

Hybrid Sterility, Haldane's Rule and Speciation in *Heliconius cydno* and *H. melpomene*

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ABSTRACT

Most genetic studies of Haldane's rule, in which hybrid sterility or inviability affects the heterogametic sex preferentially, have focused on *Drosophila*. It therefore remains unclear to what extent the conclusions of that work apply more generally, particularly in female-heterogametic taxa such as birds and Lepidoptera. Here we present a genetic analysis of Haldane's rule in *Heliconius* butterflies. Female F₁ hybrids between *Heliconius melpomene* and *H. cydno* are completely sterile, while males have normal to mildly reduced fertility. In backcrosses of male F₁ hybrids, female offspring range from completely sterile to fully fertile. Linkage analysis using the Z-linked *triose-phosphate isomerase* locus demonstrates a "large X" (Z) effect on sterility. Expression of female sterility varies among crosses in this and a previous study of *Heliconius*. Sterility may result from the production of normal but infertile eggs, production of small infertile eggs, or from a complete failure to develop ovarioles, which suggests multiple routes to the evolution of hybrid sterility in these *Heliconius* species. These results conform to the expectations of the "dominance" rather than "faster male" theories of Haldane's rule and suggest that relatively few loci are responsible. The two species are broadly sympatric and hybridize in the wild, so that female hybrid sterility forms one of several strong but incomplete barriers to gene flow in nature. The effect of female sterility is comparable to that of selection against non-mimetic hybrids, while mate choice forms a much stronger barrier to gene transfer.

HALDANE'S rule has proved an enduring generalization. It states that when one sex is absent, rare, or sterile in the F₁ offspring of a cross between two races or species, that sex is most commonly the heterogametic sex (HALDANE 1922). It holds for >95% of 324 crosses from six classes and several insect orders (LAURIE 1997) and seems to be a standard feature of the evolution of complete sterility and inviability (COYNE and ORR 1989a, 1997). However, a universal explanation has proved elusive.

Recently a degree of consensus has been reached that Haldane's rule is largely explained by the "dominance theory," with a contribution in *Drosophila* from "faster male evolution," and possibly "faster X evolution" (WU and DAVIS 1993; TURELLI and ORR 1995, 2000; LAURIE 1997; ORR 1997; TURELLI 1998). All of these theories rely on the existence of complementary genes (BATESON 1909; DOBZHANSKY 1936; MULLER 1940) that can produce hybrid inviability or sterility between two populations without either having passed through an adaptive valley. Divergent alleles become fixed at different loci between populations and cause incompatibility only

when brought together in novel hybrid genotypes. The dominance theory stems directly from this epistatic model. If members of complementary sets of genes are found on the sex chromosomes, and act as recessive alleles in their effect on hybrid sterility, the heterogametic sex will suffer incompatibilities before the homogametic sex. This will produce Haldane's rule and may be sufficient to explain it (TURELLI and ORR 1995). However, several observations from studies of Diptera have inspired other theories. Within *Drosophila*, cases of Haldane's rule for sterility vastly outnumber those for inviability (WU and DAVIS 1993). Attached-X hybrid females with homozygous X chromosomes fail to show sterility in crosses where male hybrids are sterile (COYNE 1985), but do show inviability in cases where normal male hybrids are inviable (ORR 1993). Sterility factors therefore seem to accumulate more rapidly in males and are sex specific, unlike inviability loci. Male sterility is also found in *Aedes* mosquito hybrids, which lack a hemizygous X so that the dominance theory cannot act (PRESGRAVES and ORR 1998). These observations may be explained by "faster male" evolution, that is, the faster evolution of male sterility alleles, due either to greater sensitivity of spermatogenesis to disruption or to a rapid divergence of male reproductive characters driven by sexual selection (WU and DAVIS 1993; WU *et al.* 1996). Of course, this theory cannot contribute to sterility in female-heterogametic taxa. The incidence

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of Haldane's rule may also be promoted by "faster X" evolution, where hemizyosity enhances selection of favorable recessive alleles on the X chromosome (HALDANE 1924; CHARLESWORTH *et al.* 1987). For these alleles to produce sex-limited effects in hybrids and contribute to Haldane's rule, faster X explanations must act in conjunction with either the dominance theory or a chance correlation across taxa between the sex that is heterogametic and the sex-specific expression of incompatibilities (ORR 1997). Two detailed introgression studies provide little support for a greater density of sterility factors on the X chromosome than on autosomes, ruling out a major contribution from faster X theory (HOLLOCHER and WU 1996; TRUE *et al.* 1996). They do, however, provide evidence for both dominance and faster male evolution, with many recessive autosomal sterility factors effective only when made homozygous and a greater density of male than female sterility factors. In sum, the dominance theory seems to provide a fundamental explanation of Haldane's rule across all taxa for sterility and inviability, while faster male evolution contributes to the disproportionate representation of hybrid male sterility in *Drosophila*.

