

SHORT COMMUNICATION

## Female-biased sex ratio due to male-killing in the Japanese ladybird *Coccinula sinensis*

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### Introduction

Male-killing bacteria that kill hosts early in embryogenesis have been recorded from a diverse range of insect species, with some groups, such as coccinellid beetles, being particularly prone to male-killer invasion, because female offspring of infected mothers gain a resource advantage from consuming the soma of sibling male eggs. Majerus and Hurst (1997) stated that among the coccinellids, male-killing will be most common in species that are aphidophagous, indulge in sibling egg consumption, and lay eggs in batches. These criteria hold for all seven species of coccinellid in which male-killing has been reported (Lusis, 1947; Shull, 1948; Matsuka *et al.*, 1975; Nijjima & Nakajima, 1981; Hurst *et al.*, 1996, 1997, 1999; Majerus *et al.*, 1998) and one species, *Propylea japonica*, in which it has recently been found (M. Majerus and T. Majerus, pers. comm.). In respect of oviposition, the question arises as to what constitutes a batch. Some coccinellids, for example many members of the Chillocorini, lay just two or three eggs together. Others, such as *Coccinella septempunctata*, may lay over 100 eggs in a single batch (Majerus, 1994). In all coccinellids in which male-killing has been reported, mean egg batch size exceeds 10. High egg batch size may not only be a factor facilitating invasion of male-killing endosymbionts, but may also be an evolutionary consequence of such invasion (Hurst & McVean, 1998). This paper reports investigations of the coccinellid *Coccinula sinensis*, which generally lays smaller egg batches than any species of this family known to harbour male-killers, and reports that the species does exhibit a maternally inherited female-biased sex ratio with all the characteristics of a bacterial male-killer.

### Materials and methods

#### *Treatment of initial sample*

A sample of 55 *C. sinensis* was collected by eye and use of a beating tray, from low herbage alongside the Fuefuki River, near Kōfu City, Yamanashi Prefecture, Japan, on 1 September 1998. Mating pairs were observed in the field. Both unhatched and hatched egg batches were observed in the field and were counted with use of a hand lens. Eggs in hatched batches were designated as hatched, grey (fertile and due to hatch or dead), or yellow (infertile, male-killed, and/or cannibalised). The adult sample was transported to Cambridge, U.K., where it was fed on *Acyrtosiphon pisum* (Harris) and sexed under CO<sub>2</sub> anaesthetic by examination of the posterior abdominal sternites (males, but not females, have an obvious notch in the posterior margin of the last abdominal sternite). The beetles were kept under observation in stock Petri dishes, mated females being removed to set up individual matrilines. Eggs laid by these females were collected and counted over a 3-week period. Matriline parents and the rest of the stock were then fed on an artificial food (Majerus *et al.*, 1989) for 2 days and removed to a refrigerator. Once eggs had hatched and larvae had dispersed from egg clutches, egg hatch rates were assessed, egg remnants being classed as clear (hatched normally), grey (fertile but inviable for an unknown reason including possibly male-killed), or yellow (infertile or male-killed). Some neonate larvae were noted to eat unhatched eggs before dispersing from egg clutches. Larvae were fed daily on *A. pisum* and allowed to pupate and eclose in the dishes. Adult progeny from these lines were sexed under CO<sub>2</sub> anaesthetic.

#### *Inheritance of the sex ratio trait*

Two virgin females from each of five matrilines that produced only daughters, four virgin females from the two significantly female-biased lines that produced some males

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(line numbers UN16 and UN21), and two virgin females from matriline producing a 1:1 progenic sex ratio were mated to unrelated males from normal sex ratio matriline. One male from each of the female-biased lines that produced males (UN16 and UN21) and two males from tetracycline-treated matriline that had produced only female offspring prior to tetracycline treatment but produced males thereafter (see below), were mated to virgin females from normal sex ratio matriline. Pairs were treated in the same manner as the original matriline, with egg hatch rates and progenic sex ratios being recorded.

#### Cure of the sex ratio trait

Two of the original females that had produced only female progeny (UN2, UN6) and one female that had produced a normal progenic sex ratio (UN1) were removed from the refrigerator. These were fed for 2 days on artificial food and thereafter on *A. pisum*. They were mated to unrelated males from normal sex ratio lines. Eggs were collected for 12 days, hatch rates recorded, and ensuing larvae reared to adulthood, then sexed. Each of the three females was then offered a 10% mixture of the antibiotic tetracycline in golden syrup for 1 h daily. The egg hatch rates and progenic sex ratios produced following tetracycline treatment were recorded daily. The control of feeding just golden syrup was not performed as it has been shown previously that this diet does not affect coccinellid sex ratios (e.g. Hurst *et al.*, 1992).

