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Gametic selection, developmental trajectories, and extrinsic heterogeneity in Haldane's rule

Joanna D. Bundus,[1] Ravin Alaei,[1] and Asher D. Cutter[1,2]

[1] *Department of Ecology & Evolutionary Biology, University of Toronto, Toronto, Ontario, Canada, M5S 3B2*

[2] *E-mail: asher.cutter@utoronto.ca*

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Deciphering the genetic and developmental causes of the disproportionate rarity, inviability, and sterility of hybrid males, Haldane's rule, is important for understanding the evolution of reproductive isolation between species. Moreover, extrinsic and prezygotic factors can contribute to the magnitude of intrinsic isolation experienced between species with partial reproductive compatibility. Here, we use the nematodes *Caenorhabditis briggsae* and *C. nigoni* to quantify the sensitivity of hybrid male viability to extrinsic temperature and developmental timing, and test for a role of mito-nuclear incompatibility as a genetic cause. We demonstrate that hybrid male inviability manifests almost entirely as embryonic, not larval, arrest and is maximal at the lowest rearing temperatures, indicating an intrinsic-by-extrinsic interaction to hybrid inviability. Crosses using mitochondrial substitution strains that have reciprocally introgressed mitochondrial and nuclear genomes show that mito-nuclear incompatibility is not a dominant contributor to postzygotic isolation and does not drive Haldane's rule in this system. Crosses also reveal that competitive superiority of X-bearing sperm provides a novel means by which postmating prezygotic factors exacerbate the rarity of hybrid males. These findings highlight the important roles of gametic, developmental, and extrinsic factors in modulating the manifestation of Haldane's rule.

KEY WORDS: **Caenorhabditis, Haldane's rule, postmating prezygotic isolation, sex chromosome drive, speciation.**

Intrinsic reproductive isolation owing to the inviability or sterility of hybrids, independent of extrinsic factors, is especially important to the speciation process by representing irreversible barriers to gene flow (Coyne and Orr 1998). A strikingly common outcome of intrinsic reproductive isolation is the pronounced rarity or sterility of the heterogametic sex among F_1 hybrid individuals, famously termed Haldane's rule (Haldane 1922). Moreover, the severity of this asymmetry in the sensitivity of the sexes to hybrid dysfunction frequently differs depending on the parent-of-origin sex of each parental species. That is, reciprocal interspecific crosses often differ in the strength of reproductive isolation, a phenomenon dubbed Darwin's corollary to Haldane's rule (Turelli and Moyle 2007). The widespread incidence of these two patterns suggests that speciation might proceed with fundamental similarities across different taxa, making it critical to understand the determinants of Haldane's rule and Darwin's corollary in diverse organisms.

Genetically, three main explanations are thought to underlie Haldane's rule, although currently only a handful of specific causal genes contributing to Haldane's rule have been identified (Presgraves 2010b). First and most generally, because it applies both to species with XY and ZW chromosomal sex determination, any recessive hybrid incompatibilities linked to sex chromosomes will be exposed in the heterogametic sex (the "dominance theory"; Turelli and Orr 1995). This process would be further exacerbated by more rapid evolution of sex-linked loci ("faster X" theory; Charlesworth et al. 1987). Second, in male heterogametic species, Haldane's rule could be caused by more rapid accumulation of incompatibilities involving genes associated with male functions, for example, because of stronger sexual selection acting on them (the "faster male" theory; Wu and Davis 1993; Wu et al. 1996). Third, Haldane's rule also can be caused by genomic conflicts mediated by cytonuclear interactions and sex chromosome drive (Frank 1991; Hurst and

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Pomiankowski 1991; Meiklejohn and Tao 2010; Presgraves 2010a).

Parent-of-origin asymmetries in intrinsic reproductive isolation (Darwin's corollary to Haldane's rule), also will result from incompatibilities involving uniparentally inherited factors, including the X chromosome, mitochondria and other plastids, epigenetic factors (Turelli and Moyle 2007), and *Wolbachia* infection (Shoemaker et al. 1999; Jaenike et al. 2006). Despite the unknown relative importance for each of these possible components, there are several reasons to suspect that mito-nuclear incompatibilities might be especially important. First, the mitochondrial genome accumulates substitutions per base pair faster than the nuclear genome (Lynch et al. 2008), and to maintain proper mitochondrial function, the nuclear genome can coevolve to compensate for deleterious mitochondrial mutations (Rand et al. 2004). Indeed, the relative rate of mitochondrial genome evolution predicts the direction of asymmetries in interspecific crosses of centrarchid fish (Bolnick et al. 2008), although not in toads (Brandvain et al. 2014). Second, mito-nuclear incompatibilities cause well-documented hybrid dysfunction in interspecific crosses for some systems (e.g., *Tigriopus* copepods Burton et al. 2006; *Saccharomyces* yeasts Lee et al. 2008; *Nasonia* wasps Ellison et al. 2008). A notable exception is *Drosophila*: while mito-nuclear incompatibilities involving segregating variation within and between species cause hybrid dysfunction, fixed differences do not (Montooth et al. 2010; Meiklejohn et al. 2013). The genetically intrinsic drivers of Haldane's rule and Darwin's corollary must manifest in a specific developmental context, and development can be sensitive to extrinsic conditions to modulate the magnitude of hybrid inviability or sterility.

Caenorhabditis nematodes provide a classic genetic model organism in biology, but only the recent discovery of close sister species pairs has empowered this system for studies of the genetics and evolution of reproductive isolation (Woodruff et al. 2010; Dey et al. 2012; Baird and Seibert 2013). In particular, crosses between *C. briggsae* and *C. nigoni* exhibit Haldane's rule, producing some viable and fertile XX hybrid females, and a small number of sterile hybrid XØ males (Woodruff et al. 2010; Kozlowska et al. 2012). Although many hybrid individuals suffer severe mortality between zygote and adult stages (Woodruff et al. 2010; Kozlowska et al. 2012), the developmental timing of hybrid inviability is not yet understood in detail. Moreover, these species show asymmetric isolation, with stronger hybrid F_1 inviability and infertility with maternal *C. briggsae* (Woodruff et al. 2010; Kozlowska et al. 2012). *Caenorhabditis briggsae* strains also often contain heteroplasmic mitochondrial genomes, reflecting a large deletion polymorphism that acts like a selfish genetic element with metabolic and fitness costs (Clark et al. 2012). Because the deletion is absent in *C. nigoni* (Clark et al. 2012), we surmised that mito-nuclear incompatibilities might provide a plausible source of hybrid reproductive incompatibilities between the species. Outbreeding reproduction within *C. briggsae* also exhibits the intriguing phenomenon of early-produced offspring being biased toward the hermaphrodite sex (XX), with more male offspring (XØ) produced later (LaMunyon and Ward 1997). This change in sex ratio bias from hermaphrodites to males is caused by a competitive advantage to X-bearing sperm over nullo-X sperm (non X-bearing sperm) in fertilization as a kind of sex chromosome drive, despite forming equal numbers of X-bearing and nullo-X sperm (LaMunyon and Ward 1997). We hypothesized that this mode of gametic selection might also operate in interspecies crosses to influence the manifestation of Haldane's rule in a novel way. These species thus provide a convenient and powerful entry for dissecting how distinct factors contribute to both Haldane's rule and Darwin's corollary to Haldane's rule, which we test in terms of genetic causes, developmental sensitivity, extrinsic modulation, and the potential role for noncanonical processes, such as gametic selection.