Birds and Lepidoptera may play an important role in distinguishing the contributions of the three theories. Their females are heterogametic yet both groups still display Haldane's rule. Therefore, heterogamety rather than sex is critical. Faster male theories predict the reverse of Haldane's rule, and models suggest that the dominance theory can produce Haldane effects in females even when opposed by faster evolution of male hybrid sterility (TURELLI and ORR 2000). In Lepidoptera the Z (X) chromosome represents a relatively small proportion of the genome ($\sim 1/20$ – $1/30$), which could slow the origin of Haldane's rule effects (TURELLI and BEGUN 1997). However, for unexplained reasons other genetic differences between species show a bias toward Z-linkage (PROWELL 1998). Detailed genetic analyses on rates of accumulation of male relative to female sterility factors and on Z relative to autosomal effects have yet to be performed within the Lepidoptera (HOLLOCHER and WU 1996; TRUE *et al.* 1996) and better genetic studies of the taxon are required.

Here we study the genetic basis of sterility between *Heliconius cydno* and *H. melpomene* (Lepidoptera: Nymphalidae). These sister species are sympatric across much of Central and Andean South America below 1500 m (BROWN 1979; BROWER 1996). Both are unpalatable and warningly colored, and their speciation has been accompanied by a shift in Müllerian mimicry. *H. melpomene* is black with red and yellow markings and mimics *H. erato*, while *H. cydno* is black with yellow or white markings and usually mimics *H. sapho* or *H. eleuchia* (LINARES 1997; JIGGINS *et al.* 2001b). This shift in mimetic allegiance appears to have driven speciation, resulting both in selection against hybrids due to their intermediate non-mimetic color pattern and in assorta-

tive mating through pleiotropic effects on mate recognition (JIGGINS *et al.* 2001b). Color pattern divergence probably followed a change in habitat use, bringing the two into contact with different suites of potential comimics. *H. melpomene* is found in second growth and *H. cydno* in forest understorey, but there is considerable overlap and the two species commonly fly together (BENSON 1978; SMILEY 1978b; WAAGE *et al.* 1981; MALLETT and GILBERT 1995). They also differ in their degree of specialization among *Passiflora* host plants (SMILEY 1978a). Despite these differences the species hybridize in the wild, albeit rarely: F₁ and backcross hybrids are known from across their range, although they probably form <0.1% of natural populations (MALLETT *et al.* 1998b).

A previous study has shown female hybrid sterility between *H. melpomene* races from French Guiana and those from Panama and Colombia (JIGGINS *et al.* 2001a). Here we investigate hybrid incompatibility between the genetically more divergent sister taxa, *H. cydno* and *H. melpomene*, and review what is known of the genetics of hybrid sterility in all these crosses.

MATERIALS AND METHODS

Collection localities are given in Table 1. Crosses involving *H. melpomene rosina* and *H. cydno chioneus* from Panama and *H. melpomene melpomene* from French Guiana were performed in Gamboa, Republic of Panama, between August 1998 and March 2000. Crosses involving Colombian butterflies were made in La Vega, 50 km northwest of Santafé de Bogotá, Colombia. *H. melpomene vulcanus* and *H. cydno* were collected in the west of the Cauca valley, in the Dagua Pass. In this region three races of *H. cydno* form a hybrid zone among *H. cydno zelinde*, *H. cydno cydnides*, and *H. cydno weymeri* (LINARES 1997), but with no detectable reduction in hybrid fertility or viability. Stocks of *H. melpomene melpomene* and *H. cydno cordula* were collected from the foothills of the eastern slopes of the Andes.

After mating, females were kept individually in 1 × 1 × 2-m outdoor insectaries and supplied daily with pollen sources (Lantana and Psiguria) and artificial nectar (10% sugar solution). *Passiflora* vines were provided for oviposition, mainly *Passiflora menispermifolia* and *P. edulis*. Eggs were collected daily and kept individually in small plastic pots with moist cotton wool to maintain humidity. To avoid cannibalism caterpillars were reared individually until the third instar and then in groups of two to eight on new growth of *P. biflora* in Panama and *P. edulis* in Colombia. After pupation they were transferred to baskets until eclosion. The number of eggs laid, hatch rates, and eclosing butterflies were recorded. Females were dissected to confirm that they had received a spermatophore at mating and to assess the level of ovary development. F₁ males and females and female offspring of backcrosses were tested for fertility.

