Persistence of an extreme sex-ratio bias in a natural population

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The sex ratio is a key parameter in the evolution and ecology of a species. Selfish genetic elements that bias the sex ratio of affected individuals are well known and characterized, but their effect on populations has been considered limited, because either the element does not achieve high prevalence or the host rapidly evolves resistance to the distorting element, reducing its prevalence. We tested whether the host necessarily prevails by using a butterfly system where records from the early part of the 20th century reported extreme sex-ratio bias in nature. We reexamined this population and found the bias was present today, 400 generations after the original record, with a population sex ratio of 100 females per male. The sex-ratio bias was associated with the presence of a heritable male-killing *Wolbachia* infection in 99% of adult females, against which the host butterfly has failed to evolve resistance. The resultant dearth of males causes an average 57% reduction in the reproductive output of adult females. Persistence of the population despite the very high frequency of the sex-ratio distorter appears to be associated with the ability of males to mate >50 times in their life combined with a high intrinsic rate of increase of the species.

The sex ratio is a key parameter in the population and evolutionary biology of a species, dictating patterns of mate competition and mate choice, and affecting population growth rates (1). The sex ratio produced by individuals is also an area of intragenomic conflict. Whereas selection on autosomes favors individuals that produce the Fisher (Hamilton) optimum, selection on sex chromosomes and cytoplasmic genes does not (2–4). In male heterogametic species, selection favors X chromosome variants that become overrepresented in sperm through drive against developing sperm that bear the Y chromosome. In terms of cytoplasmic genes, various cytoplasmically inherited bacteria are known to bias the primary or secondary sex ratio toward female production, and others induce parthenogenesis in their hosts. This manipulation makes evolutionary sense from the viewpoint of the cytoplasmic bacterium: they are biasing resources away from production of male hosts (which cannot transmit the bacterium) toward the production or survival of females (which can transit the bacterium). One class of these is the male-killing bacteria. Here, females that are infected produce both male and female zygotes, but males die during embryogenesis through some unknown mechanism. In hosts where there is sibling–sibling competition for resources, male killing is thought to be adaptive to the bacterium because the death of male hosts (which cannot transmit the bacterium) increases the survival prospects of sibling female hosts (which bear the same bacterium and can transmit it).

One view of selfish genetic elements is that they arise relatively frequently, but selection on autosomes to return the sex ratio to unity quickly prevails, such that the phenotypes of these elements are not commonly seen, and they do not attain high frequency in the population. Indeed, the ability of repressor genes to restore populations to Fisher equilibrium has been demonstrated in laboratory populations (5), and studies of natural populations have also revealed the presence of repressor elements both within populations and in hybrid crosses (6–9). The concept of the "parliament of the genes" was created (and largely accepted) as an explanation for the ability of the autosomes to prevail: put simply, there is more "adaptability" in the large genome of the autosomes than the restricted genome sex-ratio-distorting element. This disparity produces a power imbalance in the speed and variety of response to selection that results in suppression of the selfish element when their frequency is high, and prevents extreme bias levels (10).

Whereas it is clear that sex-ratio distorters are often repressed in natural populations, it is not clear that rapid repression is inevitable. Although various authors have noted the ability of sex-ratio distorters to attain high prevalence if unrepressed (2, 11, 12), the ability of sex-ratio distorters to remain unrepressed over significant periods of time remains a contentious issue and is unsubstantiated. To test whether sex-ratio distorters can rise to and be maintained at high prevalence without repression, we need to examine case studies where there is historical evidence of sex-ratio bias and determine whether the sex-ratio bias is maintained over time (indicating selective constraints on the autosomes) or whether it disappears (indicating that selection on the autosomes results in repression).