Methods

NEMATODE STRAINS AND REARING

To determine the effect of temperature and mito-nuclear incompatibilities on hybrid viability, as well as the proportion of hybrid inviability that occurs at each stage of development, we performed crosses between *C. briggsae* strain AF16 and *C. nigoni* strain JU1421 (formerly *C*. sp. 9; Félix et al. 2014; Table 1). Both of these strains are effectively isogenic, owing to the extreme selfing rate in nature for *C. briggsae* (and subsequent selfing of AF16 as the species reference strain) or to 25 rounds of sibling mating of an isofemale line to generate JU1421 (Kozlowska et al. 2012; M.A. Felix, pers. comm.). To quantify the effect of mito-nuclear interactions on reproductive isolation between *C. briggsae* and *C. nigoni*, we created two new mito-nuclear hybrid strains. Because mtDNA is uniparentally inherited via the maternal parent, by crossing *C. briggsae* AF16 hermaphrodites to *C. nigoni* JU1421 males, and then backcrossing the F_1 females to *C. nigoni* males for 20 generations, we created a strain that has a *C. nigoni* nuclear genome with *C. briggsae* mtDNA (strain VX0089). By performing the reciprocal cross design, we created a strain that has a *C. briggsae* nuclear genome and *C. nigoni* mtDNA (strain VX0100). The success of these mitochondrial introgressions was validated by polymerase chain reaction (PCR) and Sanger sequencing.

Caenorhabditis briggsae strain JU934 allowed us to discriminate males from females during larval development because of its expression of an X-linked green fluorescent protein (GFP) marker (*mfIs27*[*Ce-lip-1*::GFP,*Ce-myo-2*::GFP] in an otherwise AF16 genetic background), a gift from Marie-Anne Félix (http://www.justbio.com/worms). We subsequently constructed

Table 1. Strains of *Caenorhabditis briggsae* and *C. nigoni* used.

Species	Strain	Nuclear background	Mitochondrial background	X-linked GFP (±)	Experiment
C. briggsae	AF16	AF16	AF16	−	T, M, D
C. nigoni	JU1421	JU1421	JU1421	−	T, M, D
C. briggsae	VX0100	AF16	JU1421	−	M
C. nigoni	VX0089	JU1421	AF16	−	M
C. briggsae	JU934	AF16	AF16	+	D
C. nigoni	VX0226	JU1421	JU1421	+	D

All strains were created from isofemale lines.

T = temperature sensitivity of Haldane's rule; M = mitochondrial contribution to Haldane's rule; D = Developmental trajectory of Haldane's rule.

C. nigoni strain VX0226 to have this X-linked GFP marker in the homologous portion of its genome by crossing JU934 males to *C. nigoni* JU1421 females and backcrossing the hybrids for 20 generations while maintaining the GFP marker. After 20 generations, we performed sibling matings to yield a new strain homozygous for the GFP marker on a *C. nigoni* JU1421 genetic background (VX0226; Table 1).

We cultured and maintained all strains at 25°C on Petri dishes containing NGM-Lite agar seeded with *Escherichia coli* strain OP50. Before use in crosses, we cleaned strains using a standard bleaching protocol (Stiernagle 1999). We performed all crosses on 35-mm plates with a single 10-mm diameter bacterial spot. For *C. briggsae* strains, we initially induced XØ males by heat shock (6 h at 31°C), and maintained male/hermaphrodite populations through regular mating, from which we obtained males for experiments.

EGG-TO-ADULT SURVIVAL AND DEVELOPMENT

The sex of *Caenorhabditis* individuals cannot be distinguished reliably from the egg through third larval (L3) stages using morphological features. To discriminate males from females at these stages, we made use of JU934, a strain with an AF16 genetic background that has a GFP marker linked to the X sex chromosome. The promoter begins to drive GFP expression during late embryogenesis (Gerstein et al. 2010). Because XX females inherit an X chromosome from their father, whereas XØ males do not, only the female progeny will fluoresce among the offspring of males containing the X-linked GFP marker that are crossed to unmarked females. To determine the stage of development at which male and female hybrids were inviable, we crossed either a single nonfluorescent *C. nigoni* female (JU1421) to three fluorescent *C. briggsae* males (JU934) or one nonfluorescent *C. briggsae* female (AF16) to three fluorescent *C. nigoni* males (VX0226). In both cases, by virtue of inheriting the marked X chromosome from their father, only female hybrids fluoresced. After mating at 25°C for 24 h, we sacrificed parental males and transferred their mates to a new plate to allow them to continue laying eggs for a second day. We picked each F_1 egg onto a new plate to allow it to develop, then observed the developmental stage, whether it was fluorescent, and whether it was alive or dead. We continued to observe individuals until they either died or reached adulthood. Eggs that did not hatch after 72 h were considered inviable.

EFFECT OF TEMPERATURE ON REPRODUCTIVE ISOLATION AND HALDANE'S RULE

To determine whether rearing temperature can affect the incidence of hybrid males in the cross between *C. briggsae* males and *C. nigoni* females, we performed interspecific crosses at a range of temperatures (17°C, 18°C, 20°C, 23°C, 25°C, 27°C, and 29°C). We reared both parents at 25°C and picked virgin females at the L4 stage (penultimate and fourth larval stage), then allowed them to develop into adults overnight at 25°C. We mated individual *C. nigoni* adult females (JU1421) to five *C. briggsae* males (AF16) at one of the seven experimental temperatures. After 24 h of mating, we sacrificed the males, and transferred females to new plates each day until they stopped laying eggs. We allowed hybrid offspring to develop at the temperature in which their parents mated, and we counted all adult hybrids. For each temperature, we performed 30–41 replicate crosses. As a control, we performed intraspecific crosses for *C. nigoni* (JU1421) at 17°C, 20°C, 23°C, 25°C, 27°C, and 29°C.

