Sex-ratio-distorting *Wolbachia* causes sex-role reversal in its butterfly host

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Sex-role-reversed mating systems in which females compete for males and males may be choosy are usually associated with males investing more than females in offspring. We report that sex-role reversal may also be caused by selfish genetic elements which distort the sex ratio towards females. Some populations of the butterflies *Acraea encedon* and *Acraea encedana* are extremely female biased because over 90% of females are infected with a *Wolbachia* bacterium that is maternally inherited and kills male embryos. Many females in these populations are virgins suggesting that their reproductive success may be limited by access to males. These females form lekking swarms at landmarks in which females exhibit behaviours which we interpret as functioning to solicit matings from males. The hypothesis that female *A. encedon* swarm in order to mate is supported by the finding that, in release–recapture experiments, mated females tend to leave the swarm while unmated females remained. This behaviour is a sex-role-reversed form of a common mating system in insects in which males form lekking swarms at landmarks and compete for females. Female lekking swarms are absent from less female-biased populations and here the butterflies are instead associated with resources in the form of the larval food plant.

Keywords: *Wolbachia*; male-killers; sex-role reversal; sexual selection; selfish genetic elements; female lekking swarms

1. INTRODUCTION

Males typically have the ability to reproduce faster than females, with the result that males compete for access to females while females often choose between males. As Bateman (1948) famously demonstrated in *Drosophila melanogaster*, this asymmetry means that male and female fitness is determined by different factors: for males the lifetime number of matings is most important, while for females access to resources is the primary determinant of fitness. This is thought to explain the mating systems of many animals. For example, in a comparative analysis of mammals, Clutton-Brock (1989) argued that the pattern of resource distribution determines distribution of females, which in turn determines the male strategy to monopolize mating access to these females (e.g. guarding territories). Further, experimental work on voles (Ims 1988) showed that male distribution alters in response to that of females but not vice versa.

Factors which alter the strength or direction of competition for mates between the sexes are, therefore, expected to alter the mating behaviour and mating system of that species or population. This is seen in species where males invest more than females in parental investment and hence have the slower potential rate of reproduction (Trivers 1972; Clutton-Brock & Vincent 1991). For instance, male dance flies of the species *Empis borealis* provide females with a nuptial gift of a prey item on mating and this probably represents a greater investment in reproduction than is contributed by females. The females form swarms at landmarks which are then visited by males who then choose to mate with the largest females (Svensson & Petersson 1988). This contrasts with a common mating system in insects where it is males who

form lekking swarms and females who may choose between males (Sivinski & Petersson 1997).

Biases in the sex ratio of a population might similarly be expected to alter which sex competes for mates (Emlen & Oring 1977). One of the causes of alterations in sex ratio is sex-ratio distorters, genes which are predominantly or entirely inherited through one sex and distort the sex ratio towards that sex. In this paper we investigate the effects of one such selfish genetic element, a male-killing bacterium, on the sex ratio and mating behaviour of a butterfly. These bacteria reduce the proportion of males in a population and are thus expected to reduce both the intensity of male–male competition and the opportunity for female choice between males.

Male-killers are maternally inherited bacteria which kill males during their early development. Selection favours the spread of these parasites because the death of males benefits female siblings, either by eliminating antagonistic sibling interactions such as competition or by providing resources to the females who cannibalize their dead brothers (Hurst & Majerus 1993). It is these females which will pass the bacterium to the next generation.

The main focus of this study is the butterfly *Acraea encedon* (Lepidoptera: Nymphalidae), which is host to a male-killing *Wolbachia* bacterium (Jiggins *et al.* 1998; Hurst *et al.* 1999). We aimed to investigate the prevalence of this male-killer and its consequences for the sexual behaviour and mating system of the butterfly. Previous studies have shown that the proportion of females which produce only daughters can be extremely high, from 61% in Ghana (95% confidence interval: 59–80%) up to 95% in Sierra Leone (95% confidence interval: 86–99%) (Chanter & Owen 1972; Gordon 1982). Owen observed highly female-biased population sex ratios in Uganda and found that the majority of females were virgins (Owen &

