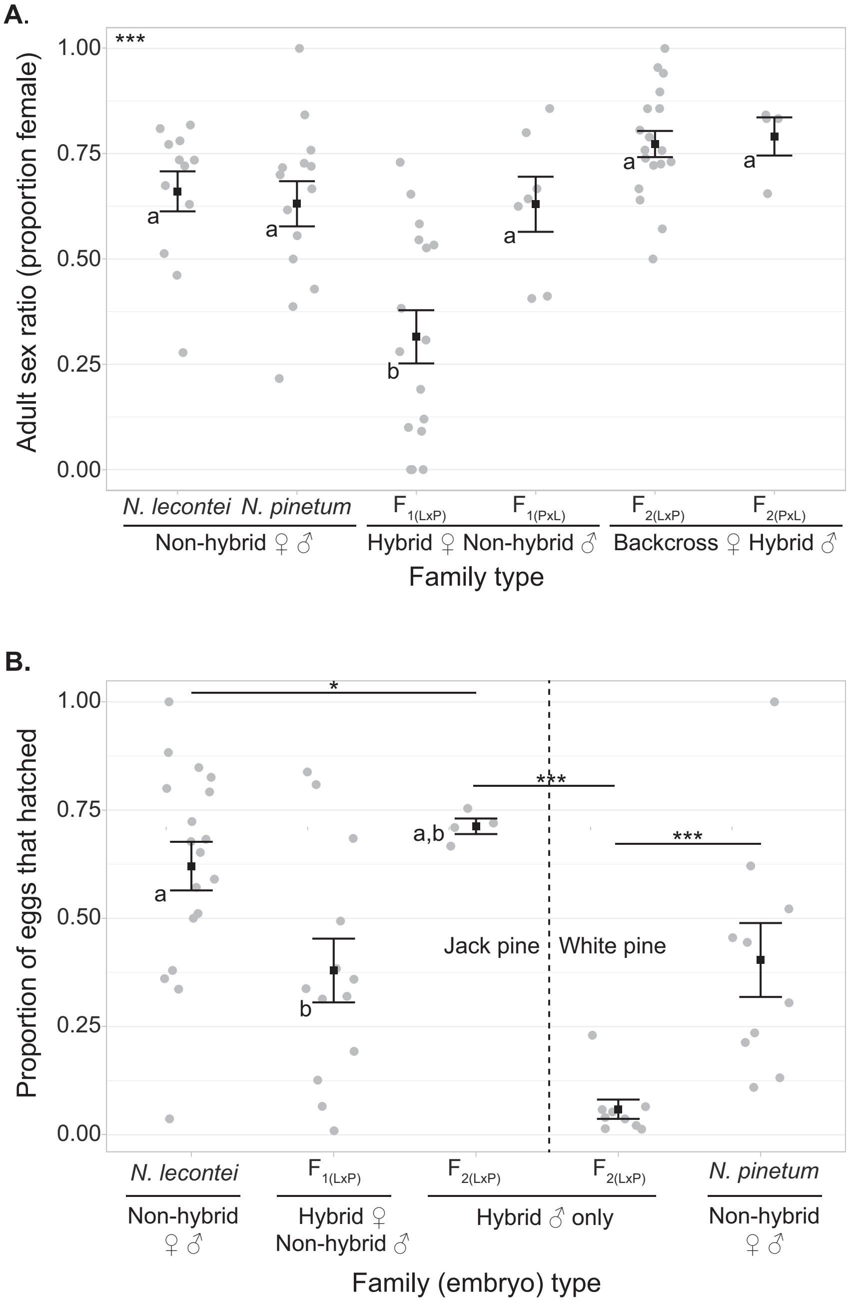

Figure 3: Viability of hybrid females and males. *A*, Adult sex ratio (proportion female) by family type. All families contain a mix of diploid females and haploid males, as indicated on the *x*-axis; family naming is as in figure 2. Only families (gray points) with $N \geq 10$ adults are included (see table S2–S5 for full results). *B*, Proportion of eggs that hatched by family type and oviposition host, with the composition of each family (gray points) indicated on the *x*-axis. The two host types (jack and white pine) are separated by a vertical, dashed line. Data were only available for one direction of the cross. In both panels, means (black points) and standard errors (lines) are given. Statistical significance of family type (*A* and within-host comparisons in *B*) and host plant (within-type comparison in *B*) is indicated by asterisks ($^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$). Different lowercase letters denote pairwise comparisons that were significant in post hoc tests.