EFFECT OF MITO-NUCLEAR INTERACTIONS ON REPRODUCTIVE ISOLATION AND HALDANE'S RULE

To isolate the effect that mito-nuclear incompatibilities might have on reproductive isolation between *C. briggsae* and *C. nigoni*, we created two new mito-nuclear hybrids strains (VX0089 and VX0100). We crossed these mito-nuclear hybrid strains to each other and to pure species (*C. briggsae* strain AF16 and *C. nigoni* JU1421) in all 16 reciprocal combinations, then counted the total adult male and female progeny produced. *Caenorhabditis briggsae* hermaphrodites are self-fertile, making a limited number of sperm before switching irreversibly to oocyte production. To exclude self-progeny from crosses involving hermaphrodites, we first performed self-sperm depletion by transferring isolated hermaphrodites to a new plate each day until they stopped

laying eggs (approximately five days), and had therefore used up all their self-sperm (Woodruff et al. 2010; Kozlowska et al. 2012). For crosses involving *C. nigoni* females, we picked females at the L4 larval stage. At this stage it is possible to distinguish females from males, but they will not have mated. We performed an average of 19 replicates for each of the 16 cross-combinations, each involving a single *C. nigoni* female or *C. briggsae* hermaphrodite mated to six males for 18 h. All experimental crosses associated with mito-nuclear hybrid strains were performed at 25°C.

To complement the phenotypic analysis of possible influence of mitochondrial background in hybrid crosses, we assessed the molecular evolutionary divergence of the 12 mitochondrially encoded genes. To estimate of divergence between the *C. briggsae* and *C. nigoni* mitochondrial genomes, we obtained sequences of the *C. briggsae, C. nigoni*, and *C. sinica* (formerly known as *C*. sp. 5; Huang et al. 2014) orthologs of each of the 12 protein coding genes located on their mitochondria from (Howe and Denver 2008; Raboin et al. 2010). We manually aligned each gene using BioEdit (Hall 1999), and computed maximum-likelihood estimates of lineage-specific rates of nonsynonymous (d*N*) and synonymous (d*S*) site substitution with the CODEML program in PAML 4.8 (Yang 1997).

Results

MOST HYBRID INVIABILITY OCCURS DURING EMBRYOGENESIS

To test whether hybrid male inviability accumulates over the course of development or is restricted to a particular developmental stage, we constructed and used strains containing an X-linked GFP marker. By providing a visible marker of sex chromosome linkage, these strains enabled us to determine the sex of hybrid animals from the first larval stage (L1) onward, as normally it is infeasible to do so reliably until the L4 stage using morphological features. Among 173 hybrid eggs derived from crosses with *C. briggsae* as the maternal species, we observed no males at any stage. However, of these embryos, 106 (61%) died before reaching L1 (Fig. S1). Reciprocal hybrid crosses involving *C. nigoni* as the maternal parent also led to substantial hybrid inviability acting during embryogenesis (Fig. 1). Among 2580 hybrid eggs, 934 (36%) died before reaching the L1 stage, a significant decline (*t*-test $P = 0.01$; Fig. 1), but only 316 (12%) died subsequently during the L1 through adult stages (293 during L1 [*t*-test $P = 0.3$], 23 at later stages). The overall egg-to-adult hybrid viability that we observed (38% for *C. briggsae* maternal vs. 52% for *C. nigoni* maternal) is comparable to hybrid viability observed in previous studies (Woodruff et al. 2010; Kozlowska et al. 2012). Moreover, the sex ratio of hybrids remained virtually constant from L2 to adult (32% male), with L1 animals having only a slightly more balanced sex ratio (39% male; Fig. 1). The limited hybrid mortality occurring after the L2 stage, which was consistent for both directions of the cross (<1% for *C. briggsae* maternal vs. 2% for *C. nigoni* maternal), indicates that the disruption of male development in hybrids is consistent with a largely discrete, early-acting developmental failure rather than gradual accumulation of problems over the full course of development. Specifically, to explain the temporal trajectory of male rarity among hybrids, it must be the case that either genetically male embryos arrest disproportionately prior to expressing the GFP marker in late embryogenesis or that primary sex ratios at fertilization are female-biased, or both.

FERTILIZATION ADVANTAGE OF X-BEARING SPERM CONTRIBUTES TO HALDANE'S RULE

Within *C. briggsae*, male sperm that contains X chromosomes has a competitive advantage in fertilization compared with nullo-X sperm that gives rise to a bias against males in early offspring production (0–24 h after mating vs. 24–72 h after mating; LaMunyon and Ward 1997). To determine whether nullo-X sperm of *C. briggsae* might also suffer a competitive disadvantage in hybrid crosses, we compared the sex ratio of progeny laid on the first day after mating to those laid on the second day after mating. We observed a lower proportion of males among hybrid progeny on the first day (29%) than the second day (39%) of egg laying (Pearson's exact test *P*-value = 0.0004, $\chi^2 = 12.45$, df = 1; Fig. 1). Provided that sex-specific embryonic mortality is equivalent for early and late hybrid embryos, these findings are consistent with an X-bearing sperm competitive advantage prior to fertilization in hybrid crosses. Moreover, we found that eggs laid on the first day are significantly more likely to survive to adulthood than eggs laid on the second day (54% survival vs. 45% survival; Pearson's exact test *P*-value = 0.014, $\chi^2 = 6.00$, df = 1). Given the generally elevated mortality of hybrid males compared to hybrid females seen in the X-linked GFP experiment (35% of L1 males died, 9% of L1 females died from *C. nigoni* maternal crosses), the greater inviability of second-day hybrid embryos is consistent with more of them being male. Thus, the elevated incidence both of males and inviability in late-produced hybrid eggs is consistent with gametic selection favoring early fertilization by sperm that contain X chromosomes.

To explore the contribution of differences in male and female inviability in combination with differences in primary sex ratio bias at fertilization across days of egg laying, we extended the model of Kozlowska et al. (2012). Specifically, we defined overall adult hybrid viabilities (v_1, v_2) and male frequencies (f_{m1}, f_{m2}) as a function of sex-specific viabilities (v_m, v_f with $v_m = pv_f$) and the abundance of zygotes of each sex (z_{m1}, z_{f1}, z_{m2}, z_{f2}) for zygotes laid on day $i = 1$ or day 2:

$$v_i = \frac{(v_{mi} z_{mi} + v_{fi} z_{fi})}{z_{mi} + z_{fi}} \quad and \quad f_{mi} = \frac{v_{mi} z_{mi}}{v_{mi} z_{mi} + v_{fi} z_{fi}}.$$