To determine whether tetracycline produced a permanent cure of the sex ratio trait, four daughters from each of the two treated sex ratio matriline, two collected before tetracycline treatment and two resulting from eggs laid at least 14 days after the commencement of tetracycline treatment, were mated to unrelated males from normal sex ratio lines.

## Results and discussion

#### Egg clutch sizes in the field and laboratory

Forty-nine clutches of eggs were observed in the field. Clutch sizes ranged from one to 13, with a mean of 5.57 (SD = 3.10). Clutches in the laboratory, from the wild-collected females, ranged from one to 14, with a mean of 6.72 (SD = 1.78) ( $n = 284$ ). These clutch sizes compare with mean laboratory clutch sizes for other species known to harbour male-killing bacteria, of 11.76 for *Propylea japonica*, 16.34 for *Cheilomenes sexmaculatus*, and 23.81 for *Harmonia axyridis* in culture under similar conditions at the same time ( $n > 200$  clutches in all cases) (M. Majerus and T. Majerus, pers. comm.). Of the clutches observed in the field, 20 were unhatched and 29 fully or partially hatched. In hatched clutches in which less than 60% of eggs had hatched ( $n = 7$ ), all but one of the unhatched eggs were classed as yellow (infertile/male-killed) ( $n = 26$ ). In hatched batches in which more than 60% of eggs had hatched, unhatched eggs were split

fairly evenly between grey (fertile and inviable or cannibalised) and yellow categories (grey = 18, yellow = 15).

#### Sex ratio of initial sample and matriline

The sex ratio of the initial adult sample was 18 male:37 female, significantly different from 1:1 ( $\chi^2 = 6.564$ , d.f. = 1,  $P < 0.05$ ).

Of the 28 matriline originally set up, two failed to produce sufficient progeny for analysis (Table 1). Of the remaining 26, five produced only daughters (designated sex ratio lines, SR), one line (UN21) produced a single male out of 24 progeny (designated incomplete sex ratio, iSR), one line (UN16) produced 15 males out of 45 progeny, which represents a significant female bias ( $\chi^2 = 5$ , d.f. = 1,  $P = 0.05$ ) (designated ?SR), the remaining 19 (73.08%) producing progenic sex ratios that were not significantly female biased.

The egg hatch rates of the sex ratio and incomplete sex ratio lines were all below 50% (Table 1) and were homogeneous ( $\chi^2 = 1.109$ , d.f. = 5,  $P > 0.95$ ). The egg hatch rates of the other

**Table 1.** The egg hatch rates and progenic sex ratios of 28 females of *Coccinula sinensis* collected from near Kōfu City, Honshu, Japan. (NSR = normal sex ratio, SR = sex ratio, iSR = incomplete sex ratio, ?SR = female biased.)

| Brood number | Egg hatch rate | Number of adult progeny | Sex ratio (proportion males) | Matriline sex ratio distribution |
|--------------|----------------|-------------------------|------------------------------|----------------------------------|
| UN1          | 0.738          | 46                      | 0.565                        | NSR                              |
| UN2          | 0.422          | 25                      | 0                            | SR                               |
| UN3          | 0.667          | 55                      | 0.436                        | NSR                              |
| UN4          | 0.6            | 33                      | 0.545                        | NSR                              |
| UN5          | 0.875          | 23                      | 0.391                        | NSR                              |
| UN6          | 0.455          | 23                      | 0                            | SR                               |
| UN7          | N/A            | 0                       | N/A                          |                                  |
| UN8          | 0.667          | 46                      | 0.435                        | NSR                              |
| UN9          | 0.848          | 60                      | 0.6                          | NSR                              |
| UN10         | 0.727          | 28                      | 0.536                        | NSR                              |
| UN11         | 0.479          | 30                      | 0                            | SR                               |
| UN12         | 0.429          | 16                      | 0                            | SR                               |
| UN13         | 0.772          | 51                      | 0.490                        | NSR                              |
| UN14         | 0.811          | 28                      | 0.429                        | NSR                              |
| UN15         | 0.846          | 37                      | 0.378                        | NSR                              |
| UN16         | 0.786          | 45                      | 0.333                        | ?SR                              |
| UN17         | 0.857          | 27                      | 0.519                        | NSR                              |
| UN18         | 0.941          | 36                      | 0.528                        | NSR                              |
| UN19         | 0.779          | 31                      | 0.742                        | Male biased                      |
| UN20         | 0.838          | 60                      | 0.583                        | NSR                              |
| UN21         | 0.453          | 24                      | 0.042                        | iSR                              |
| UN22         | 0.75           | 8                       | 0.5                          | NSR                              |
| UN23         | 0.923          | 44                      | 0.636                        | NSR                              |
| UN24         | 0.922          | 46                      | 0.587                        | NSR                              |
| UN25         | 0.390          | 15                      | 0                            | SR                               |
| UN26         | 0.66           | 25                      | 0.44                         | NSR                              |
| UN27         | 0.561          | 19                      | 0.421                        | NSR                              |
| UN28         | 0.66           | 0                       | N/A                          |                                  |

20 lines all exceeded 50%. All the unhatched eggs in the SR and iSR lines were classified as yellow, none showing external signs of embryonic development. Conversely, 74 out of 268 (27.16%) of the eggs from the other 20 lines that failed to hatch were classified as grey, indicating considerable embryonic development. Most of these grey eggs showed signs of attack by neonate siblings, although whether these attacks caused the death of the embryos is not known.