Crosses were attempted in all possible combinations, but very strong asymmetrical mate choice prevented one direction of interspecific cross (*H. melpomene* female × *H. cydno* male) from Panama populations, and this direction of cross was obtained only once each using Colombia and French Guiana females (Table 2). Crosses between the two species in Colombia were largely restricted to stocks from within a geographic

TABLE 1
Collection localities for the *Heliconius* races used in these crosses

Race	Locality and altitude	Latitude and longitude
	Panama	
<i>H. cydno chioneus</i> (CP)	Pipeline road, Soberanía National Park, 60 m	9° 08' N, 79° 42' W
<i>H. melpomene rosina</i> (MP)	Pipeline road, Soberanía National Park, 60 m	9° 08' N, 79° 42' W
	French Guiana	
<i>H. melpomene melpomene</i> (MG)	Pointe Macouria, near Cayenne Sea level	4° 54.8' N, 52° 21.6' W
<i>H. melpomene melpomene</i> (MG)	Sablance, near Cayenne Sea level	4° 57.8' N, 52° 25.2' W
	Colombia, Eastern Andean foothills	
<i>H. cydno cordula</i> (CE)	Barro Negro, Casanare 1050 m	6° 01' 06"N, 72° 05' 47"W
<i>H. melpomene melpomene</i> (ME)	Chirajara, Cundinamarca 1150-1450 m	4° 12' 48"N, 73° 47' 70"W
<i>H. melpomene melpomene</i> (ME)	Dele B (Río Charté), Casanare 1150 m	5° 25' 05"N, 72° 31' 20"W
<i>H. melpomene melpomene</i> (ME)	Pajarito, Casanare 940 m	5° 17' 30"N, 72° 42' 30"W
	Colombia, Cauca valley	
<i>H. cydno</i> (CV)	Atuncela, Valle del Cauca 1400 m	3° 44' 03"N, 76° 41' 53"W
<i>H. cydno</i> (CV)	Río Bravo, Valle del Cauca 1000 m	3° 54' 13"N, 76° 38' 18"W
<i>H. melpomene vulcanus</i> (MV)	Queremal, Valle del Cauca 1200 m	3° 31' 28"N, 76° 45' 25"W
<i>H. melpomene vulcanus</i> (MV)	Río Bravo, Valle del Cauca 1000 m	3° 54' 13"N, 76° 38' 18"W

Each race has been given a two-letter code used in Tables 2–4. Individuals of hybrid genotype are coded as maternal × paternal genotype. CP, *H. cydno* from Panama; MP, *H. melpomene* from Panama; MG, *H. melpomene* from French Guiana; CE, *H. cydno* from the Eastern Andean foothills, Colombia; ME, *H. melpomene* from the Eastern Andean foothills, Colombia; CV, *H. cydno* from the Cauca Valley, Colombia; MV, *H. melpomene* from the Cauca Valley, Colombia. Mean, variance, and standard error estimates are from a β -binomial model (for details see MATERIALS AND METHODS). The data include *H. melpomene* broods from JIGGINS *et al.* (2001a).

region. Results from interracial crosses within *H. melpomene* are described separately (JIGGINS *et al.* 2001a).

Statistical analysis: Counts of egg hatch rates were analyzed using a likelihood test based on a β -binomial model (BETA-BINO program by Ziheng Yang; see JIGGINS *et al.* 2001a). This method takes account of genetic or environmental variation in hatch rate between replicate broods within each type of cross, when the real interest is in variation between cross types. Rather than assuming a simple binomial distribution of hatch rates, the binomial parameter is allowed to vary across replicate broods within each cross type according to a β distribution, which can accommodate skewed or even bimodal distributions. Crosses can then be compared using estimates of the β -distribution parameters of mean, variance, and their standard errors. The program provides five alternative models for a data set containing replicate broods of several classes: (1) a classical binomial parameter for each class, which assumes a zero brood-to-brood variance; (2) a single β mean and variance for the entire data set; (3) different means for each class but a single variance; (4) a single mean but different variances; and (5) a different mean and variance for each class. Further comparisons can be made by fitting models after combining parts of the data set. For example, to compare the fertility of different male genotypes while controlling for female genotype, models are fitted before and after combining brood classes of the same maternal genotype but different paternal genotype. These models are then compared using likelihood (L) ratio tests, for which the test statistic $G = 2\Delta \log L$ asymptotically follows a χ^2 distribution (EDWARDS 1972). Colombian broods were analyzed separately as hatch rates of control broods were lower than those in Panama, presumably for environmental reasons. For sex ratio, where a simple binomial was the best-fitting model, support limits were obtained at the parameter values that led to a decrease in \log_e likelihood

of two units, approximately equivalent to 95% confidence intervals (EDWARDS 1972).

Linkage analysis: Intron 4 of the sex-linked *triose-phosphate isomerase* (*Tpi*) gene was amplified using primers situated in the surrounding exons. Evidence for sex linkage, primer sequences, and PCR conditions is described by JIGGINS *et al.* (2001a). This intron contains a 39-bp insertion that is common (but not fixed) in the Panama *H. cydno* population, hereafter the “*cydno* insertion,” but absent in *H. melpomene*. This length variation was used to follow segregation of *Tpi* in Panama backcross broods: alleles were separated on 6% acrylamide gels run for 4 hr at 125 V and stained using ethidium bromide. All broods proved informative with regard to the segregation of alleles in female offspring, having F_1 fathers heterozygous for the insertion.