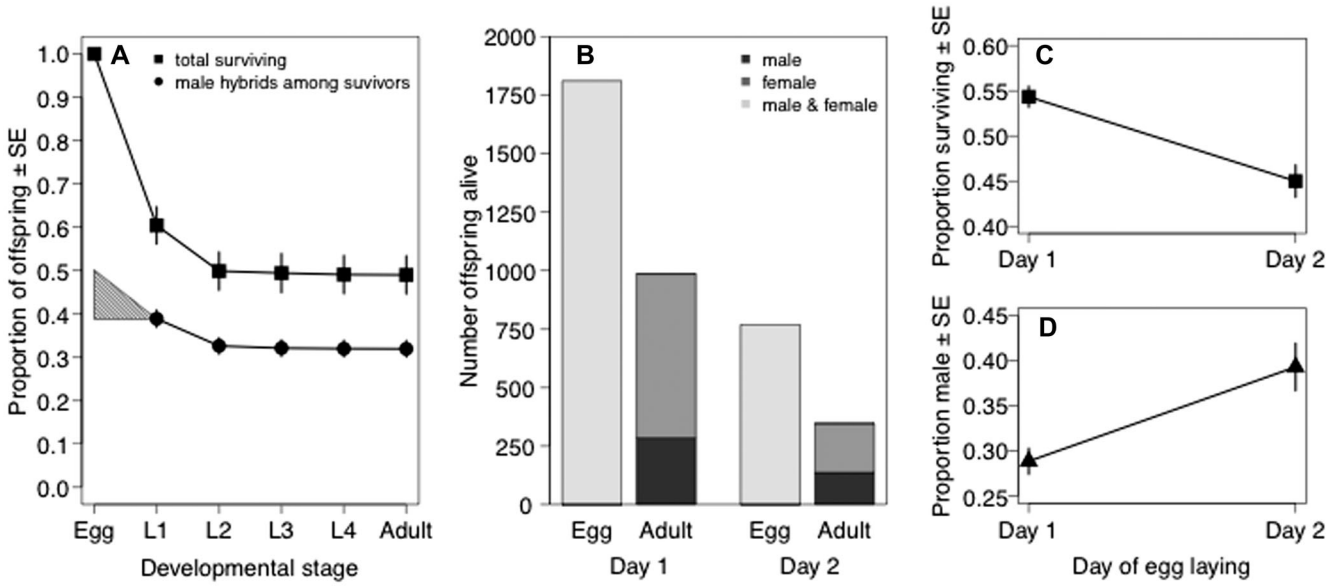

Figure 1. **Survival of hybrid offspring, and proportion of hybrid male offspring throughout development (A), and as a function of early versus late produced eggs (B). All crosses involve pure *Caenorhabditis briggsae* males and *C. nigoni* females to yield F_1 zygotes. The filled triangular area between the egg and L1 stage indicates the possible initial proportion of males, given the total number of eggs and the sex ratio at L1 (assuming a maximum 50% male primary sex ratio). The proportion of males declines significantly from L1 to L2 (*t*-test $P = 0.02$), but remains constant after the L2 stage (*t*-test $P = 0.8$). Survival to adulthood of eggs laid on the first day of egg laying is higher than day 2 egg to adult survival (Pearson's exact test *P*-value = 0.014, $\chi^2 = 6.00$, df = 1) (C). However, the proportion of males is higher among hybrids that survive to adulthood for those eggs laid on day 2 than day 1 (Pearson's exact test *P*-value = 0.0004, $\chi^2 = 12.45$, df = 1) (D). Error bars in (C) and (D) indicate ±1 SE. $n = 20$.**

We presume that sex-specific viabilities are independent of the day on which they were laid (e.g., $v_{f1} = v_{f2} = v_f$), then simplified and solved the system of equations for relevant quantities. Substituting in our empirical values of f_{m1}, f_{m2}, v_1, and v_2, we solved for the ratio of male to female zygotes for hybrid eggs laid on day 1 ($g_1 = z_{m1}/z_{f1}$) as a function of the zygotic sex ratio on day 2 ($g_2 = z_{m2}/z_{f2}$) to give: $g_1 = -0.29 + 0.71\ g_2$. The coefficient for g_2 indicates that the primary sex ratio of male:female fertilized hybrid eggs is about 30% lower on day 1 than day 2. From this analysis, we derived the range of male:female viability and zygotic sex ratios that are compatible with our empirical data (Fig. S2).

LOW TEMPERATURES DISRUPT HYBRID MALE PRODUCTION

To determine the effect of temperature, an extrinsic factor, on intrinsic hybrid incompatibilities, we performed crosses at temperatures ranging from 17°C to 29°C. We found that in both interspecific and intraspecific crosses, temperature influenced total offspring production (analysis of variance [ANOVA] interspecific $F_{1,249} = 21.8$, $P < 0.001$; intraspecific $F_{1,59} = 8.5$, $P = 0.005$; Fig. 2). Additionally, in both interspecific and intraspecific crosses, offspring production was maximal at 23°C, an intermediate temperature, and declined precipitously for temperatures below 20°C, indicating that reproduction is especially sensitive to cold perturbation. In contrast to this pattern for overall progeny production, only in interspecies crosses did temperature affect the proportion of male offspring (ANOVA interspecific $F_{1,185} = 51.5$, $P < 0.001$; intraspecific $F_{1,48} = 0.06$, $P = 0.812$; Fig. 2), indicating that hybrid male development is especially sensitive to thermal environment. Rather than exhibiting the unimodal response to temperature seen for total progeny production, male hybrids were disproportionately rare at cold temperatures, with moderate and high temperatures yielding similar incidences of hybrid males. We further considered day of egg laying in combination with rearing temperature to show that males were more common among progeny that developed from hybrid eggs laid on the second day than the first day, with no significant interaction between temperature and day (ANOVA day $F_{1,498} = 10.57$, $P = 0.001$; day–temperature interaction $F_{1,498} = 0.685$, $P = 0.408$; Fig. S3).

MITO-NUCLEAR INCOMPATIBILITIES DO NOT DRIVE REPRODUCTIVE ISOLATION

Prior to conducting experimental tests, we first sought lines of evidence that mito-nuclear incompatibilities might represent a plausible hypothesis for the strong parent-of-origin asymmetry in postzygotic isolation and Haldane's rule between *C. briggsae*

Table 2. Lineage-specific divergence between *Caenorhabditis briggsae* and *C. nigoni* mitochondrially encoded genes (*C. sinica* as outgroup).

Gene	Length	S_N	*C. briggsae* d*N*	*C. nigoni* d*N*	*C. briggsae* d*S*	*C. nigoni* d*S*
ATP6	597	1	0.002	0	0.968	<0.001
COX1	1575	0	<0.001	<0.001	0.501	0.256
COX2	693	0	<0.001	<0.001	0.978	0.041
COX3	765	0	0	0.002	0	2.641
CYTB	1110	3	0.004	<0.001	1.698	0.136
ND1	873	5	0.007	<0.001	0.616	1.238
ND2	846	8	0.010	0.004	1.863	<0.001
ND3	330	3	0.012	0	0.709	0
ND4	1227	6	0.005	0.002	1.352	<0.001
ND4L	231	0	<0.001	0	0.268	<0.001
ND5	1581	7	0.005	0.001	0.465	0.561
ND6	432	0	0.003	0.003	0.722	0.001
median	846	2	0.003	<0.001	0.716	0.021

S_N = number of observed nonsynonymous substitutions.