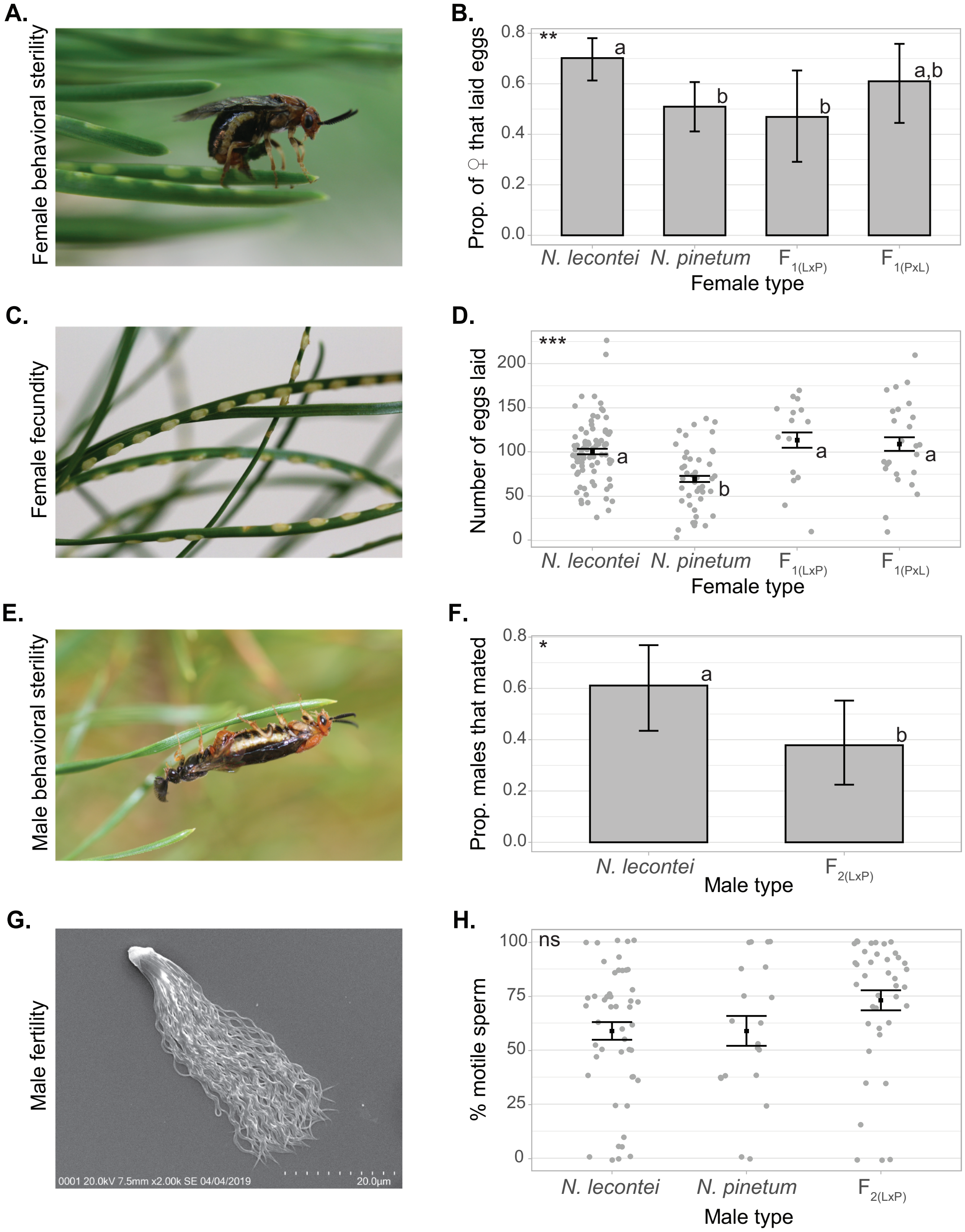

Figure 4: Fertility of hybrid females and males. *A*, Ovipositing *Neodiprion lecontei* female with swollen, egg-filled abdomen (photo: Robin K. Bagley). *B*, Proportion of females of each type that laid eggs in choice assays. *C*, Egg-bearing needles laid by *N. lecontei* (photo: Kim Vertacnik). *D*, Number of eggs laid by female type. *E*, Mating pair of *N. lecontei* adults (male on left; photo: Robin K. Bagley). *F*, Proportion of males that mated with *N. lecontei* females by male type. *G*, Scanning electron microscope image showing bundled *Neodiprion* sperm (photo: Kristin Hook). *H*, Proportion of motile sperm bundles (averaged across two testes) by male type. Statistical significance in *B*, *D*, *F*, and *H* is indicated by asterisks ($^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$; ns = not significant); different lowercase letters denote pairwise comparisons that were significant in post hoc tests. In *B* and *F*, error bars are binomial (Clopper-Pearson) 95% confidence intervals for proportions. In *D* and *H*, means and standard errors are given (black points and lines).

produced any adults, seven produced adult females, indicating that hybrid males are fertile.

Discussion

Although haplodiploid organisms represent a substantial proportion of terrestrial biodiversity (Forbes et al. 2018), they are almost entirely absent from comparative studies of speciation. Our analysis of viability and fertility in hybrids between a pair of host-specialized haplodiploid species helps fill this void and revealed several patterns of interest. First, hybrid females suffered greater mortality than hybrid males (fig. 3), a pattern opposite to that expected under Haldane's rule. Second, we also observed asymmetries in the strength of postzygotic isolation: hybrid female viability was lowest when hybrids had *Neodiprion lecontei* cytoplasm (fig. 3*A*). Similar asymmetries have been documented in diverse taxa, including haplodiploids (Breeuwer and Werren 1995; Tiffin et al. 2001; Turelli and Moyle 2007; Lowry et al. 2008; Niehuis et al. 2008; Clark et al. 2010; Koevoets et al. 2012; Matute and Cooper 2021). Third, we found no evidence of reduced fecundity in females or reduced sperm motility in males (fig. 4). Together, these results are consistent with evidence from other species pairs that suggest that intrinsic postzygotic isolation—especially hybrid sterility—may evolve