Table 1. *The location of sites sampled and the butterflies' sex ratio, behaviour and bacterial prevalence*

(*Commelina* is a larval food plant of *A. encedon*. The sex ratios and prevalences of samples collected from the same population on different dates have been summed when neither the sex ratio nor behaviour changed through time.)

location (date)	behaviour (see text)	habitat	proportion females infected (sample size)	sex ratio (proportion male) (sample size)
Kagolomolo	food plant	*Commelina* in farmland	0.81 (70)	0.34 (169)
Entebbe (7–17/4/98)	food plant	*Commelina* beside Lake Victoria 75 m from lekking site described below	0.79 (47)	0.46 (84)
Nalugala	food plant	*Commelina* in farmland 50 m from lekking site described below	0.86 (7)	0.47 (15)
Kibuyi	food plant	*Commelina* in farmland	0.78 (27)	0.50 (56)
Gangu	lekking swarm	lawn next to tall trees on hilltop	0.87 (23)	0.00 (25)
Kajansii	lekking swarm	short grass in forest clearing on hilltop	1.00 (9)	0.00 (9)
Kawanda	lekking swarm	grassy hilltop with small trees	0.70 (20)	0.00 (19)
Makerere	lekking swarm	lawn beside trees on hilltop	0.93 (215)	0.01 (2139)
Entebbe (22/3/98)	lekking swarm	short vegetation surrounded by tall trees; halfway up hillside	1.00 (50)	0.02 (48)
Entebbe (7–9/7/98)	lekking swarm	see Entebbe 22/3/98	0.96 (52)	0.02 (142)
Luya	lekking swarm	lawn beside trees on hilltop	0.92 (38)	0.02 (525)
Nalugala (7/4/98)	lekking swarm	lawn beside tall trees next to a swamp	0.89 (9)	0.00 (10)
Nalugala (27/7/98)	lekking swarm	see Nalugala 7/4/98	0.89 (9)	0.04 (24)
Lake Mburu	lekking swarm	lawn beside trees on top of ridge	0.97 (30)	0.06 (34)
Kajansii	lekking swarm	grassy pasture with *Eucalyptus* trees beside swamp	0.92 (51)	0.12 (308)

Chanter 1969; Owen *et al*. 1973); however, it is difficult to interpret the large body of data they collected since it was later realized that they were actually studying two sibling species, *A. encedon* and *Acraea encedana* (Owen *et al*. 1994). Ugandan populations of *A. encedana* have subsequently been found to be infected with the same strain of male-killing bacterium at a similarly high prevalence with 95% of females infected (95% confidence interval: 90–98%) (Jiggins *et al*. 2000).

2. MATERIAL, METHODS AND RESULTS

(a) *Sex ratio and* Wolbachia *prevalence*

Butterflies were sampled from sites in southern Uganda between February and July 1998 with the aim of recording temporal and spatial variation in male-killer prevalence and population sex ratio. The study sites listed in table 1 are all in the vicinity of Kampala, with the exception of Lake Mburu, which is 200 km to the west. Some sites were sampled at regular intervals while others were visited opportunistically on one or more occasions. *A. encedon* are often found in high density 'colonies' with few butterflies encountered outside these areas. This distribution and the slow flight of the species enabled us to catch most of the butterflies flying, as by the end of a day collecting there were usually few butterflies remaining.

The sex ratio of butterflies caught at different sites varied from 50 to 100% females (table 1) despite the proximity of the sites (with the exception of Lake Mburu, none were more than 22 km apart). These results should be treated with caution because behavioural differences commonly make field samples of butterflies sex biased, usually towards males.

Wolbachia-specific polymerase chain reactions (PCR) were used to test females for infection with the male-killer in order to

estimate its prevalence in different populations. DNA was extracted from the ovaries of female butterflies using a method modified from Walsh *et al*. (1991). A small amount of ovary tissue was incubated at 56 °C with Chelex 100^{TM} ion exchange resin (Bio-Rad, Hercules, CA), dithiothreitol and proteinase-K, boiled and the supernatant then used directly for PCR. The presence of *Wolbachia* was assayed using the PCR primers *wsp*81F and *wsp*691R, which amplify the bacterium's *wsp* gene (Zhou *et al*. 1998). The extractions which tested negative for *Wolbachia* were then tested using primers that amplify the ITS1 region of insects DNA (Hillis & Dixon 1991) to check that the DNA extraction had been successful. To confirm that this procedure reliably detects the male-killer infection, a number of females reared from all-female and normal sex-ratio broods were stored, dissected and tested for the bacterium alongside the wild-collected females.