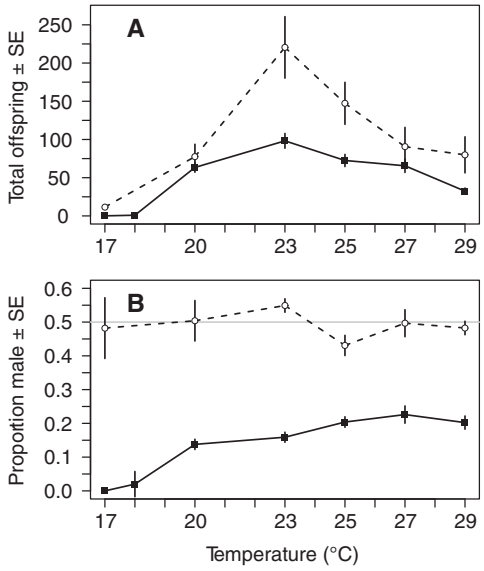

Figure 2. The effect of temperature on total offspring and the proportion male offspring within *Caenorhabditis nigoni* (white circles), and of crosses between *C. nigoni* females and *C. briggsae* males (black squares). Temperature has an effect on (A) total offspring produced (ANOVA interspecific, $F_{1,249} = 21.8$, $P < 0.0001$; intraspecific, $F_{1,59} = 8.5$, $P = 0.005$). Temperature only has an effect on (B) proportion of males in interspecific crosses (ANOVA interspecific, $F_{1,185} = 51.5$, $P < 0.001$; intraspecific $F_{1,48} = 0.06$, $P = 0.812$). Error bars indicate standard error of the mean. The horizontal gray line in (B) indicates the expected 1:1 sex ratio. Error bars indicate ± 1 SE. Crosses between *C. briggsae* hermaphrodites and *C. nigoni* males produce no males, and so were not included.

and *C. nigoni*. In addition to the motivating examples of cyto-nuclear incompatibilities in animals and plants (Burton et al. 2006; Ellison et al. 2008; Lee et al. 2008; Ellison and Burton 2010), as well as the distinctive evolution of heteroplasmic mitochondrial compositions within *C. briggsae* (Howe and Denver 2008; Raboin et al. 2010; Clark et al. 2012), we also quantified substantial sequence divergence between these two species. Upon comparing the rate of molecular evolution of 12 mitochondrially encoded genes between *C. briggsae* and *C. nigoni*, we observed a total of 33 nonsynonymous substitutions (Table 2). The majority of these substitutions have accumulated specifically on the *C. briggsae* lineage (*C. briggsae* median d$N = 0.004$, *C. nigoni* median d$N < 0.001$; median d*N*/d*S* *C. briggsae* $= 0.00305$ *C. nigoni* $= 0.0001$). The higher synonymous-site substitution rate (d*S*) along the *C. briggsae* lineage (Table 2) is consistent with it having a higher mutation rate than other *Caenorhabditis* (Howe et al. 2010). Moreover, the elevated d*N*/d*S* ratio for *C. briggsae* implicates relaxed selection in this highly selfing species with reduced effective population size (Cutter et al. 2006a, b; Thomas et al. 2015). These molecular evolutionary patterns are consistent with the possibility of mito-nuclear incompatibilities contributing to the observed asymmetric reproductive isolation (Turelli and Moyle 2007), as hybrid dysfunction also is greater among progeny derived from *C. briggsae* maternal parents (Woodruff et al. 2010; Kozlowska et al. 2012).

To determine whether mito-nuclear incompatibilities truly are involved in reproductive isolation between *C. briggsae* and *C. nigoni*, we performed crosses between pure species and mito-nuclear hybrids in all 16 reciprocal combinations. These experiments revealed, first, that interspecific crosses produce fewer offspring than intraspecific crosses, that hybrid progeny obey Haldane's rule, and that hybrid crosses involving the *C. briggsae* maternal nuclear background are more severely detrimental than

