*Evolution*, 55(8), 2001, pp. 1631–1638

# SEX-LINKED HYBRID STERILITY IN A BUTTERFLY

CHRIS D. JIGGINS,[1,2] MAURICIO LINARES,[3] RUSSELL E. NAISBIT,[4] CAMILO SALAZAR,[3]
ZIHENG H. YANG,[4] AND JAMES MALLET[4]
[1]*The Smithsonian Tropical Research Institute, Apartado 2072, Balboa, República de Panamá*
[2]*E-mail: jigginsc@naos.si.edu*
[3]*Instituto de Genética, Universidad de los Andes, Carrera 1E No. 18A-10, P.O. Box 4976, Santafé de Bogotá, República de Colombia*
[4]*The Galton Laboratory, University College London, 4 Stephenson Way, London NW1 2HE, United Kingdom*

*Abstract.*—Recent studies, primarily in *Drosophila*, have greatly advanced our understanding of Haldane's rule, the tendency for hybrid sterility or inviability to affect primarily the heterogametic sex (Haldane 1922). Although dominance theory (Turelli and Orr 1995) has been proposed as a general explanation of Haldane's rule, this remains to be tested in female-heterogametic taxa, such as the Lepidoptera. Here we describe a novel example of Haldane's rule in *Heliconius melpomene* (Lepidoptera; Nymphalidae). Female $F_1$ offspring are sterile when a male from French Guiana is crossed to a female from Panama, but fertile in the reciprocal cross. Male $F_1$s are fertile in both directions. Similar female $F_1$ sterility occurs in crosses between French Guiana and eastern Colombian populations. Backcrosses and linkage analysis show that sterility results from an interaction between gene(s) on the Z chromosome of the Guiana race with autosomal factors in the Panama genome. Large X (or Z) effects are commonly observed in *Drosophila*, but to our knowledge have not been previously demonstrated for hybrid sterility in Lepidoptera. Differences in the abundance of male versus female or Z-linked versus autosomal sterility factors cannot be ruled out in our crosses as causes of Haldane's rule. Nonetheless, the demonstration that recessive Z-linked loci cause hybrid sterility in a female heterogametic species supports the contention that dominance theory provides a general explanation of Haldane's rule (Turelli and Orr 2000).

*Key words.*—Haldane's rule, *Heliconius*, hybrid sterility, Lepidoptera, speciation.

Received September 22, 2000. Accepted April 17, 2001.

''Future experiments—especially those dissecting the genetics of speciation in taxa with heterogametic females—will reveal if the explanation of Haldane's rule championed here fares any better than its many predecessors'' (Orr 1997, p. 215).

Recent years have seen great advances in our understanding of one particular aspect of speciation, namely the genetic basis of hybrid sterility and inviability. Most studies have concentrated on the search for an explanation of two rules of speciation (Coyne and Orr 1989): The tendency for the heterogametic sex to be preferentially affected by hybrid sterility or inviability (Haldane's rule) and the large effect of the X chromosome on incompatibility (the large X effect). After a number of years of widespread disagreement, it has recently been suggested that Muller's original explanation for Haldane's rule, known as dominance theory, largely explains both patterns (Muller 1940; Orr 1997). This posits that epistatic loss-of-function alleles causing hybrid breakdown will tend to be recessive, so that the hemizygous sex is afflicted by the expression of X-linked incompatibility genes to a far greater extent than the homogametic sex.

Over the years there has been a proliferation of proposed explanations for Haldane's rule, invoking such diverse causes as the disruption of dosage compensation, meiotic drive, cytoplasmic endosymbionts, and chromosomal rearrangements (Coyne and Orr 1989). Much of the controversy arose because Muller's original hypothesis was apparently contradicted by experiments showing that attached-X females, homozygous for two identical X chromosomes, are fertile in some *Drosophila* crosses in which males are sterile. If sterility results from recessive genes, it was thought that such homozygous females should be sterile (Coyne 1985; Coyne and Orr 1989). Subsequently, however, it was realized that dominance theory could be consistent with this result. If the loci causing male and female sterility are not the same, we do not necessarily expect that an X chromosome carrying male sterility loci will also contain female sterility loci (Turelli and Orr 1995; Orr 1997). There is now convincing evidence from *Drosophila* that loci causing hybrid inviability act in both sexes, whereas loci causing sterility are sex-specific (Hollocher and Wu 1996; True et al. 1996). In addition to dominance theory, there may be other effects that exaggerate Haldane's rule, notably faster-male evolution and possibly also faster-X evolution (Charlesworth et al. 1987; Wu et al. 1996). However, dominance theory appears to provide the only generally applicable explanation of Haldane's rule for both sterility and inviability in all taxa with sex chromosomes (Muller 1940; Turelli and Orr 1995, 2000).

The genetic data that led to this consensus have come almost exclusively from studies of *Drosophila* and other Diptera (Dobzhansky 1951; Coyne and Orr 1989; Orr 1997; Presgraves and Orr 1998). Crosses in female-heterogametic taxa such as birds and butterflies follow Haldane's rule (Coyne 1992; Laurie 1997), but virtually nothing is known about the genetic basis of hybrid dysfunction in such taxa. There is thus a need for studies examining the genetic architecture of hybrid sterility in birds and butterflies. Such studies will test whether the patterns observed in *Drosophila* are indeed generally applicable, as predicted by dominance theory. Here we describe and perform genetic analyses on a previously unknown, asymmetrical Haldane's rule effect between races of *Heliconius melpomene* (Linnaeus).