The female biases in sex ratio families are deduced to be the result of distortion of the secondary sex ratio by a male-killer.

The vertical transmission efficiency of the sex ratio agent, based on the production of males in the initial sex ratio and incomplete sex ratio matriline, whose female progeny also only produced female offspring, is estimated to be 0.991 (one male out of 117 progeny). The prevalence of the trait in the sample is 0.23 (six out of 26) (prevalence range for 95% confidence limits = 0.09–0.43).

**Table 2.** Egg hatch rates and progenic sex ratios of four F1 females from matriline UN16, which had previously produced a significantly female-biased sex ratio.

| Brood  | Egg hatch rates | Number of adult progeny | Sex ratio (proportion male) |
|--------|-----------------|-------------------------|-----------------------------|
| UN16.1 | 0.844           | 35                      | 0.514                       |
| UN16.2 | 0.818           | 65                      | 0.477                       |
| UN16.3 | 0.710           | 25                      | 0.56                        |
| UN16.4 | 0.867           | 13                      | 0.548                       |

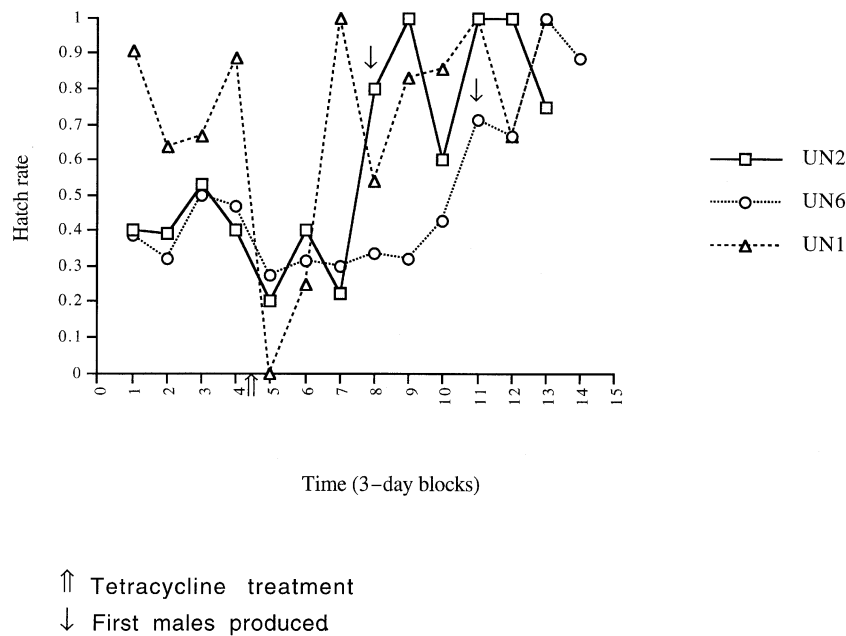
#### Inheritance of the sex ratio trait

One of the 10 families reared from the five sex ratio matriline failed to produce progeny. A second family produced just a single female. The remaining eight produced only female progeny (total progeny = 113, minimum number of progeny = 11). Similarly, three of the four females from the incomplete sex ratio line, UN21, produced only daughters (total progeny = 45, minimum progeny = 12), the fourth producing a single male out of 12 progeny. In all these families, egg hatch rates were below 50%, with the majority of unhatched eggs being classified as yellow, although some grey eggs were recorded (41 of 274, 14.96%). In contrast, the four females from UN16 each produced normal sex ratios and high egg hatch rates (Table 2). The male from UN21 mated to a normal female produced a high egg hatch rate (0.762,  $n = 84$ ) and normal sex ratio (24 males, 21 females), as did the male from UN16 (hatch rate = 0.86,  $n = 50$ ; 22 males, 21 females).

These results indicate that the sex ratio trait is maternally inherited only. The female bias in UN16 appears not to be the consequence of a heritable male-killer.