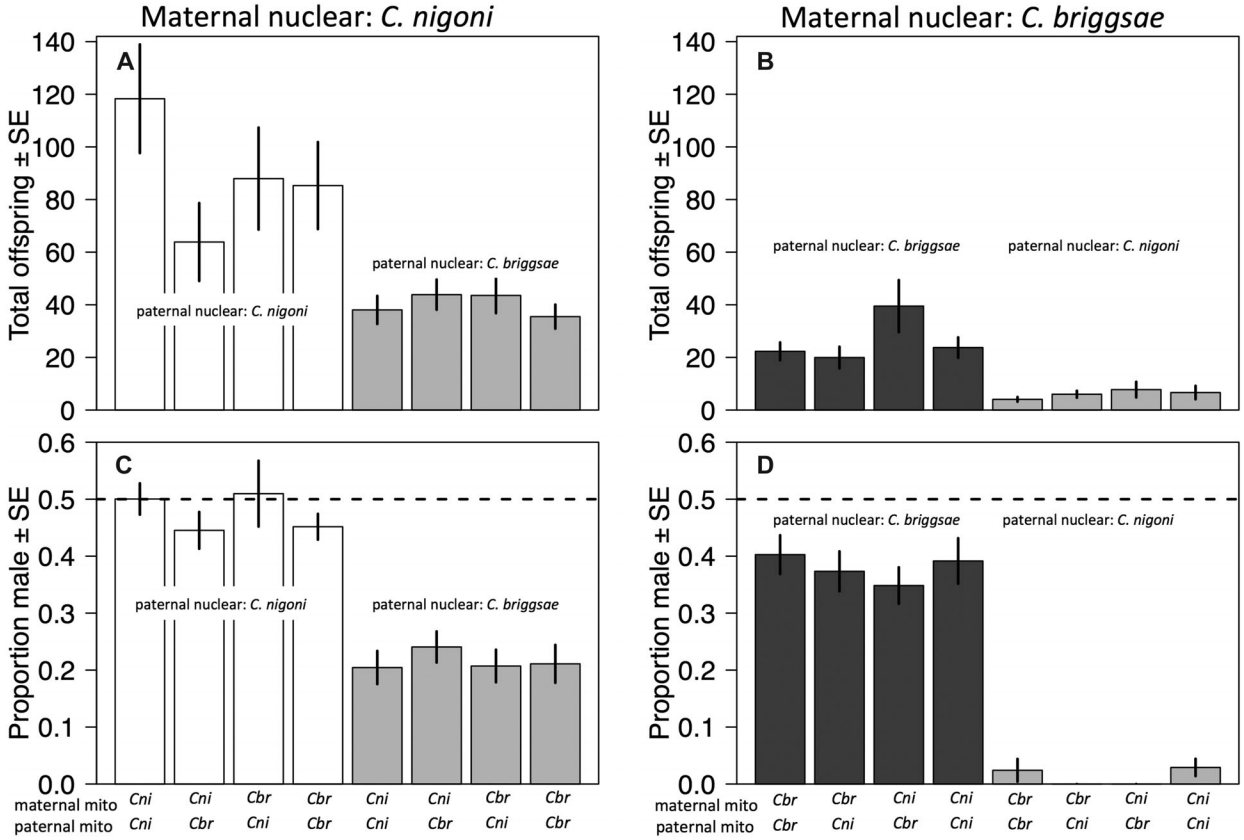

Figure 3. **Total offspring and proportion male offspring in all reciprocal cross combinations between pure and mitochondrial replacement strains. Panels (A) and (B) show total offspring production. Panels (C) and (D) show the proportion male progeny, highlighting the more severe effects on hybrids with a *Caenorhabditis briggsae* maternal parent (D cf. C). White bars indicate crosses in which both parents have a *C. nigoni* nuclear genome; dark gray bars indicate crosses in which both parents have a *C. briggsae* nuclear genome. Light gray bars indicate crosses in which parents have different nuclear genomes (i.e., nuclear hybrids). Nuclear genotype has an effect on total offspring (ANOVA, *C. briggsae* maternal: $F_{7,148} = 38.55$, $P < 0.001$; *C. nigoni* maternal: $F_{7,152} = 26.58$, $P < 0.001$), and proportion of males (ANOVA, *C. briggsae* maternal: $F_{7,148} = 340.91$, $P < 0.001$; *C. nigoni* maternal: $F_{7,152} = 116.32$, $P < 0.001$). Mitochondrial genotype does not affect total offspring production (ANOVA, *C. briggsae* maternal: $F_{7,148} = 3.446$, $P = 0.065$; *C. nigoni* maternal: $F_{7,152} = 0.097$, $P = 0.756$) or proportion of males (ANOVA, *C. briggsae* maternal: $F_{7,148} = 0.726$, $P = 0.396$; *C. nigoni* maternal: $F_{7,152} = 0.014$, $P = 0.908$). Zeroes in (D) indicate that no male offspring were observed. The dashed line in (C) and (D) indicates the expected 1:1 sex ratio. Error bars indicate ± SE. Mean $n = 19$.**

the reciprocal *C. nigoni* hybrid crosses, consistent with Darwin's corollary to Haldane's rule (Fig. 3). These observations affirm previous studies (Woodruff et al. 2010; Kozlowska et al. 2012). However, we detected no significant effect of mitochondrial background on total offspring production (ANOVA, *C. briggsae* maternal: $F_{7,148} = 3.446$, $P = 0.065$; *C. nigoni* maternal: $F_{7,152} = 0.097$, $P = 0.756$) or on the proportion of males produced (ANOVA, *C. briggsae* maternal: $F_{7,148} = 0.726$, $P = 0.396$; *C. nigoni* maternal: $F_{7,152} = 0.014$, $P = 0.908$; Fig. 3). Our results therefore indicate that, despite the molecular evolutionary patterns of mitochondrial evolution, mito-nuclear incompatibilities are unlikely to play an important role in reproductive isolation in this system.

In contrast, all crosses for which the nuclear genomes differed yielded strong reductions both in progeny production (ANOVA, *C. briggsae* maternal: $F_{7,148} = 38.55$, $P < 0.001$; *C. nigoni* maternal: $F_{7,152} = 26.58$, $P < 0.001$; Fig. 2) and in male incidence among hybrid progeny (ANOVA, *C. briggsae* maternal: $F_{7,148} = 340.91$, $P < 0.001$; *C. nigoni* maternal: $F_{7,152} = 116.32$, $P < 0.001$; Fig. 2). These findings in the F_1 generation in both directions of this cross indicate an especially potent effect of dominant nuclear–nuclear postzygotic incompatibilities on both total progeny production and the production of males. These nuclear–nuclear interactions are important for both overall postzygotic reproductive isolation and the manifestation of Haldane's rule, further suggesting a key role of X autosome interactions in generating Darwin's corollary. The X chromosome comprises approximately 20% of *Caenorhabditis* genomes (Ross et al. 2011), thus representing a substantial target to harbor incompatibility loci. Pairwise divergence of

orthologs between *C. briggsae* and *C. nigoni* (Thomas et al. 2015), however, show slightly lower rates of protein evolution for X-linked loci (median X-linked $dN/dS = 0.061$, autosomal $dN/dS = 0.074$, Wilcoxon test $P = 0.001$).

Discussion

Two patterns, Haldane's rule and asymmetric postmating isolation (Darwin's corollary), characterize the outcome of hybridization for a wide range of species, suggesting that speciation shares fundamental similarities across diverse organisms. In this study, we use the sister species *C. briggsae* and *C. nigoni* to determine how intrinsic and extrinsic factors can contribute to how these patterns manifest. We demonstrate that when hybrid inviability occurs, it occurs primarily during embryogenesis rather than late development (Fig. 1), and show that, in addition to greater mortality of hybrid male zygotes, a competitive superiority of X-bearing sperm contributes to the female-biased sex ratios seen among hybrid offspring (Fig. 1). Temperature, an extrinsic factor, also mediates the strength of Haldane's rule (Fig. 2). Finally, we demonstrate that mito-nuclear incompatibilities do not contribute to asymmetric postzygotic isolation between these species (Fig. 3). Together these observations demonstrate the important contributions of development, gametic, and extrinsic factors to intrinsic reproductive isolation.

HYBRID INVIABILITY OCCURS DURING EMBRYOGENESIS

One way to gain insight into postzygotic isolation is to determine when incompatibilities arise during hybrid development (e.g., Orr et al. 1997; Baird and Yen 2000; Moehring et al. 2007). For example, information about the genetic complexity of hybrid dysfunction can be divined from whether it occurs at a single stage or multiple stages during development and, for model organisms such as *Caenorhabditis* nematodes, might suggest corresponding candidate genes and genetic pathways involved in incompatibilities (Baird and Yen 2000). Genes acting later in development accumulate more substitutions between species than do genes acting early in development (Cutter and Ward 2005; Davis et al. 2005; Roux and Robinson-Rechavi 2008; Artieri et al. 2009), which is thought to be a by-product of stronger developmental and genetic network constraints for early-acting genes (Arthur 1988; Raff 1996) and/or the higher reproductive value of younger individuals (Medawar 1952; Charlesworth 1994; Promislow and Tatar 1998; Partridge 2001). However, it is not entirely clear how such molecular evolutionary signatures might be predictive about reproductive isolation. On the one hand, the rapid evolution of genes expressed late in development might correspondingly accumulate more substitutions that yield opportunity for incompatibilities to arise. The implications for hybrid incompatibility of "differentiation gene batteries"' and "hotspots" of evolutionary changes in genetic networks might follow a similar logic (Davidson and Erwin 2006; Stern and Orgogozo 2009), as with the seeming special sensitivity of male fertility to interspecies hybridization (Wu and Davis 1993).

On the other hand, any evolutionary changes that manage to accrue in the typically constrained early-acting genes in one lineage could affect a cascade of catastrophe in hybrid development. That is, seeming "coldspots" of evolutionary divergence might nonetheless contribute disproportionately to hybrid incompatibilities. Differences in pathway size or the robustness to genetic perturbation (Johnson and Porter 2000; Nijhout 2002) of early versus late acting gene networks might provide means of making different stages of development more or less prone to hybrid dysfunction. More canalized stages also will be capable of accumulating "cryptic" genetic variation as a basis of subsequent divergent selection or developmental systems drift (True and Haag 2001; Felix and Wagner 2008; Paaby and Rockman 2014). In any case, one would predict hybrid incompatibilities to accumulate over the course of development when many independent genetic incompatibilities contribute to reproductive isolation. This accretion could occur either because more late development genes have accumulated incompatibilities, or because early incompatibilities also exert later deleterious effects owing to pleiotropic downstream interactions.