## MATERIALS AND METHODS

*Heliconius melpomene melpomene* individuals were collected near Cayenne, French Guiana, in May 1998 and Feb-

ruary 1999 (Pointe Macouria, 4°54.8′N, 52°21.6′W; Sablance, 4°57.8′N, 52°25.2′W, elevation at sea level for both sites) and from eastern Colombia between April 1998 and January 1999 (Virgen de Chirajara, 4°12.8′N, 73°47.9′W, elevation 1150–1450 m; Pajarito, 5°17.5′N, 72°42.5′W, elevation 940 m). *Heliconius melpomene rosina* individuals were collected from Gamboa (9°7.4′N, 79°42.2′W, elevation 60 m) and the nearby Parque Nacional Soberanía, República de Panamá, during the course of the experiments.

Crosses were carried out between these races of *H. melpomene* in insectaries sited in Gamboa, Panama (August 1998–October 1999), and La Vega, Colombia (May 1998–January 2000). Brood females were kept in individual cages at least 1 × 1 × 2 m with access to ample *Psiguria* flowers, occasionally supplemented by hand-feeding with commercially available bee pollen. In Panama, larvae were reared individually from egg to third instar to prevent cannibalism, and subsequently in groups of two to five individuals from the same brood, fed on shoots of *Passiflora biflora.* Eggs were collected daily and placed individually in plastic containers with damp cotton to maintain moisture. These were inspected daily and hatch rates recorded. In Colombia, larvae were reared on *P. edulis* and *P. oerstedii* plants in the insectaries. All eggs present were collected from each female every 15 days and their hatch rates recorded. In all cases, females were provided with healthy *Passiflora* plants on which to lay, either *P. menispermifolia,* the primary host of *H. melpomene* around Gamboa, or *P. edulis* (maracuyá). Control females were kept alongside crosses and reared under identical conditions. In text and tables, the genotypes of *H. melpomene* from French Guiana, Panama, and Colombia are abbreviated as MG, MP, and MC, respectively, and all crosses are given with the female genotype first. Eggs laid by several females representing each control genotype and the sterile $F_1$ class were measured under a binocular microscope using a 5 mm miniscale. Sample sizes for egg measurements are MP, $N = 10$; MG, $N = 15$; and $F_1$, $N = 28$.

Hatch rate data were analyzed using likelihood-ratio tests based on the beta-binomial distribution. Our problem is to compare survival between broods of different types (e.g., pure vs. hybrid). Traditionally, one might use analysis of variance with percent survival as the variate. However, this method is inefficient, and variation in brood size can cause heteroscedasticity. An alternative might be to assume a binomial distribution within broods of the same type. However, this method ignores real differences in survival between replicate broods, which may be due to genetic or environmental variation. Here we assume that the count within each brood has a simple binomial distribution, while the binomial probability $p$ varies according to a beta distribution among replicate broods. This leads to a beta-binomial distribution of the counts overall (Johnson et al. 1993). The beta distribution is particularly relevant in this context because it can approximate unimodal (e.g., normal or binomial) distributions or U-shaped, L-shaped, or reverse-L-shaped distributions, any of which might be expected in extreme cases of variation within brood classes (e.g., in backcrosses involving the segregation of sterility, as here). The beta-binomial is thus useful for a very general class of problems in which variable count data is used to compare fractional parameters between data classes. We analyze our data using the program BETABINO written by Z. H. Yang. BETABINO is described more fully in the Appendix and is available via ftp://abacus.gene.ucl.ac.uk/pub/ or on request from C. D. Jiggins.

### *Genetic Analysis*

Primers for an approximately 470-bp fragment of the triose phosphate isomerase (*Tpi*) gene were developed by W. O. McMillan and D. Heckel for *Heliconius erato* from sequences of the noctuid moth *Heliothis* (Logsden et al. 1995). This locus is sex linked in *Heliconius* as in many other Lepidoptera (Turner et al. 1979). Primer sequences, sited in coding regions, are 5′-GGTCACTCTGAAAGGAGAACCATCTT-3′ (forward) and 5′-CACAACATTTGCCCAGTTGTTGCCAA-3′ (reverse), which amplify a highly variable intron of approximately 470 bp in the *Tpi* gene. Using MacVector (Eastman Kodak, Rochester, NY), sequences from individuals collected in Guiana and Panama (M. Beltrán, unpubl. ms.) were searched for diagnostic restriction enzyme sites. One site approximately in the center of the sequence was found; it differs between the two races by a single base-pair substitution and corresponds to the restriction site of the enzyme Dde1 (5′ to 3′ CTNAG). Total genomic DNA was extracted from frozen tissue (one-third of a thorax) homogenized in 500 μl CTAB buffer, and digested overnight at 55°C with 0.1 mg proteinase K. After three extractions (twice with phenol:chloroform: isoamyl alcohol and once with chloroform : isoamyl alcohol), the DNA was precipitated with ethanol (Sambrook et al. 1989). The *Tpi* fragment was amplified using a polymerase chain reaction protocol of 94°C for 7 min, followed by 10 cycles of 94°C for 45 sec, 58°C for 45 sec falling by 0.5°C per cycle, and 72°C for 105 sec and finally 25 cycles of 94°C for 45 sec, 53°C for 45 sec, and 72°C for 105 sec. Amplified DNA was digested with Dde1 (0.05 units/μl) for 4 h at 37°C. Digestion products were separated by electrophoresis on 1.5% agarose gel and stained with ethidium bromide. Sex linkage of the locus was confirmed by restriction analysis of 12 individuals from $F_1$ brood 128: All male offspring were heterozygotes, and all females appeared to be homozygotes (i.e., were actual hemizygotes) of the paternal allele (six males : six females, $G_1 = 16.6$, $P < 0.001$). Backcross broods were then analyzed to investigate possible linkage with sterility phenotypes.