The results of this screen are given in table 1. The DNA extractions which tested negative for *Wolbachia* all amplified using insect PCR. Seven females from known male-killer lines and five females from lines of normal sex ratio were all correctly identified by this procedure.

(b) *Correlates between butterfly behaviour, population sex ratio and bacterial prevalence*

The behaviour of the butterflies in different populations fell into two distinct categories on the basis of resource availability (table 2) and these correlated with the sex ratio and bacterial prevalence of the population.

The first type of site lacked larval food plants and usually contained no adult nectaring plants. Despite being devoid of resources, these sites always consisted of short vegetation next to trees, although the plant species present differed between sites. The preference of the butterflies for trees was supported by an

Table 2. *A comparison of the habitat and behaviour of butterflies at food-plant-based and resource-lacking aggregations*

site without resources ('lekking')	sites with resources ('food plant')
no larval food plant or adult nectaring plants	larval food plant *C. benghalensis* always common
sometimes located at hilltops and always on short vegetation	no distinguishing features common to all sites
butterflies present only in the afternoon	butterflies present from the first sun in the morning until evening
females patrol around small arena; when another female is encountered they engage in brief mid-air chase and often land on the ground; aggregation of females on tall grass stalks	unusual female behaviours absent
males rarely observed but when found they were often mating	mating pairs common
0–12% butterflies male	34–50% butterflies male
higher male-killer prevalence	lower male-killer prevalence

unintended experiment at Kajansii, where on three occasions felling of trees caused the butterflies to move to a position beside new trees, until, when all the trees had been removed, the butterflies disappeared. The aggregations were also commonly located on hilltops (table 1); however, it should be noted that our results are strongly biased towards hilltop sites as these are most easily located. Anywhere between two and 351 female butterflies could be found at these sites within a small arena typically measuring $10 \text{ m} \times 20 \text{ m}$. In the late afternoon, butterflies perched in tight aggregations, usually of two to 12 butterflies, on grass stalks or similar vegetation. Owen & Chanter (1969) have previously described these behaviours and provide detailed descriptions of both the habitat and behaviour of the butterflies.

The behaviour of butterflies at the second type of site was strikingly different (table 2). These sites were based around areas containing large amounts of *Commelina benghalensis*, a larval food plant of *A. encedon* which often grows in cleared fields. The butterflies were loosely associated with the food plant in an area which was larger and not so discretely demarcated as the resource-lacking arenas described above. The female butterflies exhibited none of the unusual behaviours associated with the resource-lacking arenas.

Populations where the butterflies aggregated on resource-lacking sites had both a higher bacterial prevalences (Mann–Whitney U-test: $n_1 = 11$, $n_2 = 4$, $U = 40$; $p < 0.05$) and more female-biased adult sex ratios (Mann–Whitney U-test: $n_1 = 11$, $n_2 = 4$, $U = 44$; $p < 0.01$) than the populations exhibiting the 'food-plant' behaviour (table 1). Two populations showed changes in behaviour that were associated with changes in sex ratio. At Entebbe the bacterial prevalence was initially high and the females aggregated on a resource-lacking site. The bacterial prevalence then dropped and the number of males caught increased. This change in sex ratio was associated with a behavioural change; the resource-lacking site was now deserted but the butterflies were now associated with a patch of food plant approximately 75 m away. Bacterial prevalence then increased and the butterflies once again swarmed at the resource-lacking site and were scarce around the food plant. Similar changes were observed in a smaller population at Nalugala (table 1).