RESULTS

Crosses between the sister species in Panama: Female hybrids between *H. cydno* and *H. melpomene* were completely sterile (Table 2). In the sympatric Panamanian cross 25 F_1 females were tested, producing 209 apparently normal eggs, not one of which hatched. Dissections confirmed that these females had received a spermatophore at mating and had developed normal ovaries (Table 3). Fifteen of the females failed to lay eggs despite surviving >15 days, by which time fertile control broods had invariably begun laying. Male hybrids were fully fertile, and there was no significant difference in hatch rate between their offspring and those of control broods ($G = 2.90$, d.f. = 4, $P > 0.05$).

TABLE 2
Hatch rate of control, F₁, and backcross broods

Cross type	Maternal genotype	Paternal genotype	No. of broods	No. of eggs	Mean hatch rate	SE	Variance	SE
Panama and French Guiana crosses								
Pure	CP	CP	16	820	0.859	0.039	0.021	0.011
	MP	MP	22	943	0.945	0.013	0.002	0.002
	MG	MG	18	881	0.901	0.027	0.011	0.007
F ₁ intraspecific	MP	MG	7	316	0.915	0.022	0.001	0.002
	MG	MP	7	200	0.946	0.022	0.001	0.002
F ₁ interspecific	CP	MP	5	516	0.955	0.007	0.000	0.000
	CP	MG	5	250	0.932	0.019	0.000	0.002
	MG	CP	1	15	1.000	—	—	—
Fertile backcrosses	CP	CP × MP	5	594	0.887	0.046	0.010	0.009
	MP	CP × MP	5	451	0.977	0.011	0.000	0.000
	CP	CP × MG	2	143	0.615	0.041	0.000	0.000
	MG	CP × MG	4	178	0.611	0.124	0.059	0.033
Sterile backcrosses	CP × MP	CP/MP/MG/F ₁	25	209	0	—	—	—
	CP × MG	CP/MG	10	0	0	—	—	—
	MG × CP	MG	2	23	0	—	—	—
Colombia crosses								
Pure	CE	CE	8	386	0.588	0.099	0.078	0.028
	ME	ME	11	416	0.520	0.065	0.038	0.017
	CV	CV	14	791	0.665	0.073	0.073	0.023
F ₁ interspecific	CE	ME	3	81	0.574	0.105	0.025	0.024
	CV	MV	2	68	0.925	0.085	0.013	0.028
	ME	CV	1	82	0.600	—	—	—
Fertile backcrosses	CE	CE × ME	3	241	0.629	0.101	0.025	0.021
	CV	CV × MV	5	83	0.894	0.041	0.003	0.008
Sterile backcrosses	ME × CV	CV/F ₁	7	257	0.172	0.036	0.003	0.004
	CE × ME	CE/ME/MV/F ₁	15	51	0	—	—	—
	CV × MV	CV/MV	8	0	0	—	—	—

Sex ratio across all broods was best described by a simple binomial model with an average proportion of females of 0.516 (support limits 0.491, 0.542), across 1553 adults from 48 broods. There was no evidence for differences in the sex ratio of adults emerging from nine Panama and French Guiana control, F₁, and Panama backcross brood classes ($G = 9.15$, d.f. = 8, $P > 0.05$), suggesting an absence of Haldane's rule on female inviability.

TABLE 3

Sterility phenotypes of female F₁ hybrids from interspecific and interracial crosses

Cross	Phenotype of ovary	Egg phenotype	Fertility
CP × MG	Undeveloped	—	Sterile
MG × CP	Normal	Normal	Sterile
CP × MP	Normal	Normal	Sterile
MP × CP	Unknown	Unknown	Sterile ^a
MP × MG	Normal	Small	Sterile ^b
MG × MP	Normal	Normal	Fertile ^b

^a Inferred (see DISCUSSION).

^b JIGGINS *et al.* (2001a).

When fertile F₁ males were backcrossed to females of the two parental species, female offspring were recovered showing the full range of fertility but with pronounced bimodality between complete sterility and apparently normal fertility (Table 4, A and B, and Figure 1). In the backcross to *H. cydno*, complete sterility was usually manifested as a failure to lay eggs, while from the backcross to *H. melpomene*, sterile females typically laid eggs that did not hatch.

There was strong linkage between sterility and Z-linked *Tpi* in female offspring from the backcross to *H. melpomene* (Table 4A). All six females that were completely sterile had the *cydno Tpi* insertion, while all five females that showed at least some fertility lacked the insertion and therefore had that region of the *H. melpomene* Z chromosome ($G = 15.16$, d.f. = 1, $P < 0.001$). In contrast, in the backcross to *H. cydno* there was no association between *Tpi* genotype and sterility (Table 4B, $G = 1.98$, d.f. = 1, $P > 0.05$).