## RESULTS

### *Panamanian Crosses*

Female hybrids between *H. melpomene* from French Guiana (MG) and Panama (MP) show asymmetrical sterility. The female offspring of a cross between a female from Panama and a male from Guiana (MP × MG) lay eggs that are significantly smaller than normal (egg sizes with standard errors are: MG, 1.3 ± 0.02 mm × 0.8 ± 0.01 mm; MP, 1.3 ± 0.01 mm × 0.9 ± 0.02 mm; MP × MG, 0.9 ± 0.02 mm × 0.5 ± 0.01 mm), and never hatch (250 sterile eggs laid from 19 broods). In contrast, the reciprocal cross (MG × MP) produces female offspring that lay fertile eggs with a hatch rate of 0.88 ± 0.02 when mated to MG and 0.90 ± 0.04 when mated to MP (Table 1). This compares with control hatch

TABLE 1. Hatch rates in control, $F_1$, and backcross broods. MP are *Heliconius melpomene* from Panama, MG are from French Guiana, and MC are from Colombia. Cross types are given with the female genotype first. Values shown are derived from a beta-binomial model fitted to the data over all broods, excluding females that laid no eggs and brood classes that were completely sterile. Broods with high variance and intermediate mean are primarily backcross classes that show segregation of sterility between individuals (see Results). In some cases, broods sharing one parental genotype have been combined in the analysis because hatch rate did not differ significantly between crosses (shown as MG/MP or ?). Note that where the number of broods in a crosstype is low, exact parameter estimates may vary slightly between runs of the BETABINO program.

| Female genotype | Male genotype | No. broods | No. eggs | Mean hatch rate | SE | Variance | SE |
|---|---|---|---|---|---|---|---|
| Panama crosses | | | | | | | |
| MG | MG | 18 | 863 | 0.90 | 0.03 | 0.012 | 0.007 |
| MP | MP | 14 | 577 | 0.93 | 0.02 | 0.003 | 0.002 |
| MG | MP | 6 | 180 | 0.94 | 0.02 | 0.001 | 0.001 |
| MP | MG | 8 | 265 | 0.85 | 0.06 | 0.020 | 0.018 |
| MG/MP | MG × MP | 7 | 275 | 0.71 | 0.14 | 0.120 | 0.056 |
| MG/MP | MP × MG | 4 | 347 | 0.95 | 0.01 | 0.000 | 0 |
| MG × MP | MG | 3 | 257 | 0.88 | 0.02 | 0.000 | 0 |
| MG × MP | MP | 3 | 501 | 0.90 | 0.04 | 0.004 | 0.005 |
| MP × MG | MG | 13 | 144 | 0 | — | — | — |
| MP × MG | MP | 6 | 106 | 0 | — | — | — |
| (MG × MP) × MG | MG | 18 | 383 | 0.45 | 0.08 | 0.115 | 0.023 |
| (MG × MP) × MG | MP | 5 | 52 | 0.10 | 0.04 | 0.000 | 0 |
| (MG × MP) × MP | MG | 7 | 205 | 0.94 | 0.02 | 0.001 | 0.002 |
| MP × (MG × MP) | MG/MP | 10 | 225 | 0.49 | 0.14 | 0.206 | 0.023 |
| MP × (MP × MG) | MP | 6 | 174 | 0.57 | 0.16 | 0.164 | 0.041 |
| Colombia crosses | | | | | | | |
| MC | MC | 7 | 285 | 0.55 | 0.09 | 0.051 | 0.023 |
| MG | MG | 3 | 159 | 0.60 | 0.10 | 0.023 | 0.021 |
| MC | MG | 2 | 81 | 0.77 | 0.20 | 0.080 | 0.081 |
| MG | MC | 1 | 41 | 0.15 | 0.05 | 0.000 | 0 |
| MC × MG | ? | 2 | 31 | 0.36 | 0.08 | 0.000 | 0 |
| MC × MG | ? | 8 | 0 | — | — | — | — |
| MG × MC | MC | 6 | 255 | 0.65 | 0.11 | 0.050 | 0.031 |
| MC | MC × MG | 3 | 115 | 0.53 | 0.15 | 0.068 | 0.040 |
| MC × (MC × MG) | ? | 6 | 140 | 0.57 | 0.09 | 0.017 | 0.024 |

rates of 0.90 ± 0.03 for MG and 0.93 ± 0.02 for MP (Table 1).