(c) *Why do females aggregate?*

The hypothesis that females aggregate on resource-lacking sites in order to mate was tested by release–recapture of virgin and mated females. Captive-reared females were numbered and either put into a cage with males present or lacking males. Siblings from the same brood were split in equal numbers between the two treatments. These cages were checked regularly

through the afternoon to record which butterflies mated and the following morning the virgin and mated females were released at the Makerere hilltop site. The butterflies were then recaptured on the hilltop during the afternoons of the subsequent one to six days. Virgins showed a higher recapture rate than mated females; 48% (67 out of 141) of virgins were recaptured compared with 17% (12 out of 72) of mated females ($\chi^2 = 9.74$, d.f. = 1; $p < 0.005$). This supports the hypothesis that females swarm in order to mate.

Behavioural observations at these aggregations also support the hypothesis that females were soliciting matings. Captive-reared males which were released in these female aggregations in order to observe their behaviour quickly mated, apparently with the first female encountered. The mating behaviour was typical of the genus *Acraea*, the male grapples the female in mid-air and the pair fall to the ground. The male then clambers onto the underside of the female's hind wing, both butterflies curl their abdomens ventrally outwards and they mate.

This behaviour of females towards males resembles some aspects of the behaviour between females in these aggregations. Females land on top of other females when they are resting on the ground and tend to hold their abdomens curled ventrally outwards in a manner similar to that observed during mating. This is a typical mate-acceptance behaviour in butterflies usually only seen in male–female interactions.

(d) *The mating frequency of infected and uninfected butterflies*

Wild female butterflies collected over two days from the Makerere hilltop aggregation were dissected and examined for the presence of a spermatophore to determine whether or not they had mated. To check that the dissections were reliable, control dissections were carried out on mated and virgin captive-reared females. All dissections were conducted without knowledge of whether the butterflies were infected and, in the case of the controls, whether they had mated. These butterflies were then tested for the presence of *Wolbachia* to determine whether the infected and uninfected butterflies were equally likely to have mated.

Eleven mated and two virgin control females were all correctly identified. Of the butterflies collected at Makerere, 94% (203 out of 215) were virgins (note that, given the behaviour described above, this is not a random sample of mated and virgin butterflies from the population). Uninfected females were more likely to have mated than infected females (four out of 14 uninfecteds had mated compared with eight out of 201 infecteds, Fisher's exact test: $p < 0.01$).

(e) *Behaviour of* A. encedana

The butterfly *A. encedana*, which is closely related to *A. encedon*, is infected by the same strain of male-killing bacterium (Jiggins *et al.* 2000). In the Kampala region, 95% are infected with the male-killer and only 6% of wild-caught butterflies were male (Jiggins *et al.* 2000). These butterflies aggregated at the same site as *A. encedon* at Kajansii and exhibited virtually identical 'unusual' behaviours. Within the grassy arena (approximately $20\,\text{m} \times 10\,\text{m}$) the species mixed but *A. encedon* was noticeably more common at one end of the site and *A. encedana* at the other end. Similarly, when the females aggregated on vegetation the two species rarely mixed.

3. DISCUSSION

In this study, we observed strongly female-biased sex ratios in certain populations which were associated with high prevalences of a male-killing bacterium. Two types of population were observed. First, there were strongly female-biased populations, in which swarms of females formed in small arenas lacking both larval food plants and adult nectaring plants but characterized by the presence of certain landmarks. Second, there are populations containing more males, where the butterflies were associated with the larval food plant. Here, up to 15% of butterflies were in mating pairs, suggesting that females probably mate on emergence from their pupae. The sex ratio of two populations was observed to change through time, and these changes correlated with switches between the two behavioural strategies.

The strongly female-biased sex ratios we observed are likely to cause female reproduction to be sperm limited. *A. encedon* takes a full day to mate and butterflies which feed only on nectar have a limited life span; under our unnatural rearing conditions males rarely lived longer than a fortnight, although they may live longer in the wild. Taken together, these figures suggest that many females will never mate in the most female-biased populations. This is supported both by the large numbers of virgin females in these populations and Owen & Chanters' (1969) observation that, in a mixed *A. encedon* and *A. encedana* population, an increase in the proportion of males was followed one generation later by an increase in the population size. We would therefore expect females in these populations to actively search and compete for males, whilst in populations where males are abundant, it is males which are expected to compete for mates.

