Volume 6, Issue 1, February 2014

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Sudan Journal of Science (SJS)

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Estimation of the population sex ratio in butterflies infected with bacterial male-killers: a case study

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Abstract

Female-biased population sex ratios in butterflies arise as a consequence of infection by maternally-transmitted endosymbionts that manipulate host sex ratio in order to maximize their transmission down the generations. A critical step toward understanding the host/male-killer relationship as well as the evolutionary consequences of male-killing is to provide accurate estimations for the population sex ratios in the host species. There are two ways to perform this: first, through counting the numbers of males and females in wild collections, and second, through the molecular screening of collected females for bacterial infection, using the PCR. In this paper, we compare the estimations of the two methods in the case of the queen butterfly *Danaus chrysippus* at Uganda and Sudan. We concluded that molecular screening is the most accurate approach to assess the population sex ratio. Moreover, a theoretical argument has been presented at the end of this article to explain why the bacterial prevalence approach avoids the inherent collection bias that is suffered by the sex ratio method.

Keywords:

Molecular screening, prevalence, *Danaus chrysippus*, *Spiroplasma*, Uganda, Sudan

Introduction

The cytoplasmic endosymbionts of arthropods apply a wide variety of reproductive manipulation strategies in order to improve their vertical transmission through the maternal line. In the early-acting male-killing strategy, the male-offspring of the infected mother are killed, at an early developmental stage, so that their female offspring benefit from the reduced sibling competition and/or the increased cannibalistic opportunities in the egg brood. Through killing the males in the brood, the endosymbiont improves the fitness of its identical clones that infect the females of that same brood. Natural selection would thus favor the spread of the male-killer in the host population; because of the resource-reallocation fitness advantage, infected females produce more daughters than uninfected ones and the proportion of infected females in the female population would subsequently rise. As a consequence, the host populations would experience a growing state of sex ratio distortion toward females (O'Neill *et al*., 1997; Majerus *et al*., 2003).

The population dynamics of male-killing endosymbionts are determined by four major factors: First, The magnitude of the resource-reallocation fitness advantage gained by infected females; second, the physiological cost of endosymbiont infection suffered by infected females; Third, the efficiency of the endosymbiont vertical transmission and fourth, the efficiency of host resistance mechanisms. The overall prevalence achieved by a male-killer in a host species is determined by the combined effect of these four factors (Hurst, 1991). For example, if the male-killer is transmitted to all the offspring of the infected mother (i.e. perfect vertical transmission), provides high fitness compensation and no fitness cost to infected females and has 100% expression in infected males (i.e. no suppression for the male-killing phenotype), then it is expected that the male-killer would spread to fixation, ultimately driving the host species toward extinction. On the other side, if the cost of infection is too great or the vertical transmission rate is too low so that they are

not compensated by the resource reallocation advantage, natural selection would eliminate the male-killer from the host species. Moderate fitness compensation maintains the male-killer at equilibrium prevalence. The result would be a stable polymorphism with respect to the male-killing trait in which both infected and uninfected females co-exist in the host species populations.

In Africa, the nymphalid butterflies *Danaus chrysippus*, *Acraea encedon* and *A. encedana* show female-biased population sex ratios due to the occurrence of all-female egg broods that are produced by a considerable proportion of females (e.g. Owen, 1971). The cause was found to be the infection with maternally inherited, male-killing bacterial endosymbionts; *Danaus* is infected with *Spiroplasma* while the two sibling *Acraea* species are infected with *Wolbachia*. In *Danaus chrysippus*, bacterial prevalences were found to display considerable fluctuations with space and time and were limited to a specific zone in East and Central Africa. In the sibling species *A. encedon* and *A. encedana*, extremely high prevalences were reported from the wild; in certain populations, more than 95% of females were infected with the male-killer (Jiggins *et al*., 1998; Hurst *et al*., 1999; Jiggins *et al*., 2000a, b; 2002).