The Independent Samoan population of the butterfly *Hypolimnas bolina* represents just such a test. The attractive wing pattern polymorphism exhibited in female *H. bolina* led to this species being extensively collected and bred by entomologists in the early 20th century. Alongside records of color patterns come records of sex-ratio bias in natural populations, both at the individual and the population level, that were easily obtained given the sexual dimorphism in color pattern exhibited by this species. In Fiji, some females were seen to produce all-female broods in a heritable fashion, and recent work has shown that this is associated with a cytoplasmically inherited *Wolbachia* infection that kills males (13, 14). Pertinently, Hopkins (15) recorded the presence of extremely female-biased population sex ratios, of the order of 100 females per male, in Independent (then Western) Samoa in 1927. He stated ". . . the male is very rare, much less than one percent." He further noted that "the rarity of the male is so marked as to lead inevitably to a suspicion that parthenogenesis must occur in this race," and that the bias recapitulated that found by Rechinger in 1905.

We used this record to test whether selection has produced a rapid return of maladaptive sex ratios toward a 1:1 sex ratio in nature, as has been seen in laboratory experiments. Our first aim was to examine whether the sex-ratio bias was present today as in 1927 and 1905. To do this, we examined the proximate cause of the sex-ratio bias at the individual level, specifically examining the role of two factors, the occurrence of parthenogenesis (as conjectured by Hopkins) and the presence of a male-killing *Wolbachia* infection (as occurs in Fiji *H. bolina*; ref. 14), as causes of the sex-ratio bias. We also examined the effect of the bias at a population level. We investigated the potential effects of the

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Table 1. Sex ratios produced by females collected on Independent Samoa, with embryonic survival of eggs laid and infection status with B-group *Wolbachia*

Class	No. of crosses	Sex ratio, male, female	Fertilized eggs that hatch, %	B group *Wolbachia*
All female	61	No males	57 (52–61)	Yes
Female biased	2	2, 12	48 (29)	Yes
		2, 14	73 (26)	Yes
Normal	1	22, 22	100 (48)	No

Hatch rate data for ''all female'' class represents median, with interquartile range in parentheses. For others, the figure is for egg hatch rate from an individual female, with numbers of eggs in parentheses. Survival rate of eggs is given as the proportion of viable eggs (i.e., those that showed signs of development) that hatched.

sex-ratio bias on the intrinsic rate of increase of the species, mediated via reductions in mate availability.

Our results indicated that the extreme bias had persisted, despite 400 generations of selection for resistance, and that the bias was not associated with parthenogenesis but with a high prevalence of male-killing *Wolbachia* infection. Comparison of this island with others with lower prevalence of infection led us to conclude that the low frequency of males was leading to a 57% loss of reproductive output. However, the population clearly remains viable.

Methods

Population Sex-Ratio Bias in Independent Samoa. Population sex-ratio bias was assessed by walking census on Upolu Island, Samoa, during July 2001. The assessment took place on several afternoons over a 2-week period. On each occasion, a walk was made across human-disturbed ground on the edge of settlements, and the sex of butterflies observed was recorded. Sites were assessed once. Males and females could easily be distinguished on the basis of different wing color patterns.

Individual Sex-Ratio Bias Variation in Independent Samoa. Individual females were caught from a fallow garden in Apia, Upolu Island, during July 2000 and July 2001, presented with cuttings of *Sida rhombifolia*, and allowed to oviposit. Egg clutches were collected, and egg hatch rates were recorded for the first 50 eggs laid by an individual female. Eggs were classified thusly: those that hatch successfully, those that do not hatch but show development (a gray embryo is seen through the chorion), and those that do not show signs of development/fertilization. Larvae were reared through to adulthood on *Sida rhombifolia* leaves, and the F_1 sex ratio was recorded. Individual parental females were preserved in 95% ethanol, and F_1 females were returned to the field.

Cause of Variation in Sex-Ratio Bias Between Individuals. The role of parthenogenesis in the origin of the sex-ratio bias was examined by assessing the mating status of the females bred above. The dead females were dissected and scored for the presence or absence of a spermatophore within the abdomen. This score was then related to their ability to produce fertile eggs.