slowly in haplodiploids and other animals that lack sex chromosomes (Koevoets and Beukeboom 2009; Johnson and Lachance 2012; Matute and Cooper 2021). However, our data also revealed that hybrid females have dramatically reduced oviposition success on one host plant (fig. 3*B*) and that hybrid males have reduced mating success with *N. lecontei* females (fig. 4*F*), demonstrating the potential importance of extrinsic sources of postzygotic isolation (Linn et al. 2004; Matsubayashi and Katakura 2009; Clark et al. 2010; McBride and Singer 2010; Koevoets et al. 2012; Turelli et al. 2014; Bendall et al. 2017). Here, we discuss these patterns in more detail, considering limitations of our data and contrasting our patterns to those observed in other haplodiploids. We conclude with a provisional tally of taxa obeying Haldane's rule in haplodiploid taxa and compare these numbers with those observed in diploids.

Asymmetric Reduction of Hybrid Female Viability in Neodiprion *Sawflies*

Despite considerable genetic, morphological, and ecological divergence between *N. lecontei* and *Neodiprion pinetum* (Bendall et al. 2017, 2022), we recovered viable adult hybrids of both sexes in both cross directions (fig. 3). However, families with hybrid females ($F_{1(L\times P)}$) from one cross direction (*N. lecontei* females × *N. pinetum* males) had markedly lower female-to-male sex ratios, on average, than all other nonhybrid and hybrid families (fig. 3*A*; tables S2–S5). There are multiple non-mutually-exclusive explanations for reduced sex ratios in families containing $F_{1(L\times P)}$ hybrid females, including postmating sources of reproductive isolation (e.g., females mated to heterospecific males could reduce the proportion of eggs they fertilize), intrinsic postzygotic isolation due to incompatible allele combinations, and extrinsic postzygotic isolation due to hybrid-host interactions. Although we do not yet have any data on fertilization rates or larval survival rates on different hosts in hybrid and nonhybrid families, our egg hatch data clearly indicate that reduced viability in $F_{1(L\times P)}$ hybrid females manifests early in development (fig. 3*B*). By contrast, there was minimal evidence for reduced hybrid viability for $F_{1(P\times L)}$ females and $F_{2(L\times P)}$ and $F_{2(P\times L)}$ males (fig. 3*A*; tables S2–S5); this runs counter to the prediction that the latter two groups would suffer the greatest mortality.