Crosses between *H. melpomene* from French Guiana and *H. cydno* from Panama: Interspecific hybrid females of this cross were also sterile (Table 2). None of the 10 female offspring of *H. cydno* female × *H. melpomene* male laid any eggs. Dissections showed that the failure to lay eggs in these females was due to a complete failure to

TABLE 4

Hatch rate of eggs laid by the female offspring of backcross broods

Female parent no.	Brood no. of female parent	No. of days in cage	No. of eggs laid	Hatch rate (%)	<i>Tpi</i> genotype
A. Offspring of backcross to Panama <i>H. melpomene</i> MP × (CP × MP)					
354	341	6	27	0	C
355	341	(10)	(3)	(100)	—
358	341	6	12	0	C
365	345	14	13	0	—
366	345	14	32	97	M
367	341	12	31	0	C
368	345	18	26	81	M
372	345	16	33	0	C
374	341	7	52	83	M
381	345	10	31	0	C
385	345	(11)	(0)	(—)	(M)
386	345	6	31	19	M
396	341	12	32	13	M
397	345	13	18	0	C
B. Offspring of backcross to Panama <i>H. cydno</i> CP × (CP × MP)					
308	304	57	0	—	M
310	304	53	0	—	M
321	304	41	0	—	C
323	304	18	3	0	C
326	304	23	58	64	M
357	342	16	15	40	C
359	342	26	0	—	—
360	342	(9)	(1)	(0)	—
363	347	20	0	—	M
383	342	34	0	—	M
388	347	(14)	(0)	(—)	(M)
389	347	6	31	81	M
390	347	5	31	84	C
391	347	(14)	(0)	(—)	(M)
393	342	20	0	—	M
394	347	21	0	—	M
395	347	21	5	0	M
399	347	(5)	(0)	(—)	—
402	347	9	28	11	C
C. Offspring of backcross to Colombia eastern <i>H. cydno</i> CE × (CE × ME)					
65	C-630	(12)	(0)	(—)	—
63	C-630	22	0	—	—
10	C-630	15	13	0	—
1	C-630	12	6	0	—
30	C-630	22	7	0	—
66	C-630	5	13	15	—
16	C-630	51	108	10	—
21	C-637	16	0	—	—
3	C-637	20	0	—	—
2	C-637	25	26	0	—
1	C-637	20	6	66	—
12	C-637	65	15	26	—
20	C-637	34	7	42	—
30	C-637	56	31	92	—
10	C-634	88	41	17	—
15	C-634	19	12	0	—

(continued)

TABLE 4
(Continued)

Female parent no.	Brood no. of female parent	No. of days in cage	No. of eggs laid	Hatch rate (%)	<i>Tpi</i> genotype
D. Offspring of backcross to Colombia Cauca valley <i>H. cydno</i> CV × (CV × MV)					
1	C-656	31	0	—	
13	C-656	17	0	—	
3	C-656	(5)	(1)	(0)	
11	C-656	61	23	100	
25	C-629	21	0	—	
1	C-626	13	7	100	
3	C-624	(12)	(0)	(—)	
18	C-624	(12)	(0)	(—)	
8	C-624	17	0	—	
1	C-619	18	0	—	
2	C-619	16	0	—	
2	C-618	19	0	—	
4	C-618	48	35	60	

A dash in the *Tpi* genotype column indicates cases in which the individual was not preserved for genetic analysis, while data in parentheses were excluded from the analysis of linkage and sterile:fertile ratios as the female died before sterility could be assigned confidently. Colombian material was not genotyped for *Tpi*.

develop ovaries (Table 3). Only two females could be produced from the reciprocal cross and, although both were sterile, one actually laid eggs. There was also a reduction in F₁ male fertility (Table 2). Broods fathered by F₁ males had hatch rates of 0.615 ± 0.041 with a *H. cydno* female and 0.611 ± 0.124 with *H. melpomene*, significantly lower than the rates of 0.859 ± 0.039 and 0.901 ± 0.027 for control broods of *H. cydno* and Guiana *H. melpomene*, respectively ($G = 16.94$, d.f. = 4, $P < 0.01$). This is hybrid male sterility, rather than incompatibility when crossing parents of different genotypes, since there was no evidence of a reduction in hatch rate among eggs fertilized by a heterospecific male when F₁ and control broods were compared ($G = 3.58$, d.f. = 1, $P > 0.05$).

Crosses between Colombian sister species: In both the Eastern Andean foothills and Cauca valley crosses, female F₁ hybrids of *H. cydno* female × *H. melpomene* male were completely sterile, either failing to lay eggs or laying eggs that never hatched (Table 2). Hybrid males were fully fertile: hatch rates of their offspring were higher than those of control broods, although not significantly so ($G = 6.235$, d.f. = 4, $P > 0.05$). Some partially fertile F₁ females were produced in the reciprocal cross of an Eastern Andes *H. melpomene* female × *H. cydno* male from the Cauca valley (Table 2). Females were tested from a single brood, with three laying few eggs that never hatched, and the remaining four producing eggs with hatch rates of ~20%.

Fertile F₁ males backcrossed to *H. cydno* produce female offspring with the full range of fertility (Table 4, C and D, Figure 1B). The ratio of completely sterile to fertile females in backcrosses does not differ significantly between the eastern foothills, Cauca valley, or

Panama backcrosses to *H. cydno* ($G = 0.880$, d.f. = 2, $P > 0.05$).