In *Heliconius* the ovarioles develop fully only after emergence from the pupa. Females were therefore dissected to investigate the morphology of sterile phenotypes once they had begun laying or after several days of access to pollen (Dunlap-Pianka et al. 1977). Sterile $F_1$ females showed no obvious abnormalities in ovarian development, except that eggs entering the oviduct were considerably smaller than in normal females (C. D. Jiggins, pers. obs.). Unfortunately, this meant that sterility was not reliably identifiable in dissections of backcross females and had to be estimated directly using hatch rates. However, the abnormal egg size observed prior to fertilization demonstrates that failure to hatch is due to $F_1$ female sterility rather than gametic incompatibility or zygote inviability.

Male $F_1$ hybrids are fertile (Table 1). One $F_1$ male genotype (MP × MG), when backcrossed to parental females, has a high hatch rate of 0.95 ± 0.01, which is similar to controls. The reciprocal $F_1$ male (MG × MP) has a reduced hatch rate of 0.71 ± 0.14. Combining these two male $F_1$ classes results in a significant reduction of fit of the model ($G_2 = 8.4$, $P < 0.02$), implying a difference in male fertility between $F_1$ classes. However, this results from a drastic reduction of hatch rate in just two of seven broods (brood 199, 16/39 hatched; brood 277, 0/16 hatched). If these two broods are excluded, the 220 eggs from the remaining five broods gave a normal hatch rate of 0.95 ± 0.02. It is therefore unclear whether this reduction in hatch rate is due to partial male sterility or other environmental or genetic variation.

Thus, female $F_1$ hybrids are completely sterile in only one direction of cross, implying sex-linked or cytoplasmic incompatibility. Maternal effects might also cause asymmetry, but generally affect early life stages and are therefore not considered likely to cause sterility (Turelli and Orr 2000). More specifically in this case, the sterile females have cytoplasm and a W chromosome from MP, but a Z chromosome from MG. Backcrosses can be used to differentiate between cytoplasmic (or W-chromosome) and Z-chromosome effects. When the fertile $F_1$ females (MG × MP) are backcrossed to MG males, their female offspring possess the MG cytoplasm ultimately from an MG mother, an MG Z chromosome from their father, and on average 75% MG autosomes. Because sterility in the $F_1$ generation is associated with the MP cytoplasm, these females should be fertile if sterility results from cytoplasmic effects. In fact, this cross results in segregation of sterility phenotypes, ranging from fully fertile to fully sterile females with a number of intermediate partially sterile phenotypes (Fig. 1a). The expression of sterility in this cross implies that interactions between the MG Z chromosome and MP autosomal genes, and not cytoplasmic or W-chromosome factors, are the primary cause of the incompatibility.

The fertile female $F_1$ (MG × MP) was also backcrossed in the other direction, to MP males, whereby all female offspring inherit the MP Z chromosome. The offspring of this

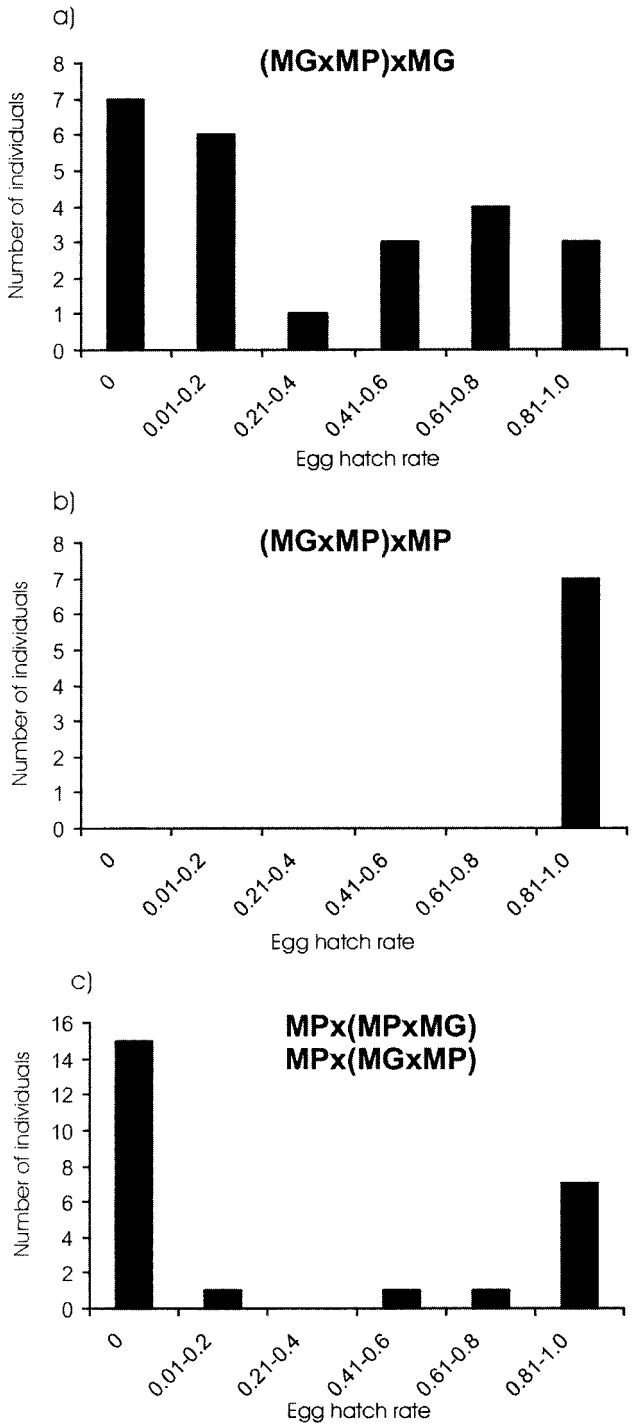


FIG. 1. Segregation of sterility phenotypes in backcross broods. The distribution of hatch rates for the female offspring of each cross type are shown. Females shown either survived ≥ 20 days after mating and laid no eggs or laid at least five eggs (although the majority laid far more than this). Female genotypes are shown first. MG is *Heliconius melpomene* from Guiana and MP is *H. melpomene* from Panama.

cross were all fully fertile (Fig. 1b), in spite of the presence of MG cytoplasm and W chromosomes on MP chromosomal backgrounds. The lack of sterility in these females is expected if MG W chromosomes and cytoplasm are not causes of the sterility phenotype.