One limitation of our viability data is that our sample sizes for the adult sex ratio analysis were smaller in one direction of the cross (*N. pinetum* female × *N. lecontei* male). Likewise, for embryo viability data, we were missing one cross direction and had a limited number of families for $F_{2(L\times P)}$ males. Thus, it is possible that increasing sampling would reveal reduced viability in additional hybrid types besides $F_{1(P\times L)}$ females. However, according to our current data, it seems unlikely that additional data would alter our finding that $F_{1(P\times L)}$ females have lower viability than other hybrid types. Moreover, our egg hatch data (fig. 3*B*) should be biased toward overestimating hybrid female viability relative to hybrid male viability for two reasons. First, egg clutches containing hybrid females were laid by *N. lecontei* mothers with egg-laying traits well suited to their preferred host (*Pinus banksiana*). In contrast, egg clutches containing hybrid males were laid by F_1 hybrid mothers that differ in morphology and behavior from both parental species (Bendall et al. 2017). Overall, maternal-host interactions would be expected to reduce egg hatch rates for F_2 hybrid male families but not F_1 hybrid female families. Second, egg clutches containing hybrid female embryos also contained some unknown fraction of nonhybrid male embryos that developed from unfertilized eggs; egg clutches containing hybrid male embryos were laid by virgin females and contained only hybrid males. Therefore, the presence of nonhybrid embryos could have increased observed hatch rates for hybrid female families but not hybrid male families.

Overall, our data suggest that *Neodiprion* hybrids do not obey Haldane's rule. One potential explanation for this pattern is that incompatible allele combinations that reduced viability in hybrid females in the F_1 generation were eliminated by selection before the production of hybrid males in the F_2 generation. Only recessive incompatibility alleles would have escaped selection in the F_1 generation, and these would have had an opportunity to recombine into viable allele combinations, reducing their overall impact on hybrid male viability (fig. 1). A non-mutually-exclusive explanation is that expression of incompatibilities tends to be female limited in these hybrids, possibly stemming from divergent selection on female oviposition traits (Bendall et al. 2017) or efficient purging of deleterious recessive alleles that are expressed in haploid males (Crozier 1976; Avery 1984; Werren 1993; Hedrick and Parker 1997). Evaluating these hypotheses will require characterizing the genetic architecture of hybrid viability in both female and male hybrids.

Although our hybrid viability patterns are not consistent with Haldane's rule, they do conform to "Darwin's corollary" to Haldane's rule: the observation that postmating isolation is often asymmetric in interspecific crosses (Darwin 1859; Tiffin et al. 2001; Turelli and Moyle 2007; Lowry et al. 2008; Matute and Cooper 2021). One explanation for asymmetries in hybrid inviabilty or sterility is that they are the product of incompatibilities involving autosomal loci and uniparentally inherited loci (e.g., those located on the mitochondria, chloroplasts, or sex chromosomes; Turelli and Moyle 2007). In *Nasonia* wasps, reduced viability and fertility in F_2 hybrid males from crosses between *N. giraulti* females and *N. vitripennis* males and between *N. longicornis* females and *N. vitripennis* males relative to the reciprocal crosses have been linked to mitochondrial-nuclear (mitonuclear) incompatibilities (Ellison et al. 2008; Niehuis et al. 2008; Gibson et al. 2010, 2013; Koevoets et al. 2012). Mitonuclear incompatibilities have also been implicated in hybrid breakdown in a lineage that independently evolved haplodiploidy, *Tetranychus evansi* spider mites (Knegt et al. 2017).

Although we have not yet mapped hybrid inviability loci in *Neodiprion*, a population genetic analysis of eastern North American *Neodiprion* species revealed pervasive mitochondrial introgression (Linnen and Farrell 2007). Several hypotheses have been proposed to explain this finding (Linnen and Farrell 2007; Patten et al. 2015; Glover et al. 2023). Our results raise an additional possibility: mitochondrial introgression could have been promoted by mitonuclear incompatibilities (Sloan et al. 2017; Burton 2022). Consistent with this hypothesis, demographic modeling suggests that gene flow between *N. lecontei* and *N. pinetum* is asymmetric, with higher rates of migration from *N. lecontei* to *N. pinetum* (Bendall et al. 2022), the same direction of the cross that had higher hybrid viability (i.e., *N. lecontei* alleles paired with *N. pinetum* cytoplasm). Taken together, these data highlight the potential power of combining lab-based estimates of reproductive isolation with field-based estimates of historical gene flow for deepening our understanding of speciation (Sobel and Chen 2014; Moyle 2022; Westram et al. 2022).