The role of male-killing *Wolbachia* infection in the sex-ratio bias was in the first place assessed by PCR assay. Sections from the abdomen of specimens above were taken and dried of alcohol, and tissues were macerated in 5% Chelex 100 (Bio-Rad) in the presence of 7 mM DTT and proteinase K. This macerate was incubated overnight at 37°C before being boiled to inactivate the proteinase K. It was then used as a template in a PCR assay for B-group *Wolbachia*, using the primer pair 81f/522r, which amplify all B-group *Wolbachia*, of which the male-killer bacteria of *H. bolina* is one (16). The quality of template giving no band was confirmed by a PCR based on the *COI* gene of the host mtDNA (17). Infection status was correlated with egg hatch rate and sex ratio produced to indicate the role of the male-killer bacteria in the production of all female or female-biased broods. The similarity of this *Wolbachia* strain to that previously found to kill males in Fiji was assessed through sequencing of the *wsp* and *fts*Z genes. In brief, these genes were amplified from four individuals by using the primer pairs 81f/691r (for *wsp*) and ftsZf/ftsZr (for *fts*Z) (16, 18). The PCR products were cleansed of nucleotides and unincorporated primers by using Amicon PCR purification microcolumns and then directly sequenced through both strands by using the original PCR primers. The sequence was then compared with the known *wsp* and *fts*Z sequences of the *Wolbachia* male-killer bacteria from Fiji.

Parasite Prevalence and Fertility of Female Butterflies from Independent Samoa Compared with Other Islands. Females from Independent Samoa were collected and dissected to produce a matedness rate across the island. The prevalence of the male-killing *Wolbachia* infection was ascertained by using the B-group-specific PCR above. A similar sample was collected and analyzed from American Samoa (100 km from Independent Samoa), where the male-killing infection does not exist. Egg hatch rates were also obtained for wild-collected females from American Samoa to allow fertility as well matedness to be compared between islands. Data were also compared with existing data from Fijian *H. bolina*.

To assess the role of male biology (as opposed to female biology or infection status) in fertility differences between islands, spermatophores were dissected out of females and measured, and the diameter of spermatophores from Independent Samoa, American Samoa, and Fiji was compared. To further investigate the role of the male in fertility reduction, Independent Samoan virgin females raised within the laboratory were crossed to males from either Fiji or American Samoa, and female fertility was recorded over the next 50 eggs laid. A single virgin male from Independent Samoa was also crossed to a virgin Independent Samoan female, and fertility was recorded.

Results

The population sex ratio, as measured by walking census in Independent Samoa was 1.1% male ($n = 368$). This contrasts with the population sex ratio measured by walking census on other Polynesian islands of 70–80% male (there is generally a bias to seeing males by virtue of their territorial defense of nectaring plants by the wayside). Breeding of female butterflies taken from the natural population revealed that the cause of the population sex-ratio bias on Independent Samoa was the production of all-female broods (Table 1). Of 64 females successfully bred, only one gave rise to a normal 1:1 sex ratio. Of the remainder, males were seen in broods from only two females, where they comprised 15% of the progeny. Significantly, only females that were mated, as determined post hoc by dissection for the presence of a spermatophore, produced viable eggs, making parthenogenesis an inadequate explanation for the data.

The cause of the female bias produced by individual females in Independent Samoa was identical to that observed in a previous study examining female-biased sex ratios produced by

Table 2. The matedness and fertility of field-collected females from Independent Samoa compared with Fiji and American Samoa, and size of spermatophores transferred to the female, along with similar data for laboratory crosses

	Prevalence of male-killing *Wolbachia*, % (*n*)	Mated in field, % (*n*)	Median fertile eggs laid by mated individuals, %	*n*	Mean spermatophore diameter, mm (95% C.I.)	*n*
Females from field						
Independent Samoa	99.2 (257)	54 (214)	72.9 (65–80.8)	88	1.14 (1.09–1.21)	28
Fiji	53.9 (76)	93.8 (130)	97 (92.6–100)	21	2.04 (1.92–2.03)	11
American Samoa	0 (29)	96.5 (29)	98 (96.9–100)	2	1.95 (1.71–2.18)	10
Independent Samoa female ×						
Independent Samoa male	N/A	N/A	68.3	1	1.09	1
Fiji male	N/A	N/A	99 (97–100)	14	2.02 (1.92–2.11)	14
American Samoa male	N/A	N/A	98 (98–98)	7	1.96 (1.71–2.18)	7