To confirm the role of the Z chromosome, male $F_1$s were backcrossed to MP females, producing offspring in which segregation of MG and MP Z chromosomes occurs on a largely Panama genetic background. There was striking bimodality in the incidence of sterility in this cross, with a ratio of 15: 1 : 9 sterile : partially sterile : fertile females (combining crosses involving both $F_1$ male genotypes; see Fig. 1c). Sterile females were either kept for at least 20 days after mating and laid no eggs or laid at least five eggs that failed to hatch. Partially sterile and fertile females laid at least five eggs and showed a hatch rate of $< 0.2$ and $> 0.5$, respectively.

Eight additional females from these crosses survived between 10 and 20 days but never laid any eggs. Fertile females almost invariably began egg laying within five to 10 days of mating, but very occasionally only began to lay after 15 to 20 days (pers. obs.). Thus, the females that survived more than 10 days are most likely to have been sterile but cannot with certainty be assigned to any class. The above ratio of sterile : fertile females may therefore underestimate the number of steriles.

The segregation of sterility phenotypes in these backcrosses showed a highly significant association with *Tpi*, a Z-linked protein-coding gene. Using a restriction digest diagnostic for the MG allele, segregation of this marker could be followed in backcross broods. Of 16 individuals genotyped, all seven fertile phenotypes had the MP genotype at *Tpi*, whereas of nine sterile phenotypes eight had an MG and one an MP genotype (test of heterogeneity: $G_1 = 15.9$, $P \ll 0.001$).

### *Colombian Crosses*

Crosses between *H. m. melpomene* from Colombia and French Guiana show a hybrid sterility effect similar to that observed between Panama and French Guiana (Table 1). When a male from French Guiana and a female from Colombia are crossed, the female $F_1$ offspring are largely sterile. The reciprocal cross produces fertile female offspring and all $F_1$ males are fertile, following Haldane's rule (Table 1). In contrast to the Panamanian crosses, most sterile $F_1$ females never laid eggs. However, Panama × Guiana sterile females were also reluctant to lay eggs unless maintained with abundant pollen supplies, so it seems likely that the sterility phenotype in Colombian crosses was similar to that of the Panamanian crosses. The egg hatch rate is also considerably lower, averaging only $0.55 \pm 0.092$ and $0.60 \pm 0.10$ in MC × MC and MG × MG pure broods, respectively, presumably for environmental reasons. Two of 10 $F_1$ females that were expected to show sterility laid some fertile eggs (females 861 and 9 laid a total of 31 eggs, 11 of which hatched), implying reduced incompatibility in Colombia × Guiana hybrids as compared to Panama × Guiana. Hatch rates of seven backcross females, offspring of an $F_1$ male backcrossed to a Colombia *melpomene* female, were also measured. These crosses gave a ratio of 6:0 fertile/partially sterile : sterile (excluding

one sterile female that lived only for nine days), differing significantly from the ratio of 10:15 shown in the equivalent Panama × Guiana cross; $G_1 = 9.3$, $P < 0.01$). Additional crosses will be needed to study how the genetic architecture of sterility in Colombia × Guiana crosses differs from that shown for Panama × Guiana.

## Discussion

The genetic basis of hybrid incompatibility is one of the best understood aspects of speciation. In particular, there is now a general consensus over the cause of Haldane's rule—the tendency for hybrid breakdown to affect preferentially the heterogametic sex (Orr 1997; but see Wu and Davis 1993). Nonetheless, there have been virtually no studies dissecting hybrid incompatibility in female-heterogametic species. Here we have identified a novel example of hybrid sterility in a butterfly, *H. melpomene,* which follows Haldane's rule (Haldane 1922). Including this example with the results of a recent literature survey (Laurie 1997) makes a total of 44 known examples of sex-specific hybrid sterility in birds and Lepidoptera, 14 of which are in Lepidoptera. Of these 44 cases, only one does not follow Haldane's rule. Therefore hybrid sterility overwhelmingly follows the rule in these taxa.

### *Sterility Involves a Large Z Effect*

Sterility of *H. melpomene* backcross hybrids is strongly associated with the Z-linked *Tpi* gene. This provides the best evidence yet for a large Z (or X) effect on hybrid sterility in female-heterogametic taxa.