DISCUSSION

Female F₁ hybrids between *H. cydno* and *H. melpomene* are completely sterile in five of the crosses described here, and their fertility is dramatically reduced in the sixth. In contrast, males have normal fertility in all but one of the crosses, with fertility reduced only in hybrids between *H. cydno* from Panama and *H. melpomene* from French Guiana. The lack of variation in sex ratio across control and F₁ broods suggests that there are no sex differences in viability. Counting as three examples the Heliconius studies (the interracial *melpomene* cross and reciprocal *cydno* × *melpomene* crosses) brings the total to 17 cases of sex-limited sterility in Lepidoptera, all but one of which conform to Haldane's rule, compared to 71 lepidopteran examples of unisexual hybrid inviability, of which only three do not conform (DAVIES *et al.* 1997; LAURIE 1997; JIGGINS *et al.* 2001a). This is the reverse of the pattern seen in *Drosophila*, where sterility predominates (WU and DAVIS 1993; LAURIE 1997), although it may be that in Lepidoptera too few species pairs have been studied carefully enough to detect hybrid sterility since inviability is more easily demonstrated. The dominance theory can explain why both taxa conform to Haldane's rule for sterility and inviability regardless of which sex is heterogametic (TURELLI and ORR 1995), while faster male evolution may explain the strong excess of sterility in *Drosophila* (WU and DAVIS 1993; TURELLI 1998).

The association between sterility and *Tpi* genotype shown here provides evidence of a large Z (X) effect

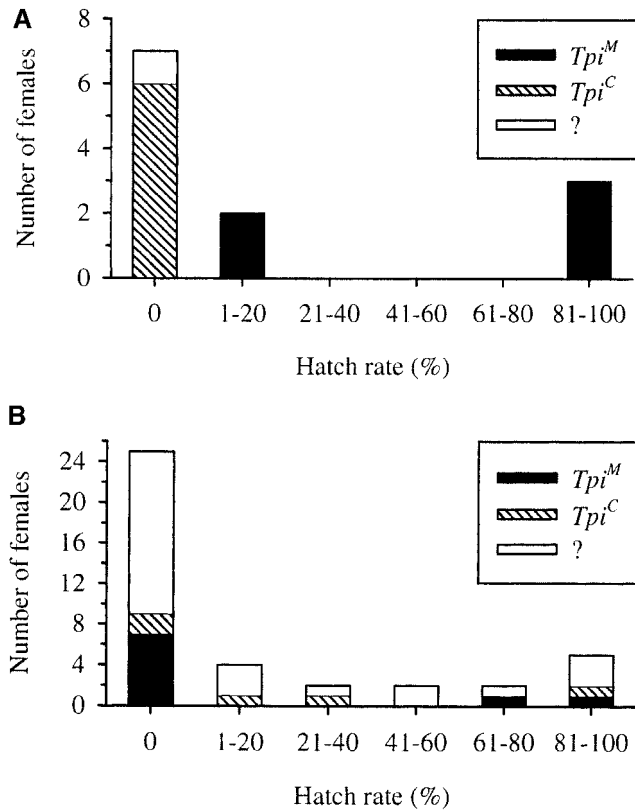


FIGURE 1.—Hatch rates of eggs laid by female offspring of the (A) backcross to *H. melpomene* MP × (CP × MP) and from (B) all backcrosses to *H. cydno* C × (C × M). Hatched bar, genotype at the *triose-phosphate isomerase* locus, with superscripts for the alleles of *H. melpomene* and *H. cydno*; ?, for individuals that were not analyzed.

on sterility, as is common in *Drosophila* (COYNE and ORR 1989b) and similar to that shown in interracial crosses in *H. melpomene* (JIGGINS *et al.* 2001a). The locus forms a terminal marker in linkage maps of the Z chromosome in *H. erato* and *Helicoverpa armigera* (N. FLANAGAN, personal communication; D. HECKEL, personal communication) so that linkage suggests factor(s) in that region able to produce complete sterility. The effect of introgression of this region of the Z chromosome is asymmetric. Introgression of the *H. cydno* region into a predominantly *H. melpomene* genotype produces complete sterility, while there is no detectable linkage between sterility and the *Tpi* locus of *H. melpomene* when introgressed into a predominantly *H. cydno* genetic background. Such asymmetric effects are predicted by Muller's model of complementary gene evolution (MULLER 1942). Sterility in the backcross to *H. cydno* must be produced by different factors, which must be less tightly linked to *Tpi* if they are on the *H. melpomene* Z chromosome. However, dominant-dominant and dominant-recessive interactions between autosomal loci cannot be ruled out for this direction of cross.