#### Cure of the sex ratio trait

In the period after removal from the refrigerator before tetracycline was administered, sex ratio females UN2 and UN6 and the control female UN1 produced progenic sex ratios consistent with those they had produced prior to refrigeration. Treatment with tetracycline produced an initial decline in egg



**Fig. 1.** Egg hatch rates (averaged over 3-day blocks) in three families of *Coccinula sinensis* prior to and following treatment with tetracycline. Families UN2 and UN6 showed the female-biased sex ratio trait prior to tetracycline treatment, while UN1 produced a normal progenic sex ratio prior to treatment. Tetracycline was administered on day 12. The first males produced in each SR family, subsequent to tetracycline treatment, are indicated by arrows.

hatch rates in all three lines treated, including the control (Fig. 1), but hatch rates increased after  $\approx 6$  days. The control, UN1, continued to produce approximately equal numbers of males and females. One of the sex ratio lines, UN2, produced only female offspring for 9 days, hatch rates to this point being consistently less than 50%. From day 10 after commencement of tetracycline treatment, hatch rates increased to over 50% (Fig. 1) and males were produced with almost equal frequency as females (16 males : 21 females). For UN6, the lag following tetracycline treatment, before an increase in egg hatch rates and the production of males was 18 days (Fig. 1). In this period, 16 female progeny were produced. Thereafter, again both males and females resulted at approximate parity (nine males : 12 females).

The F1 females from UN2 and UN6 produced prior to tetracycline treatment all produced low hatch rates (between 0.317 and 0.472) and only female progeny ( $n=45$ , minimum=8), while the four post-tetracycline females from UN2 and UN6 produced hatch rates between 0.684 and 0.832, and progenic sex ratios that did not differ significantly from 1:1 (proportion males = 0.429,  $n=28$ ; 0.405,  $n=37$ ; 0.421,  $n=19$ ; 0.577,  $n=26$ ).

These results indicate that tetracycline cures the sex ratio trait, that the sex ratio trait is not inherited from cured individuals, and that the agent responsible for the sex ratio trait is probably a bacterium.

These observations lead to the conclusion that *Coccinula sinensis* bears a male-killing endosymbiont, probably a bacterium, which is maternally inherited with a high vertical transmission efficiency ( $>0.99$ ). This brings the number of coccinellid species in which female-biased progenic sex ratios have been reported to eight.

The prevalence of the trait in the collected sample (0.23) is in the range of prevalence levels of male-killers reported from other coccinellid species. On the basis of the adult sample collected, the population sex ratio is significantly female biased. As sex-related differences in the activity and dispersal of adult coccinellids have been reported (e.g. Brakefield, 1984), however, a more reliable assessment of population sex ratio, based on collection of pupae, would be beneficial.

The lack of grey eggs among those eggs that failed to hatch in the sex ratio and incomplete sex ratio laboratory lines, and the presence of an appreciable proportion of such eggs among the unhatched eggs from the other lines, provides circumstantial evidence that the level of sibling egg cannibalism of female progeny in sex ratio lines is reduced compared to that suffered by female progeny from other lines. This reduction in cannibalism of infected females has been suggested as an additional advantage to male-killing (Hurst *et al.*, 1992), in addition to the resource advantage previously demonstrated in the coccinellid *Adalia bipunctata* (Hurst, 1993; Majerus, 1994). Further, the lack of grey eggs in egg batches from wild-caught females subsequently shown to bear the male-killing trait suggests that male embryos are killed early, before signs of embryonic development are discernible externally.

In the hatched batches observed in the field, the rarity of grey eggs in batches in which less than 60% of eggs had hatched may indicate that such batches represent male-killed

batches. Supposing this to be the case, and that all other batches were laid by females not bearing the male-killer, the prevalence of the trait based on observed hatched batches would be 0.24, which is very close to the figure of 0.23 obtained from the adult sample.

From the results presented here, it appears that the behavioural and ecological criteria of coccinellids said to facilitate invasion of a male-killing bacterium (Majerus & Hurst, 1997) hold for *C. sinensis*, despite the small mean egg batch size produced by this species. It is probable that the small batch size in this species is a consequence of the relatively large size of the eggs laid by this small coccinellid (egg weight = 1.749% of adult female weight, compared with 1.089% in *Propylea japonica* and 0.569% in *Cheilomenes sexmaculatus*; D. Fakhouri and M. Majerus, pers. comm.). This relatively large egg size may be required to allow neonate larvae sufficient resources (nutrients and space) to attain a minimum size necessary to give newly dispersing larvae a reasonable chance of finding and subduing prey. This requirement, allied to a limited daily energy budget for egg production, is likely to be of primary import in the evolution of clutch size in *C. sinensis*. Evolutionary pressure to increase egg clutch size consequent on invasion of the species by a male-killer, as suggested by Hurst and McVean's (1998) hypothesis, is likely to have limited effect in this species. Given the large relative size of *C. sinensis* eggs, investigations of the level of resource advantage accruing to female neonate larvae from the consumption of male-killed eggs would be valuable. In addition, field assessments of benefits that may accrue to infected females through the reduction in local competition for resources or through reduced predator or parasitoid pressure due to lower densities of the coccinellid, would be timely.

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