We tested for hybrid incompatibilities across all stages of development that manifest as inviability in reciprocal crosses between *C. briggsae* and *C. nigoni.* Nearly all hybrid inviability occurs during embryogenesis, with little mortality during larval stages (Fig. 1), which is inconsistent with a prediction of continually accumulating incompatibilities over the course of development. Instead, our results suggest the possibility that catastrophic incompatibilities acting almost exclusively during embryogenesis might underlie the inviability of *C. briggsae* × *C. nigoni* hybrids. We rule out a major influence of temporal pleiotropy masking the existence of late-acting inviability, assuming independence of penetrance in different developmental stages, because F1 individuals that manage to survive past L1 also are almost certain to survive to adulthood, despite being genetically identical to F1 animals that die as embryos. Embryonic arrest is seen in many other *Caenorhabditis* interspecific crosses (Baird and Yen 2000; Baird and Seibert 2013; Dey et al. 2014), suggesting embryogenesis could be particularly sensitive to disruption in *Caenorhabditis* and, along with hybrid male sterility (Dey et al. 2014), might be among the earliest intrinsic postzygotic barriers to arise during speciation in these organisms. If cell division is a crucial phase for the manifestation of incompatibilities, then embryogenesis in *Caenorhabditis* might be especially susceptible to feeling their effects. More than half of all of development's somatic cell divisions occur in the *C. elegans* embryo throughout its

deterministic cell lineage, with mature embryos comprising 558 of the 959 adult hermaphrodite's somatic cells, only 51 of which go on to divide further (Sulston et al. 1983; Kipreos 2005).

Numerous genes contribute to inviability during embryogenesis in *C. briggsae* (Verster et al. 2014), making them plausible candidates for involvement in genic Dobzhansky–Muller incompatibilities (DMIs; Dobzhansky 1937; Muller 1942). Additionally, the differential regulation and loss of sex-biased genes in *C. briggsae* (Thomas et al. 2012) could contribute to dysfunction of hybrids, especially in males, as a result of evolution in their contrasting reproductive strategies (*C. nigoni* is dioecious, male and female sexes; *C. briggsae* is androdioecious, male and hermaphrodite sexes). Finally, it is possible that large-scale rearrangements between the genomes of *C. briggsae* and *C. nigoni* could contribute importantly to their intrinsic reproductive isolation, as their genomes likely differ in size by 30% or more (Thomas et al. 2012). Viewing the genomic shrinkage and male-gene loss in *C. briggsae* as a genomic manifestation of the "selfing syndrome" (Cutter 2008; Thomas et al. 2012), we might generally expect an exaggerated Haldane's rule in hybrid crosses between selfing and outbreeding species.

HALDANE'S RULE IS TEMPERATURE SENSITIVE

By exposing individuals to distinct environments, what are typically hidden phenotypic differences can be revealed as cryptic genetic variation (Felix and Wagner 2008; Paaby and Rockman 2014) that can be important in the speciation process (Cutter 2012). Understanding the influence that environmental changes exert on speciation genes is thus important both for understanding how speciation proceeds in nature and for comparing studies of speciation across laboratories.

Temperature stress, in particular, can exert strong effects on the strength of postzygotic inviability, sterility, and courtship behavior of hybrids (Bordenstein and Drapeau 2001; Koevoets et al. 2012). In some cases, one sex is more strongly affected by environmental changes (e.g., Hutter and Ashburner 1987; Wade et al. 1999). Temperature differences also appear to act as an important selective agent within *C. briggsae* (Prasad et al. 2011; Stegeman et al. 2013) and between *C. briggsae* and *C. nigoni* (Woodruff et al. 2010). We therefore examined the effect of temperature on total offspring production and sex ratio of both pure *C. nigoni* crosses and *C. nigoni* hybridized with *C. briggsae* males (virtually no F1 hybrid males are produced in the reciprocal hybrid cross, precluding evaluation of their temperature sensitivity). In both cases, we found that extreme temperatures caused a decrease in total offspring production (Fig. 2), consistent with a previous report on viability (Woodruff et al. 2010). We also found that while intraspecific sex ratio was unaffected by temperature, hybrid males were especially sensitive and almost completely absent at extreme cold temperatures (Fig. 2B). These results are consistent with the hypothesis that at least some of the genetic basis to hybrid male fitness is regulated in response to temperature. If this phenomenon is general across organisms, it suggests that some conditions might show Haldane's rule while others do not, particularly if male developmental inviability acts as a threshold trait. Genes involved in stress responses can evolve rapidly (Zou et al. 2009), and might therefore be particularly likely to be involved in hybrid incompatibilities. Many genes regulated in response to temperature stress have been identified in *C. elegans* (Rodriguez et al. 2013) and could be evaluated for the possibility of expression disruption in hybrid animals (Landry et al. 2005), although less is known about the molecular genetic underpinnings of cold sensitivity than heat sensitivity.

Our finding that Haldane's rule for male inviability appears strongest at low temperatures (Fig. 2B) stands in contrast to previous studies in insects, where Haldane's rule is exacerbated at high temperatures (Hutter and Ashburner 1987; Hutter 1997; Wade et al. 1999; Koevoets et al. 2012; Hoekstra et al. 2013). It is possible that the exceptional rarity of hybrid males at low temperatures (Fig. 2B) results from a generalized cold sensitivity of development, as *C. nigoni* in particular shows low reproductive performance when cold (Woodruff et al. 2010). Alternatively, cold temperatures could exacerbate gametic selection favoring X-bearing sperm (see below), because the slower pace and reduced amount of female egg laying at low temperatures might disproportionately exclude nullo-X sperm from fertilization events before ovulation ceases. However, we detected no significant interaction between temperature and day of egg laying that would have been indicative of differing gametic selection at cool versus warm rearing conditions.