Here we find sterility in females with the *H. cydno* *Tpi* allele on a Panama *H. melpomene* autosomal background. Interestingly, in hybrids between geographic races of

H. melpomene from Panama and French Guiana, sterility is associated with the same Z-linked *Tpi* marker: in this case it is the French Guiana *Tpi* that is associated with sterility on a Panama autosomal background while the reverse cross produces no sterility and therefore no association with *Tpi* (JIGGINS *et al.* 2001a). According to recent molecular evidence, the two *H. melpomene* races are most closely related, with *H. cydno* as an outgroup (BELTRÁN *et al.* 2002; V. BULL, unpublished results) in spite of previous evidence for paraphyly of *H. melpomene* relative to *H. cydno* (BROWER 1996). Two possible explanations exist for the observed pattern of sterility in the three taxa. First, sterility is due to a Z-linked region involved in female fertility and ovary development, which has diverged rapidly between populations. Independent substitutions in the *Tpi* regions of both *H. cydno* and Guianan *H. melpomene* must then have occurred to cause sterility on a Panamanian *H. melpomene* autosomal background. Second, and more parsimoniously, sterility in both cases is due to the same derived Z-linked and autosomal alleles substituted within *H. melpomene* in Panama. In both crosses, sterility is found in females with an autosomal background predominantly derived from Panamanian *H. melpomene*, but lacking the Z-linked *Tpi* region of that population. Sterility arises when an individual lacks the epistatic sex-linked genes of Panama *H. melpomene* needed to complement the autosomes of that population, providing good evidence for complementary genes (MULLER 1940, p. 203). Sterility caused by the absence of this Z-linked region would therefore make the *H. cydno* and French Guiana *H. melpomene* regions behave as recessive "loss-of-function" alleles, as predicted under the dominance theory. However, loci causing sterility are likely to be sex specific, as in *Drosophila*. Ideally, therefore, we would need fertility data from the equivalent of attached-Z males to test between sex specificity (faster female evolution in this case) and the dominance theory as the principal cause of Haldane's rule in this example. However, no such strains exist and it is not even certain that the resulting \widehat{ZZW} genotype would be phenotypically male under lepidopteran sex determination.

Three different sterility phenotypes appear in female hybrids involving *H. melpomene* (Table 3):

1. In the cross described here between female Panamanian *H. melpomene* and male *H. cydno*, F₁ females lay normal-sized eggs that never hatch. In this cross alone it is possible that "sterility" is actually produced by zygote inviability or failure of fertilization despite normal spermatophore transfer.
2. The sterile eggs of hybrids are much smaller in interracial crosses between female *H. melpomene* from Panama and male *H. melpomene* from French Guiana (JIGGINS *et al.* 2001a). (In the reciprocal cross, egg size and fertility are normal.)
3. The third sterility phenotype is more extreme: hybrid

females between French Guiana *H. melpomene* and Panama *H. cydno* fail to develop ovaries at all.

These distinct sterility phenotypes back up evidence from *Tpi* linkage to suggest that at least some different loci are involved in each case, although a parsimonious explanation of the results involving Panamanian *H. melpomene* (see previous paragraph) implies some genes in common.

At least four features of these crosses suggest that relatively few loci each of major effect are important in producing sterility:

1. The asymmetry seen in crosses between French Guiana and Panama or Colombia *H. melpomene* implies that few loci are involved, so that, by chance, incompatibilities have arisen in one but not both reciprocal crosses (MULLER 1942).

It has been asserted on the basis of an unpublished analysis that such asymmetry might arise even if large numbers of loci produce sterility (TURELLI and ORR 1995, p. 395). However, many other examples of heterogeneity between the crosses studied here add to an impression that a few different major-effect loci, rather than many loci of individually small phenotypic effect, are important in each cross. For instance,

2. there are strong effects of loci linked to *Tpi* in some crosses but not others, and
3. the sterility phenotypes are very different between the different crosses. Neither would be expected if sterility were a polygenic effect of steadily accumulating genetic differences.

Finally,

4. the distribution of sterility in the backcrosses is strongly bimodal, with fertile females recovered after only a single generation of backcrossing.

If many loci combine to produce sterility, one might expect to see a range of intermediate fertility levels. Alternatively, if major-effect sterility factors were scattered throughout the genome, most offspring should have low fertility because in the backcrosses there is the opportunity for many additional interactions between dominant and recessive autosomal factors. *Tpi*-linked sterility on the *H. cydno* Z chromosome is therefore unusual in comparison with the rest of the genome. While some of these heterogeneities might be explained by multiple genes and threshold effects, the combination of effects seems most easily explained if several (rather than many) genomic regions are important, at least some of which have major effects on sterility.