Two previous studies have demonstrated Z effects on incompatibility in Lepidoptera. Crosses between two species of *Colias* butterflies show asymmetric female sterility similar to that described here (Grula and Taylor 1980). In this case the segregation of Z chromosomes in backcrosses could be followed using a sex-linked color-pattern trait, showing that the Z chromosome was associated both with reduced male mating vigor and with inviability. Unfortunately, sample sizes were too small to provide much information on the inheritance of female sterility, although both autosomal and Z-linked loci are likely involved. Similarly, two races of *Papilio glaucus* differ at a number of Z-linked loci including allozyme, color pattern, and life-history characters (Hagen and Scriber 1989). These traits showed significant deviations from Mendelian ratios in backcrosses, associated with reduced female pupal survival. Deficits of certain genotypes are most readily explained by a reduction in hybrid viability due to introgression of the foreign Z chromosome. Thus, in the handful of studies where backcross analysis has been carried out, a large Z effect seems to be the rule in Lepidoptera as it is in *Drosophila* (Coyne and Orr 1989).

### *Number of Loci Involved in Sterility*

In common with a number of cases of hybrid incompatibility, the effect demonstrated here is asymmetric (Coyne and Orr 1997). Thus, a Guiana male crossed with a Panama female produce sterile hybrid females, but in the reciprocal cross the $F_1$ females are fertile. Muller (1942) argued that the common occurrence of asymmetry implies that relatively few genes are involved in causing incompatibility. If many loci were involved, there should be greater similarity in the number of loci that accumulate in the two diverging taxa. However, depending on the form of the interactions, asymmetry could theoretically occur even if up to 100 loci were involved in causing incompatibilities (Turelli and Orr 1995; M. Turelli, pers. comm.). Thus, although asymmetry is more probable when a few major loci are involved, asymmetric hybridizations such as ours provide only weak evidence that this is the case.

A better way to determine the number of loci involved would be by linkage analysis, and the close association of sterility with the *Tpi* gene would seem to imply that this region of the Z chromosome has a strong effect on sterility. However, Maside and Naveira (1996a,b) have cautioned against inferring single genes with major effects on sterility using linkage analysis. In crosses between *Drosophila buzzatii* and *D. koepferae* sterility results if greater than 40% of any autosome is introgressed (Naveira and Maside 1998). Introgressions of less than 30% result in fertile males. Thus, many interacting loci cause sterility and any one locus alone has little or no effect. Similar results are seen in analysis of X-linked factors such as the Odysseus gene, a sterility factor of major effect detected in crosses between *D. simulans* and *D. mauritiana* (Perez et al. 1993). Subsequent analysis has shown that if smaller fragments of the Odysseus region are introgressed, no one fragment alone can cause sterility. Again, many interacting genes are clearly involved (Wu and Hollocher 1998).

In *H. melpomene,* the backcross showing segregation of Z chromosomes has a 1:1 ratio of sterile : fertile females (16: 9 when sterile and partially sterile females are combined; $G_1 = 1.99$, n.s.; see Fig. 1c), compatible with a single-gene hypothesis. This is a weak test, however, and sterility might still be polygenic, with a threshold fraction of the Guiana Z chromosome required for sterility. In first generation backcrosses an association of sterility with *Tpi* would be expected if the marker were situated near the center of the Z chromosome, such that the Guiana *Tpi* allele was commonly associated with a predominantly Guiana Z chromosome. In both *Heliconius erato* and *Helicoverpa armigera*, the *Tpi* gene is at one end of the Z-chromosome linkage group (D. Heckel, pers. comm.; N. Flanagan, pers. comm.). If this is also the case in *H. melpomene*, then the association of sterility with *Tpi* suggests that sterility factor(s) are localized toward one end of the chromosome. However, more Z-linked markers are needed to confirm this.

The backcross of the female $F_1$ to Guiana provides some information on the main autosomal factors in the MP genome that interact with the MG Z chromosome (Fig. 1a). The situation is simplified as there is no recombination in females (Turner and Sheppard 1975), so in this cross introgressed chromosomes are inherited intact. All females therefore possess a complete MG Z chromosome and the segregation of phenotypes depends on which MP chromosomes are inherited. The observed variation from sterile to fertile phenotypes implies that many autosomes are involved, several of which are needed for complete sterility.

*Evidence in Support of the Dominance Theory*

The large Z (or X) effect demonstrated here suggests that sterility factors are recessive as predicted by dominance theory. Similarly, in *Drosophila* the introgression of hemizygous X-linked fragments often leads to far greater hybrid dysfunction than introgression of similar-sized heterozygous fragments on the autosomes. Because many introgressed autosomal regions also cause male sterility when homozygous, this implies that the large X effect is due mainly to the recessiveness of sterility genes, rather than a greater preponderance of loci on the X chromosome (Hollocher and Wu 1996; True et al. 1996). The large X effect therefore provides support for dominance theory (Turelli and Orr 2000) and is not just an artifact of backcross analysis (Wu and Davis 1993; Wu et al. 1996).

Some Panama × Guiana backcross females have a more extreme sterility phenotype than is ever expressed in $F_1$ crosses. Sterile $F_1$ females invariably laid small eggs that never hatched. In contrast, some backcross female genotypes never laid eggs, despite living for more than 50 days in some cases. One likely explanation is that more incompatible genetic interactions are expressed in these individuals than in the $F_1$s. Interactions between two or more incompatible homozygous (or hemizygous) loci ($H_2$ interactions in the terminology of Turelli and Orr 2000), occur in backcross but not $F_1$ genotypes. In this case, increased sterility in a backcross might result from interactions between the hemizygous Guiana Z chromosome and homozygous Panamanian autosomal loci. This would imply that the Panama genome contains autosomal recessive alleles not expressed in the $F_1$ that contribute to sterility. This provides a further line of evidence supporting the recessive nature of sterility factors.