Release–recapture experiments demonstrated that virgin females are more likely than mated females to remain in the swarms on resource-lacking sites, indicating that the function of this behaviour is to acquire mates. Behavioural observations also support the hypothesis that these females are attempting to solicit matings, females not only mating immediately with males released in the swarms but also appearing to adopt a mate-acceptance posture when interacting with other females.

We therefore conclude that high prevalences of the male-killing bacterium cause these butterflies to form sex-role-reversed lekking swarms. Ideally future research should confirm this conclusion by experimentally altering the sex ratio and looking for changes in the mating system. While male lekking swarms are widespread among insects, female lekking has previously been recorded in only two species, one insect, the dance fly *Empis borealis* (Svensson & Petersson 1988) and one bird, the Eurasian dotterel *Eudromias morinellus* where female leks are ground based (Owens *et al.* 1994). The cause of sex-role reversal in both these cases is male parental investment rather than a distorted population sex ratio.

The lekking behaviour of birds and some insects is thought to enable females to choose between males. Could the leks of *A. encedon* allow males to choose between females? There is strong selection on males to mate with uninfected females as they will produce sons who will have a reproductive success approximately 20 times greater than daughters. There is some tantalizing evidence that, within swarm sites, males do choose uninfected females as uninfected females are more likely than infected females to have mated.

However, there are two reasons to caution against firm acceptance of this conclusion. First, in straight behavioural terms, the males do not appear to exhibit any choice behaviour; there is no elaborate courtship or assessment. Second, there are alternative explanations for the association between females having mated and being uninfected. Both brother–sister mating (which will be confined to uninfected females, as infected females have no brothers) and the presence of females migrating from low prevalence populations to high, could also explain the association between infection and virginity. It is therefore interesting to speculate that other changes to the mating system, such as mating in the natal population and sibling mating, could evolve as male behaviours to choose uninfected females, even if these behaviours carry costs such as inbreeding depression.

Whatever the means by which the uninfected females gain a mating advantage (male choice, inbreeding or migration) it is clear that it may act as an important stabilizing feature within the dynamics of the male-killing bacteria. It will result in frequency-dependent selection against infected females because uninfected females will only benefit from having a mating advantage when males are scarce (i.e. the prevalence of the male-killer is high). The mating advantage of uninfected females may therefore prevent the infection spreading to fixation and driving the population extinct (similar scenarios have been modelled for cytoplasmic male sterility in plants, selfing was considered by Lloyd (1975) and geographical variation in prevalence by McCauley & Taylor (1997)). That is to say, not only has the presence of the male-killing bacterium altered the mating system, but changes in the mating system have altered the dynamics of the bacterium.

In summary, we conclude that there is strong evidence that the butterfly *A. encedon* forms female lekking swarms, a sex-role-reversed mating system, in response to high prevalences of a male-killing bacterium. Sex-ratio distorters may be common in many arthropods and have far reaching consequences for the evolution of their host organisms. Coevolution between such genetic parasites and their hosts is thought to have led to female stalk-eyed flies choosing males which carry genes which suppress sex chromosome meiotic drive (Wilkinson *et al.* 1998). In this paper we have extended this observation by demonstrating that a selfish genetic element is associated with changes in

the mating system itself. Future research should address both the causes of variation in parasite prevalence which we have observed and why the butterflies switch from a resource-based mating system to swarming as competition between females increases (male choice, mate location at landmarks, predator avoidance etc.).

REFERENCES

Bateman, A. J. 1948 Intra-sexual selection in *Drosophila*. *Heredity* **2**, 349–368.

Chanter, D. O. & Owen, D. F. 1972 The inheritance and population genetics of sex ratio in the butterfly *Acraea encedon*. *J. Zool.* **166**, 363–383.

Clutton-Brock, T. H. 1989 Mammalian mating systems. *Proc. R. Soc. Lond.* B **236**, 339–372.

Clutton-Brock, T. H. & Vincent, C. J. 1991 Sexual selection and the potential reproductive rates of males and females. *Nature* **351**, 58–60.