Evidence of Extrinsic Postzygotic Isolation, but Not Hybrid Sterility, in Both Females and Males

Hybrid females ($F_{1(L\times P)}$ or $F_{1(P\times L)}$) did not have reduced fecundity (fig. 4*D*). We also found no evidence of "host confusion"—a form of extrinsic postzygotic isolation (behavioral sterility) that results from hybrid females failing to oviposit on either parental host because of conflicting host preference or host avoidance behaviors (Linn et al. 2004; fig. 4*B*). However, consistent with previous work (Bendall et al. 2017), we did find evidence of ecological sterility via trait mismatch in $F_{1(L\times P)}$ hybrid females. When given a choice, these hybrid females tend to choose the thin-needled pine (*Pinus strobus*) but had very low hatching success on this host compared with both *N. pinetum* (white pine specialist) and $F_{1(L\times P)}$ females that chose the thicker-needled pine (fig. 3*B*). Previous work suggests that this source of extrinsic postzygotic isolation occurs in both cross directions (Bendall et al. 2017). Extrinsic postzygotic isolation related to divergent host adaptation in parental species has also been invoked to explain reduced F_1 hybrid female viability in crosses between *N. vitripennis* and *N. longicornis* (Koevoets et al. 2012).

For hybrid males, $F_{2(L\times P)}$ males did not have reduced sperm motility relative to nonhybrid males (fig. 4*H*). We also discovered that *Neodiprion* males have cooperatively swimming sperm bundles (fig. 4*G*). This observation is consistent with the prediction that the absence of sperm competition in haploid males, which produce genetically identical sperm, facilitates the evolution of sperm cooperation (Immler 2008; Normark 2009). Although we confirmed that hybrid males ($F_{2(L\times P)}$) had motile sperm bundles and can produce diploid daughters when mated with *N. lecontei* females, we did not quantify sperm production. We therefore cannot rule out reduced sperm production in hybrid males, such as in hybrid males produced by crosses between *N. vitripennis* and *N. giraulti* (Clark et al. 2010). We also did not quantify fertilization rates of hybrid and nonhybrid males. Thus, it remains possible that there is a quantitative reduction in hybrid male fertility that went undetected in our assays, as in hybrid males produced by crosses in *N. vitripennis* and *N. longicornis* (Koevoets et al. 2012). Also, because we were not able to obtain fertility data for one direction of the cross, we cannot assess potential cross asymmetries in hybrid male fertility (e.g., as in Clark et al. 2010; Koevoets et al. 2012). In short, although hybrid males ($F_{2(L\times P)}$) were generally fertile, more work is needed to fully characterize patterns of hybrid male sterility in hybrids between *N. lecontei* and *N. pinetum.*

As was the case in hybrid females, we found evidence of extrinsic postzygotic isolation in hybrid males. Compared with nonhybrid males, hybrid males were less likely to mate successfully, indicating some behavioral sterility in males (fig. 4*F*). Although we were unable to include *N. pinetum* males in our hybrid mating assay, recent work using the same source populations that were used for our hybrid mating assays indicates that *N. pinetum* pairs are just as likely to mate as *N. lecontei* pairs (Glover et al. 2023). On the basis of this previous work and the data in figure 4*F*, we conclude that hybrid males are less likely to mate than nonhybrid males from both species.

Behavioral sterility has also been reported in *Nasonia* crosses and appears to be due to disrupted courtship displays (Clark et al. 2010; Koevoets et al. 2012). In *Neodiprion*, courtship behaviors are much less elaborate than those described for *Nasonia*. In general, a *Neodiprion* male will repeatedly attempt to mount a female that may display varying levels of resistance, from no resistance at all to wing buzzing to attacking or killing the male (Glover et al. 2023). Because proper body alignment is essential for establishing a secure mating connection (fig. 4*E*), there is strong size-based assortative mating within and between both species. Additionally, *N. lecontei* males and females are larger, on average, than their *N. pinetum* counterparts (Glover et al. 2023). Therefore, if hybrid males tend to be smaller than *N. lecontei* males, this may explain why they had reduced mating success with *N. lecontei* females. If this is the case, hybrid males may fare better when paired with smaller *N. pinetum* females. Alternatively, reduced mating in hybrid males may result from disruptions to other mating cues (e.g., chemosensory signals). Additional mating experiments—including ones with the reciprocal hybrid and *N. pinetum* adults—will be needed to determine the cause of reduced mating in male hybrids.