While female sterility is complete in F₁ hybrids between a *H. cydno* female and *H. melpomene* male, it remains uncertain if sterility is normally present in hybrids from the reciprocal cross since few were produced due to strong behavioral isolation. There is sterility in both

directions of cross between *H. cydno* from Panama and *H. melpomene* from French Guiana. In the Colombian crosses there is variation among females from the one brood of *H. melpomene* female by *H. cydno* male, with some showing complete sterility and others having low fertility with ~20% of eggs hatching. This variation within F₁ females is difficult to explain, but both parents come from regions where interspecific hybridization occurs, *H. melpomene melpomene* with *H. heurippa* in the Eastern Andean foothills and *H. cydno* with *H. melpomene vulcanus* in the Cauca valley and Pacific slopes (LINARES 1989; MALLETT *et al.* 1998b). Populations may therefore carry introgressed polymorphisms for loci causing interspecific sterility, which then segregate in our F₁ cross. On the basis of the linkage between sterility and *Tpi*, we predict that female offspring from the unobtainable *H. melpomene* female × *H. cydno* male Panama cross should also be sterile. These would have a *H. cydno* Z chromosome and heterozygous *H. melpomene* autosomes. This is similar to the interaction that causes sterility in the backcross to *H. melpomene* shown by *Tpi* linkage, but which consists of the *H. cydno* Z together with on average 50% homozygous and 50% heterozygous *H. melpomene* autosomes. These females were completely sterile.

Closely related species of *Heliconius* typically have several incomplete barriers to gene flow that can include assortative mating, hybrid sterility, selection against hybrids due to increased predation on their non-mimetic color patterns, and the divergent ecology of the parental species (MALLETT *et al.* 1998b). Speciation is probably most closely associated with the origin of assortative mating and ecological divergence in *Heliconius* and other taxa (MALLETT *et al.* 1998b; JIGGINS and MALLETT 2000; KIRKPATRICK and RAVIGNÉ 2002). Hybrid sterility is neither necessary nor sufficient for speciation. For example, *H. erato* and *H. himera* show no evidence for a reduction in viability or fertility of interspecific hybrids, and yet the two remain distinct in hybrid zones characterized by assortative mating and probably strong ecological selection against hybrids (JIGGINS *et al.* 1997; McMILLAN *et al.* 1997; MALLETT *et al.* 1998a). In contrast, unidirectional female hybrid sterility has been found between the French Guiana and Panama races of *H. melpomene* (JIGGINS *et al.* 2001a), although these are traditionally considered part of the same species and are thought to be connected via continuous populations (BROWN 1979; MALLETT 1986). Hybrid sterility may therefore arise as a consequence of reproductive or geographic isolation more than as a direct cause of speciation. If spatial isolation or assortative mating limit gene flow, even weak divergent selection in the two populations may lead to the accumulation of epistatic incompatibilities in hybrids. Sterility can of course promote further divergence by reducing gene flow still further and providing a selection pressure for increased assortative mating through reinforcement. In *H. cydno* and *H. melpomene* sterility currently forms one of three strong

barriers reducing the fitness of F_1 and later generation hybrids. Genomic incompatibility resulting in female sterility produces strong selection against F_1 hybrids, of 50% when averaged across both sexes, and backcrosses produce females with an average reduction in fertility of $\sim 80\%$, so that the total barrier due to F_1 and backcross sterility is ~ 0.7 (i.e., $0.5 + 0.5 \times 0.5 \times 0.8$). Ecological incompatibility causing selection by predators against hybrid color patterns is of a similar strength, $\sim 50\%$ if similar to that estimated from a *H. erato* hybrid zone (MALLETT and BARTON 1989). Nearly half of the backcross offspring should escape this increased predation on hybrids, because their color patterns are similar to those of the parentals due to modifier loci and linkage of several major color pattern loci (R. E. NAISBIT, unpublished results), so that the overall barrier due to mimicry is $\sim 64\%$. Mate choice between the two species is by far the strongest barrier to gene transfer, since the species hybridize extremely rarely in nature, only with great difficulty in no-choice tests, and never in laboratory choice tests (NAISBIT *et al.* 2001). This is accompanied by disruptive sexual selection against hybrids causing reduced mating success, forming an overall mating barrier of $>99.9\%$ (MALLETT *et al.* 1998b; NAISBIT *et al.* 2001).

A consequence of these multiple incomplete barriers to gene flow is that certain hybrid genotypes will suffer disproportionately while others escape, creating a selectively permeable species boundary. As in hybrid zones, different regions of the genome will cross the species boundary and introgress with varying ease, with some halted by selective barriers and others relatively unrestricted (BARTON 1979, 1983; RIESEBERG *et al.* 1999). Since backcrossing occurs with both parental species (NAISBIT *et al.* 2001), introgression should be possible in both directions (MALLETT *et al.* 1998b; GILBERT 2003). Selection will oppose the transfer of some genes, such as color pattern genes or sterility factors. Strongly selected loci will also affect associated regions, for example, the *Tpi* locus or mitochondrial DNA, the introgression of which is prevented by female sterility (SPERLING 1993). However, universally advantageous alleles should be barely affected if loosely linked. Even the strong Haldane's rule sterility found here between a pair of sympatric *Heliconius* species may not result in complete evolutionary independence in nature. In *Heliconius*, hybrid sterility clearly contributes to speciation, but it seems neither entirely necessary for speciation nor very effective at preventing gene flow after speciation has occurred.

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