Emlen, S. T. & Oring, L. W. 1977 Ecology, sexual selection and the evolution of mating systems. *Science* **197**, 215–223.

Gordon, I. J. 1982 *Biology of* Danaus chrysippus *L.* (*Lepidoptera, Danaidae*) *and its mimics in Ghana*. University of Ghana.

Hillis, D. M. & Dixon, M. T. 1991 Ribosomal DNA: molecular evolution and phylogenetic inference. *Q. Rev. Biol.* **66**, 411–453.

Hurst, G. D. D. & Majerus, M. E. N. 1993 Why do maternally inherited microorganisms kill males? *Heredity* **71**, 81–95.

Hurst, G. D. D., Jiggins, F. M., Schulenberg, J. H. G., Bertrand, D., West, S. A., Goriacheva, I. I., Zakharov, I. A., Werren, J. H., Stouthamer, R. & Majerus, M. E. N. 1999 Male-killing *Wolbachia* in two species of insect. *Proc. R. Soc. Lond.* B **266**, 735–740.

Ims, R. A. 1988 Spatial clumping of sexually receptive females induces space sharing among male voles. *Nature* **335**, 541–543.

Jiggins, F. M., Hurst, G. D. D. & Majerus, M. E. N. 1998 Sex ratio distortion in *Acraea encedon* (Lepidoptera: Nymphalidae) is caused by a male-killing bacterium. *Heredity* **81**, 87–91.

Jiggins, F. M., Hurst, G. D. D., Dolman, C. E. & Majerus, M. E. N. 2000 High prevalence male-killing *Wolbachia* in the butterfly *Acraea encedana*. *J. Evol. Biol.* (In the press.)

Lloyd, D. G. 1975 The maintenance of gynodioecy and androdioecy in angiosperms. *Genetica* **45**, 325–339.

McCauley, D. E. & Taylor, D. R. 1997 Local population structure and sex ratio: evolution in gynodioecious plants. *Am. Nat.* **150**, 406–419.

Owen, D. F. & Chanter, D. O. 1969 Population biology of tropical African butterflies. Sex ratio and genetic variation in *Acraea encedon*. *J. Zool.* **157**, 345–374.

Owen, D. F., Owen, J. & Chanter, D. O. 1973 Low mating frequencies in an African butterfly. *Nature* **244**, 116–117.

Owen, D. F., Smith, D. A. S., Gordon, I. J. & Owiny, A. M. 1994 Polymorphic Mullerian mimicry in a group of African butterflies: a reassessment of the relationship between *Danaus chrysippus*, *Acraea encedon* and *Acraea encedana* (Lepidoptera: Nymphalidae). *J. Zool.* **232**, 93–108.

Owens, I. P. F., Burke, T. & Thompson, D. B. A. 1994 Extraordinary sex roles in the Eurasian dotterel: female mating arenas, female–female competition and female mate choice. *Am. Nat.* **144**, 76–100.

Sivinski, J. M. & Petersson, E. 1997 Mate choice and species isolation in swarming insects. In *Mating systems in insects and arachnids* (ed. J. C. Choe & B. J. Crespi), pp. 294–309. Cambridge University Press.

Svensson, B. G. & Petersson, E. 1988 Non-random mating in the dance fly *Empis borealis*: the importance of male choice. *Ethology* **79**, 307–316.

Trivers, R. L. 1972 Parental investment and sexual selection. In *Sexual selection and the descent of man, 1871–1971* (ed. B. Campbell), pp. 136–179. Chicago, IL: Aldine.

Walsh, P. S., Metzger, D. A. & Higuchi, R. 1991 Chelex 100 as a medium for simple extraction of DNA for PCR based typing of forensic material. *Biotechniques* **10**, 506–513.

Wilkinson, G., Presgraves, D. & Crymes, L. 1998 Male eye span in stalk-eyed flies indicates genetic quality by meiotic drive suppression. *Nature* **391**, 276–279.

Zhou, W. F., Rousset, F. & O'Neill, S. 1998 Phylogeny and PCR based classification of *Wolbachia* strains using *wsp* gene sequences. *Proc. R. Soc. Lond.* B **265**, 509–515.