Haldane's Rule for Hybrid Viability in Haplodiploids versus Diploids

In a 2009 compilation of postzygotic isolation studies in nine haplodiploid taxa (including three *Nasonia* species pairs), seven taxa had evidence of cytoplasmic incompatibility related to *Wolbachia* infections (Koevoets and Beukeboom 2009). Only five taxa were evaluated for additional forms of postzygotic isolation. Four of these taxa were not informative for testing Haldane's rule: two taxa because they did not show any evidence of postzygotic isolation and two taxa because F_1 females were inviable, making it impossible to evaluate F_2 male viability. Indeed, it is for this reason that inviable or sterile F_1 females create a built-in bias against detecting exceptions to Haldane's rule in haplodiploids (Koevoets and Beukeboom 2009). Only one species pair—*N. vitripennis* and *N. giraulti*—could be evaluated for Haldane's rule and was found to obey this rule for hybrid inviability.

Following Koevoets and Beukeboom's (2009) compilation, evidence for Haldane's rule for inviability was found in an additional *Nasonia* pair, *N. vitripennis* and *N. longicornis* (Koevoets et al. 2012). More recently, multigenerational crosses in haplodiploid spider mites—one among differentiated lineages of *T. evansi* (Knegt et al. 2017) and another among three host races of *Tetranychus urticae* (Villacis-Perez et al. 2021)—reported hybrid viability patterns that appear to be consistent with Haldane's rule, but this rule and possible alternative explanations for the data (e.g., extrinsic postzygotic isolation) were not tested explicitly. If indirect evidence of Haldane's rule (e.g., patterns of introgression or ancestry in natural populations) is included, at least two additional haplodiploid species pairs appear to obey Haldane's rule (*Formica* ants [Kulmuni et al. 2010; Kulmuni and Pamilo 2014]; *Tetramorium* ants [Cordonnier et al. 2020]). However, this indirect evidence should be interpreted with caution because there are multiple mechanisms that could explain observed asymmetries in introgression and ancestry (Schilthuizen et al. 2011). Caveats aside and including the *Neodiprion* data presented here, 85.7% (6/7) of haplodiploid taxa obeyed Haldane's rule for inviability, with *Neodiprion* sawflies as the single exception to this pattern. We note however that our literature search was not exhaustive, and there could be additional data buried in descriptive studies or hybrid zone analyses.

For diploid animals with male or female heterogamety, a 2011 compilation revealed that 85% (381/448) of tests obeyed Haldane's rule for hybrid inviability (table 2 in Schilthuizen et al. 2011, with four haplodiploid data points removed; this dataset also includes both indirect and direct tests). Thus, despite several mechanisms that could differentially impact adherence to Haldane's rule (table 1) and the counterexample we have uncovered here, diploid and haplodiploid taxa appear to be surprisingly similar in their tendency to obey Haldane's rule for inviability. Of course, seven data points are still far too small a sample size to draw strong conclusions about patterns of speciation in haplodiploids. While the dearth of haplodiploid speciation studies may be due in part to some added difficulties in the lab—including the nontrivial effort involved in having to rear two generations of hybrids in nonmodel taxa—investment in the development of additional haplodiploid systems has the potential to yield many new insights into our understanding of speciation (Nouhaud et al. 2020).

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Statement of Authorship

E.E.B., A.J.M., and C.R.L. conceptualized the study; E.E.B. and K.M.M. collected the data; E.E.B. and C.R.L. analyzed the data and wrote initial drafts of the manuscript. All authors contributed to methods development and reviewing and editing manuscript drafts.

Data and Code Availability

The data and code used to produce the results in this study have been deposited in the Dryad Digital Repository (https://doi.org/10.5061/dryad.qrfj6q5j3; Bendall et al. 2023).

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