*Lepidoptera and Alternative Causes of Haldane's Rule*

In addition to dominance, two further effects are considered likely to contribute to Haldane's rule (Orr 1997), and studies of female heterogametic taxa such as the Lepidoptera may hold the key to understanding their relative importance. First, genes for male sterility appear to diverge faster than those that affect females (Wu and Davis 1993), perhaps due to rapid evolution of the male reproductive system driven by sexual selection (Chapman et al. 1995). Indeed, introgression experiments have shown many more male than female sterility factors in *Drosophila* crosses (Hollocher and Wu 1996; True et al. 1996), which likely explains the frequent occurrence of male sterility in *Drosophila* (Wu et al. 1996). However, faster-male evolution should cause anti-Haldane effects in female-heterogametic taxa such as butterflies and would therefore seem to be contradicted by the observation that Haldane's rule for sterility is overwhelmingly obeyed in Lepidoptera and birds (Laurie 1997). This is most likely because male sterility factors are not expressed in homogametic $F_1$ males if they are recessive, and faster-male evolution is therefore masked by dominance in female-heterogametic taxa (Turelli 1998). To confirm this, future studies need to investigate whether there is a similar preponderance of male versus female sterility factors in Lepidoptera as has been observed in *Drosophila*.

If the X chromosome were to evolve faster than the autosomes, this could also contribute to Haldane's rule (Charlesworth et al. 1987). Faster evolution of the X chromosome might result from fixation of favorable recessive alleles, although such beneficial mutations must be extremely recessive for this effect to be strong (Charlesworth et al. 1987). Alternatively, mutation rates likely vary between sex chromosomes and autosomes, because males have higher per-generation mutation rates than females (Miyata et al. 1987). In male-heterogametic taxa, X chromosomes spend 75% of their time in females, and are thus expected to show lower mutation rates than autosomes, but in female-heterogametic taxa this situation is reversed. In Lepidoptera, therefore, either of these effects might cause faster divergence of X (or Z) chromosomes, which could exaggerate Haldane's rule (Orr 1997). There is tentative evidence that lepidopteran Z chromosomes do diverge faster than the autosomes, as many species-diagnostic traits are Z linked, including 24% of differentiated allozyme loci across 11 species comparisons (Sperling 1994; Prowell 1998). However, in Lepidoptera, which generally have chromosome numbers around 30, between 10% and 25% of all polymorphic allozyme loci are Z linked (Mallet et al. 1993; Raijmann et al. 1997; D. Heckel, pers. comm.). This might mean that sex-linked loci are both more polymorphic and diverge faster, but could reflect a sampling bias in the enzyme loci studied. In conclusion, it seems plausible but by no means proven that faster-X evolution contributes to Haldane's rule. Direct estimates of the relative abundance of Z-linked versus autosomal sterility factors are needed in Lepidoptera.

*Genetic Compatibility Is Not Concordant with Color Pattern*

Species of *Heliconius* consist of multiple geographic populations, typically separated by incompatibility in mimetic pattern, rather than hybrid fertility (Sheppard et al. 1985; Mallet and Barton 1989). In the case of mimicry, the adaptive peaks can be clearly identified as color patterns that are adapted to local mimicry rings. In contrast, we know nothing about the adaptive value or otherwise of the genes causing hybrid sterility. Nonetheless the effects are analogous, in that both represent divergent adaptive peaks separated by troughs of reduced hybrid fitness. Interestingly, hybrid sterility is not concordant with color pattern: Crosses between Colombian and French Guianan populations of the race *H. m. melpomene* produce sterile hybrids (Table 1). All previous races of *H. melpomene* that have been crossed are genetically compatible. Sheppard et al. (1985) crossed *H. melpomene* from Belem, to the east of French Guiana, with populations from Venezuela, eastern Brazil, Trinidad, central Amazonia, and eastern Ecuador and found no evidence for hybrid breakdown. Similarly, crosses of Costa Rican females with a male from eastern Colombia and another from Trinidad both produced fertile female offspring (J. Mallet and L. E. Gilbert, unpubl. data). These latter crosses in particular might have been expected to show sterility if the incompatible Z chromosome were more widespread. Notably, *H. m. melpomene* from Trinidad are similar in appearance to *H. m. melpomene* from French Guiana, but show no incompatibility with either Be-

lem or Costa Rica. Thus, the distribution of hybrid sterility does not obviously correspond to color pattern boundaries.

Hybrid compatibility seems more concordant with the geographic structure of mitochondrial DNA haplotypes. Apart from divergence across the Andes, most adjacent color-pattern races are little differentiated at mitochondrial DNA (Brower 1996) or allozymes (Turner et al. 1979; Jiggins et al. 1997). In contrast, the French Guiana populations form the most divergent mitochondrial lineage within *H. melpomene* (Brower 1996). Hybrid female sterility, even in only one direction of cross, will strongly impede mitochondrial gene flow (Sperling 1994). It is perhaps unsurprising, therefore, that the incompatible Z-chromosome distribution corresponds with mitochondrial DNA rather than color-pattern differentiation.