POSTMATING PREZYGOTIC ISOLATION CONTRIBUTES TO HALDANE'S RULE

Postmating prezygotic (PM–PZ), or gametic, barriers act after mating, but before fertilization. PM–PZ barriers can contribute substantially to overall reproductive isolation (Simmons 2001; Howard et al. 2009; Palumbi 2009) through a variety of mechanisms, including sperm competition in *Drosophila* (Price 1997; Chang 2004; Castillo and Moyle 2014), cryptic female choice in scorpionflies (Thornhill 1983), and sperm-induced harm in *Caenorhabditis* (Ting et al. 2014). However, none of these mechanisms are known to contribute to Haldane's rule. Within *C. briggsae* conspecific matings, X-bearing sperm from males enjoy a competitive advantage over those male-derived sperm that lack an X chromosome, although the specific mechanism responsible for this mode of sex chromosome drive remains unresolved (LaMunyon and Ward 1997). This phenomenon leads to more hermaphrodite progeny among early-fertilized eggs and, as X-bearing sperm are used up, more male progeny from late-fertilized eggs, so the overall sex ratio remains close to 1:1 (LaMunyon and

Ward 1997). We find that this competitive superiority of *C. briggsae* X-bearing sperm also holds for interspecies matings to female *C. nigoni*, and the magnitude of the effect seems to be similar, although we did not evaluate a direct comparison. In particular, we find that the ratio of female to male hybrid offspring is higher among zygotes produced on the first day after mating than on the second day after mating (Fig. 1). Offspring production in the cross between *C. briggsae* males and *C. nigoni* females declines rapidly 24 h after mating, such that on the third day after mating extremely few offspring are produced at all (Woodruff et al. 2010). This means that by the time nullo-X sperm would normally gain the opportunity to fertilize eggs in the intraspecific cross, in interspecific crosses, ovulation would have ceased or sperm would have been lost from the female reproductive tract. As a result, the competitive X-bearing sperm remains in abundance throughout the fertile period following interspecies matings and contributes to the overall bias against male hybrid zygotes.

This phenomenon of an X-biased fertilization advantage is analogous to certation in plants, whereby competition in pollen tube growth occurs between genotypes within the style. Within the dioecious plant *Rumex*, the pollen tubes of female (X-bearing) microgametophytes are thought to have a growth advantage within the style over the pollen tubes of male (Y-bearing) microgametophytes (Stehlik and Barrett 2006; Pickup and Barrett 2013). It is therefore possible that sex chromosome drive mediated by gametic competition could contribute to Haldane's rule in interspecific plant crosses (Brothers and Delph 2010), as we observe in *Caenorhabditis*.

MITOCHONDRION-INDEPENDENT ASYMMETRIC POSTZYGOTIC ISOLATION

Asymmetric postzygotic isolation results from incompatibilities involving uniparentally inherited factors (Turelli and Moyle 2007). One such factor, the mitochondrial genome, has been implicated in asymmetric postzygotic isolation in several organisms, including the marine copepod *Trigriopus* (Burton et al. 2006), *Nasonia* wasps (Breeuwer and Werren 1995), and the eel *Anguilla* (Gagnaire et al. 2012). A possible role for mitochondria in asymmetric isolation in *Caenorhabditis* also was motivated by the unusual mitochondrial evolution involving a heteroplasmic deletion of NADH dehydrogenase subunit 5 (ND5). This deletion is not found in *C. nigoni*, but in *C. briggsae* confers a fitness reduction when present (Howe and Denver 2008; Raboin et al. 2010; Clark et al. 2012). Moreover, 33 nonsynonymous substitutions distinguish the mitochondrial genomes of *C. briggsae* and *C. nigoni*, a disproportionate number of which arose on the *C. briggsae* lineage. We therefore examined the role that the mitochondrial genome plays in the asymmetric isolation seen between *C. briggsae* and *C. nigoni.* First, we created mitochondrial substitution strains via 20 generations of backcrossing. All else being equal, such extensive backcrossing should eliminate all but approximately 1 millionth of the parental nuclear genome, unless extremely strong mito-nuclear interactions drag nuclear loci through the backcross procedure. We then crossed these strains to both pure species and to each other in every possible reciprocal combination. Our measures of postzygotic reproductive isolation from these crosses, however, eliminate mito-nuclear incompatibilities as a potential cause of asymmetric isolation in this system; reciprocal cross patterns also rule out the possibility of having unintentionally cointrogressed nuclear partners in mito-nuclear interactions. Thus, despite the more extensive molecular evolution of the mitochondrial genome along the *C. briggsae* lineage having correctly "predicted" that *C. briggsae* maternal parents will generate greater hybrid dysfunction, we find no evidence of mito-nuclear incompatibility in the reproductive isolation between these species. Instead, our findings implicate nuclear–nuclear incompatibilities involving the X chromosome or, potentially, epigenetically inherited factors as causes for the striking asymmetric reproductive isolation between *C. briggsae* and *C. nigoni*. Recent work using marked introgressions between these species (Bi et al. 2015), as well as for *Drosophila* (Masly and Presgraves 2007; Lachance and True 2010), indeed show a large contribution of X-linked factors in dysfunctional hybrid phenotypes.

CONCLUDING REMARKS

We have demonstrated that clearly defined sources govern variation in the manifestation of rare hybrid males, Haldane's rule, in *Caenorhabditis*. The most obvious disparity in how Haldane's rule expresses itself in this system is the parent-of-origin asymmetry (Darwin's corollary), such that nearly 0% of hybrids develop to be male from *C. briggsae* mothers compared to 20% or more from *C. nigoni* mothers. However, we showed that mito-nuclear incompatibilities cannot explain this asymmetry (Fig. 3), and so we conclude instead that dominant nuclear–nuclear incompatibilities involving the X chromosome most likely are responsible. Disproportionate hybrid male inviability occurs primarily during embryogenesis, with little difference between hybrid male and female mortality at later stages of development (Fig. 1). This developmental heterogeneity in the expression of Haldane's rule suggests that the robustness of genetic networks operating at different times during development might provide insight into the predictability of targets in the evolution of intrinsic reproductive incompatibilities. Extrinsic factors also influence the magnitude of Haldane's rule, with cold temperatures inducing stronger reproductive isolation between *C. briggsae* and *C. nigoni* (Fig. 2). This observation illustrates the oft-neglected interaction of abiotic, or biotic, extrinsic variables with genetically intrinsic barriers to reproduction between species. In some systems, this effect could conceivably erase negative fitness consequences of hybridization

under particular environmental conditions. Finally, we discovered that PM–PZ gametic selection can contribute to the realization of Haldane's rule (Fig. 1). The competitive advantage of X-bearing sperm generates a bias against males in the primary sex ratio of hybrids at fertilization with what appears to be a form of sex chromosome drive, exacerbating the rarity of males among hybrid offspring. This observation, and its conceptual connection to meiotic drive (Coyne et al. 1991; Frank 1991; Hurst and Pomiankowski 1991; Coyne and Orr 1993) and to certation in plants (Stehlik and Barrett 2006; Pickup and Barrett 2013), suggests that chromosome drive mechanisms operating at the gametic level might represent an underappreciated contributor to the rarity of males among interspecific hybrid offspring.

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DATA ARCHIVING

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Survival and sex ratio of *C. briggsae* hermaphrodites crossed with *C. nigoni* males hybrid offspring throughout development.

Figure S2. Experimentally observed frequencies of adult male hybrids and overall egg-to-adult viabilities from eggs laid on day 1 versus day 2 are consistent with a range of sex-specific viabilities (v_m/v_f) and primary sex ratios (z_m/z_f).

Figure S3. Total offspring production (A) and proportion of males (B) among hybrids produced by crossing *C. briggsae* males with *C. nigoni* females on days 1, 2, and 3 after mating at different temperatures.