Fertility is given as percentage of eggs laid showing some sign of development, with interquartile range in parentheses.

certain *H. bolina* females from Fiji. In Fiji, around half of the females produce all-female broods associated with a *Wolbachia* infection that kills males during embryogenesis. All 63 Independent Samoan females producing female-biased clutches exhibited ≈50% embryonic mortality and were infected with *Wolbachia* bacteria (Table 1). This *Wolbachia* strain was identical over 557 bp of *wsp* and 945 bp of *ftsZ* sequence to that observed in Fiji (GenBank accession nos. AJ307075 and AJ307076) in the four individuals sequenced. The one Independent Samoan female that produced a normal sex ratio had no embryonic mortality and was uninfected with *Wolbachia* (Table 1).

An assay for the presence of the male-killing *Wolbachia* bacteria across wild-collected females revealed that 99.2% of female individuals were infected ($n = 257$; 95% C.I. on prevalence, 97.2–99.9%). This figure is consistent with the observed population sex ratio of 1.1% male (from parasite prevalence and data on sex ratios produced: 0.8% of females are uninfected and produce a 1:1 sex ratio, 3.1% are infected and produce 15% males, and 96.1% produce no males). Three males also collected from the wild were tested for *Wolbachia* presence. Two of these males were uninfected with *Wolbachia*, and one was infected. This observation is consistent with the breeding results: males can derive occasionally from infected females when male killing fails but also come from the rare uninfected females in the population.

We then examined whether the sex-ratio bias affects the reproductive biology of the population by comparing the Independent Samoan population with those of American Samoa and Fiji (where 0% and 50% of females, respectively, bear the male-killing *Wolbachia* infection). We scored three aspects of reproductive biology: the rate at which females were mated, the fertility of mated females, and the size of spermatophore produced by males.

Independent Samoan females were less likely to have mated ($\chi^2 = 66.5$, df = 2, $P < 0.0001$) and fertilized fewer eggs when mated (Kruskal–Wallis test: $\chi^2 = 44.2$, df = 2, $P < 0.0001$), than females from American Samoa or Fiji (Table 2). Laboratory crosses in which Independent Samoan females showed full fertility when mated to males from outside the country indicate the role of the male in the reduction in fertilization rate within the wild Independent Samoan population (Table 2) (difference between laboratory-mated Independent Samoan females and naturally mated Fijian and American Samoan females: $\chi^2 = 1.54$, df = 1, not significant). Overall, there is an increase in unmatedness from 6% to 46% from Fiji/American Samoa to Independent Samoa and a decrease in the fertility of mated females from 97% egg fertilization to 73%. These data mean that the reproductive output of female *H. bolina* from Independent Samoa is 57% of that found on neighboring islands, measured in terms of females producing eggs that hatch into females.

The low fertility of mated female butterflies on Independent Samoa correlates with the production of smaller spermatophores by Independent Samoan males. Females from Independent Samoa contained much smaller spermatophores than comparators from Fiji and American Samoa ($F_{2,46} = 112$, $P < 0.001$), and spermatophores from Fiji and American Samoa did not differ in size ($P = 0.44$) (Table 2). Because Independent Samoan females mated to males from Fiji/American Samoa in the laboratory contained spermatophores that did not differ in size from those found in wild mated Fijian/American Samoan females ($F_{3,38} = 0.36$, $P = 0.78$), we conclude that this, like the reduced fertility, is a product of the male butterfly rather than the female (Table 2).