### *Implications for Speciation*

To demonstrate that any process contributes to speciation, it must be shown to play a role in reproductive isolation between species that still hybridize and exchange genes. At least one *Heliconius* species pair, *H. himera* and *H. erato,* show no hybrid incompatibility, demonstrating that hybrid sterility is not essential for speciation (Jiggins et al. 1996; McMillan et al. 1997). Nonetheless, *H. melpomene* and its sister species, *H. cydno,* hybridize occasionally in the wild to produce sterile $F_1$ female hybrids (Linares 1989; Mallet et al. 1998; Gilbert 2000; R. E. Naisbit, C. D. Jiggins, M. Linares, C. Salazar, and J. Mallet, unpubl. ms.). Male hybrids are fertile, and there is evidence of recent gene flow (V. Bull, unpubl. ms.). Thus, hybrid sterility must play a role as a current barrier to gene flow in this case. However premating isolation is probably more important: There is strong assortative mating between *H. melpomene* and *H. cydno,* using color pattern as a mating cue (Jiggins et al. 2001), and habitat segregation of mimicry rings leading to ecological isolation (Smiley 1978; Srygley and Ellington 1999). As in hybrid zones, premating isolation is likely to be more effective than hybrid incompatibility in maintaining species differences despite gene flow (Jiggins and Mallet 2000). Thus, in *Heliconius* butterflies, ecology and mimicry likely play the key roles in speciation (Jiggins et al. 2001), such that studies of genomic incompatibility alone cannot be considered sufficient to understand the process.

## Acknowledgments

We thank M. Turelli for comments on the manuscript and D. Heckel for valuable discussion; M. Beltrán, C. Paresce, C. Estrada, L. O'Donovan, and R. Coe, who reared butterflies in Panama; G. Fajardo, who reared butterflies in Colombia; B. Hermiers for help in French Guiana; and W. O. McMillan, D. Heckel, and L. Gahan for use of the *Tpi* primers. This work was carried out in Panama at the Smithsonian Tropical Research Institute with permission from the Autoridad Nacional del Ambiente and funded by the Natural Environment Research Council, and in Colombia by grants from Colciencias and Banco de la República to ML (Grants CO-1204-05-510-93 and 734 respectively).

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Corresponding Editor: C. López-Fanjul

## Appendix

The BETABINO program, written by Z. H. Yang, fits beta-binomial models to data of count (available via ftp://abacus.gene.ucl.ac.uk/pub/). We want to compare the probability of an event (e.g., success of hatching) across different classes (e.g., mating types). In each class, multiple families are tested, serving as multiple replicates. The problem with using a binomial model is that the success rate varies among families in each class. In a beta-binomial model, the probability of observing $k_{ij}$ successes in $n_{ij}$ trials in the $j$th family of the $i$th class is given by the binomial distribution Bino($k_{ij}$; $n_{ij}$, $p_{ij}$):

$$\text{Prob}(k_{ij};\, n_{ij},\, p_{ij}) = \binom{n_{ij}}{k_{ij}} p_{ij}^{k_{ij}}(1 - p_{ij})^{k_{ij}},$$

$$0 \le k_{ij} \le n_{ij}. \qquad \text{(A1)}$$

However, the probability parameters $p_{ij}$ (i.e., success rates) vary according to a beta distribution Beta($\alpha_i$, $\beta_i$) with shape parameter $\alpha_i$ and scale parameter $\beta_i$. The density of the beta distribution is

$$f(p;\, \alpha,\, \beta) = p^{\alpha-1}(1 - p)^{\beta-1}/B(\alpha,\, \beta), \qquad 0 \le p \le 1, \qquad \text{(A2)}$$

where $B(\alpha, \beta)$ is the beta function. The counts of successes among families from the same class then follow a beta-binomial distribution, also known as the negative-hypergeometric distribution (Johnson et al. 1993, pp. 242, 264–266):

$$\text{Prob}(k_{ij};\, n_{ij},\, \alpha_i,\, \beta_i) = \binom{-\alpha_i}{k_{ij}}\binom{-\beta_i}{n_{ij} - k_{ij}}\Big/\binom{-\alpha_i - \beta_i}{n_{ij}},$$

$$0 \le k_{ij} \le n_{ij}. \qquad \text{(A3)}$$

The likelihood is calculated as the product of probabilities of the counts over families. To facilitate comparison among different classes, we use the mean of the $p_{ij}$ values, $m = \alpha/(\alpha + \beta)$, and their variance, $v = \alpha\beta/[(\alpha + \beta)^2 (\alpha + \beta + 1)]$ as parameters instead of $\alpha$ and $\beta$, and thus specify the beta distribution as Beta($m$, $v$). Parameters *alpha;* and $\beta$ are given as

$$\alpha = m(m - m^2 - v)/v \quad \text{and} \qquad \text{(A4a)}$$

$$\beta = (1 - m)(m - m^2 - v)/v, \qquad \text{(A4b)}$$

with $0 \le m \le 1$, and $0 \le v \le m(1 - m)$.

The BETABINO program obtains maximum-likelihood estimates of parameters and calculates the optimum log likelihood under four models concerning differences among classes: the same mean and variance among classes (model 0), the same mean but different variances (model 1), different means and the same variance (model 2), and different means and variances (model 3). The models can be compared using the likelihood-ratio test to examine whether the different classes have the same average probability of success. The analysis is rather similar to a one-way analysis of variance, where the class is the main effect and the multiple families with each class are the replicates.