Discussion

This study has demonstrated that autosomal genes do not necessarily prevail in conflicts between sex-ratio distorters and their hosts over population genetic time scales. In our system, 100 years (≈400 generations) have passed since the first observation of extreme sex-ratio bias, and the population today is found at the same extreme sex ratio despite intense selection on the host to prevent the action or transmission of the parasite. Either the host has failed to evolve resistance at all over this period or host defense has arisen but been successfully (and nearly completely) countered. The sex-ratio bias arises from >99% of females being infected with a male-killing *Wolbachia* bacteria, making this infection the single most important factor shaping the reproductive (and indeed population) biology in this butterfly population.

This study contrasts with previous laboratory selection experiments on *Drosophila*. When sex-ratio drive was released at high frequency in *Drosophila* population cages, resistance to X chromosome drive evolved relatively rapidly, returning the population to a 1:1 sex ratio within 400 generations (5). Therefore, it is important to ask whether the data on *H. bolina* represents a special case deriving from the fact that they come from an island population. Island populations may have lower levels of standing genetic variation because of bottleneck events and small standing population size. Additionally, restricted population size will affect the time taken for a new mutation to arise and spread. Thus, the island nature of the species may have slowed down the host response to this parasite, leading to the situation observed where the parasite is clearly "on top."

Could this, therefore, be a unique curiosity? We would argue that it is not. In the first place, many insects do exist in "habitat islands" that restrict population number. Thus, although our conclusion may not be true of some species, it probably does reflect a wide variety of systems. Further, it should also be noted that although our study is unique in its combination of extreme bias and historical data, other studies have also failed to find resistance to selfish genetic elements in natural populations

(19–22). Most pertinently, there is the case of sex-ratio meiotic drive in *Drosophila pseudoobscura*. Records indicate that the driving X chromosome in this species has been at a stable equilibrium frequency for many generations, with no evidence for host repression of the trait in natural populations (19, 23). Indeed, molecular evidence suggests an origin of this element 25,000 years in the past (24). Collectively, these findings indicate that sex-ratio distorters can be maintained unrepressed over population genetic time scales in a variety of species, and our study indicates that they can do this even when at high prevalence.

The survival of this population despite the extreme sex-ratio bias also warrants discussion. Selfish genetic elements that distort the sex ratio have been suggested as agents that may cause host extinction (2, 25). The only parallel system for contrast is the decline of the saiga antelope, *Saiga tartarica.* Here, male biases in hunting patterns have resulted in severe female-biased population sex ratios (26) that are associated with the decline of the species, and with decline beginning when the population exceeded 97.5% females. In the *H. bolina* system, where the adult population is 99% female, we estimated the loss in female fertility due to the lack of adult males to be ≈57%. However, female *H. bolina*, like other colonizing butterflies, is very likely to have a high intrinsic rate of increase, with $R_0 > 3$ in the absence of sex-ratio constraints (27, 28). The level of fertility loss in our system would not, therefore, translate into a reduction below that required for population persistence. This robustness is in contrast with the situation in the saiga antelope, where limitations on fecundity make R_0 likely to be much closer to unity, and thus loss of fertility in female-biased populations causes decline.

The final feature of this system that bears comment is the difference in the size of the spermatophores transferred by male *H. bolina* in the different islands. The spermatophores produced by Independent Samoan *H. bolina* males are around half the diameter of spermatophores produced by *H. bolina* males in American Samoa and Fiji. This difference appears unlikely to be due to "tiredness" on the part of Independent Samoan males after multiple mating, because the largest spermatophore found in Independent Samoa was considerably smaller than the smallest spermatophore observed in Fiji/American Samoa, and the one virgin *H. bolina* male from Independent Samoa that mated produced a spermatophore with the same size as the population mean on first mating. Rather, this may represent adaptation to the continuing sex-ratio bias in the form of ejaculate partitioning. If so, this would represent an intriguing compensatory change that may have ameliorated the effect of lack of males at the population level. The effect of the parasite on the evolution of the host certainly bears more investigation.

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1. Emlen, S. T. & Oring, L. W. (1977) *Science* **197,** 215–223.
2. Hamilton, W. D. (1967) *Science* **156,** 477–488.
3. Cosmides, L. & Tooby, J. (1981) *J. Theor. Biol.* **89,** 83–129.
4. Hurst, G. D. D. & Werren, J. H. (2001) *Nat. Rev. Genet.* **2,** 597–606.
5. Carvalho, A. B., Sampaio, M. C., Varandas, F. R. & Klazco, L. B. (1998) *Genetics* **148,** 719–731.
6. Carvalho, A. B. & Klazcko, L. B. (1993) *Heredity* **71,** 546–551.
7. Rigaud, T. & Juchault, P. (1992) *Heredity* **68,** 47–52.
8. Rigaud, T. & Juchault, P. (1993) *Genetics* **133,** 247–252.
9. Merçot, H., Atlan, A., Jacques, M. & Montchamp-Moreau, C. (1995) *J. Evol. Biol.* **8,** 283–300.
10. Leigh, E. G. (1971) *Adaptation and Diversity* (Freeman, San Francisco).
11. Carvalho, A. B. & Vaz, S. C. (1999) *Heredity* **83,** 221–228.
12. Hurst, G. D. D., Jiggins, F. M. & Majerus, M. E. N. (2003) in *Insect Symbiosis*, eds. Bourtzis, K. & Miller, T. A. (CRC, Boca Raton, FL), pp. 177–197.
13. Simmonds, H. W. (1923) *Proc. R. Entomol. Soc. London* **1923,** ix–xii.
14. Dyson, E. M., Kamath, M. K. & Hurst, G. D. D. (2002) *Heredity* **88,** 166–171.
15. Hopkins, G. H. E. (1927) *Insects of Samoa and Other Samoan Terrestrial Arthropoda* (Brit. Mus. Nat. Hist., London), Vol. 3.
16. Zhou, W., Rousset, F. & O'Neill, S. (1998) *Proc. R. Soc. London Ser. B* **265,** 509–515.
17. Brunton, C. F. A. & Hurst, G. D. D. (1998) *Biol. J. Linn. Soc.* **63,** 69–79.
18. Werren, J. H., Zhang, W. & Guo, L. R. (1995) *Proc. R. Soc. London Ser. B* **261,** 55–71.
19. Policansky, D. & Dempsey, B. (1978) *Evolution (Lawrence, Kans.)* **32,** 922–924.
20. James, A. C. & Jaenike, J. (1990) *Genetics* **126,** 651–656.
21. Hurst, G. D. D., Jiggins, F. M. & Robinson, S. J. W. (2001) *Heredity* **86,** 161–166.
22. Jiggins, F. M., Randerson, J. P., Hurst, G. D. D. & Majerus, M. E. N. (2002) *Evolution (Lawrence, Kans.)* **56,** 2290–2295.
23. Kovacevic, M. & Schaeffer, S. W. (2000) *Genetics* **156,** 155–172.
24. Dobzhansky, T. (1958) *Evolution (Lawrence, Kans.)* **12,** 385–401.
25. Hatcher, M. J., Taneyhill, D. E., Dunn, A. M. & Tofts, C. (1999) *Theor. Popul. Biol.* **56,** 11–28.
26. Milner-Gulland, E. J., Bukreeva, O. M., Coulson, T., Lushchekina, A. A., Kholodova, M. V., Bekenov, A. B. & Grachev, I. A. (2003) *Nature* **422,** 135.
27. Thomas, C. D. & Janski, I. A. (1997) in *Metapopulation Biology: Ecology and Genetics and Evolution*, eds. Hanski, I. A. & Gilpin, M. E. (Academic, London), pp. 359–386.
28. Thomas, C. D., Jordano, D., Lewis, O. T., Hill, J. K., Sutcliffe, O. L. & Thomas, J. L. (1998) in *Conservation in a Changing World*, eds. Mace, G. M., Balmford, A. & Ginsberg, J. R. (Cambridge Univ. Press, Cambridge, U.K.), pp. 107–138.