The female-biased population sex ratios that result from male-killers' infection have the potential to induce deep evolutionary changes in host biology (Charlat *et al*., 2003; Majerus, *et al*., 2003), affecting the colour polymorphism, the mating behaviour, the population dynamics, the reproductive isolation between sibling species and the probability of extinction in the long term (Jiggins *et al*., 2000c; 2002; Smith *et al*., 1997; 1998; 2010). Critical for understanding the causes and the consequences of male-killing is the ability to estimate the overall ratio of males to females in the wild populations as well as the spatial variations and temporal changes in the sex ratio. There are two approaches to estimate the sex ratio of natural populations: the field-based and the lab-based approaches. In the field-based approach, the sex ratio of the population is directly estimated from the ratio of males to females in a wild collection. In the lab-based approach, the ratio of infected to uninfected females in the population is determined from the molecular screening for the male-killing bacteria. This ratio is then used as indirect indicator of the population sex ratio. In this paper, we address the technical issue of which method is more reliable, using comparative data on the sex ratios and the *Spiroplasma* prevalences in *D. chrysippus* specimens collected from Uganda and Sudan.

Materials & Methods

Collection sites

Danaus chrysippus butterflies were collected at fifteen sites that are distributed throughout Uganda, between March 2005 and May 2007. The collection at Khartoum, Sudan was limited to the agricultural farms along the Southern Bank of the Blue Nile River (GPS reading: 15.612931 N, 32.540259 E). Collection was conducted at two brief visits during January 2005 and April-June 2010.

Collection of Samples

Adult butterflies were collected from the wild by using a standard butterfly net. Collection was limited to butterflies that bear the characteristic morphology of *D. chrysippus*. Specimens obtained were killed by exerting sufficient pressure to the thorax. A fine spring entomological scissors, thoroughly sterilized with absolute ethanol, was used to detach the abdomen of each butterfly from behind the junction with the thorax. The detached abdomens were then placed in absolute ethanol within eppendorf tubes maintained at 4°C, for later molecular tests.

Morphological Investigations

The sex of each *D. chrysippus* specimen was described and recorded using morphological cues. Investigation of sex was done according to the pattern of spots on the butterfly hind wings, with males having three black spots on the hind-wing in addition to a fourth bigger, slightly bulged, white spot with thick black border (Ackery & Vane-Wright, 1984).

Molecular investigations

For the collection at Uganda, all females were tested for *Spiroplasma* infection. For the collection at Sudan, only 2005 females were subjected to the molecular test. DNA was extracted from the preserved abdomens using the Chelex-100

extraction method and the Wizard® Genomic DNA Purification Kit. The resulting DNA was amplified using general bacterial primers, 27f and 1495r (Weisburg *et al.*, 1991), for the 16S rDNA gene, to check for the presence of any bacterium. All samples were also checked specifically for the presence of the *Spiroplasma* previously reported by Jiggins *et al.* (2000a), using the *Spiroplasma*-specific primers HA-IN-1-f (Hurst *et al.*, 1999) and MGSOr (Van Kuppeveld *et al.*, 1992). General insect primers, C1-J-1751f and C1-N-2191r (Simon *et al.*, 1994), were used with all the DNA samples to check for the success of extractions. All the PCR premixes were cross-linked using a UV light illuminator to destroy any contaminant DNA.

Statistical analysis

Statistical data analysis was performed using the software package Microsoft® Excel 2007. The deviation of observed sex ratios from the 50% ratio has been assessed for statistical significance through the application of the Chi-squared test of heterogeneity (χ^2).

Results

During this study, a total of 963 samples of *Danaus chrysippus* were collected in Uganda during 2005-2007 period. In Sudan, a total of 201 samples were collected during 2005 and 2010 collections. The overall population sex ratios at the two countries were estimated using the field-based method (i.e. the ratio of males to females in the collection) as well as the lab-based method (i.e. the *Spiroplasma* prevalence).

***Danaus chrysippus* at Khartoum, Sudan**

The sex ratio

In 2005, 101 samples were collected, from which 53 were males and 48 were females. The overall ratio of males to females in the collection was 52.5% that did not differ significantly from the 50% ratio ($\chi 2 = 0.25$, d. f. = 1, $P > 0.05$). In 2010, 100 samples were collected, from which 67 were males and 33 were females. The ratio of males to females in the collection was 67% that is significantly higher than the 50% ratio ($\chi 2 = 11.56$, d. f. = 1, $P < 0.001$).

The Spiroplasma prevalence

All 2005 samples that were tested (females = 48, males = 10) were negative for *Spiroplasma* or any other bacteria.

***Danaus chrysippus* in Uganda**

The sex ratio

From the 963 samples obtained from Uganda (2005-2007), 507 were males. The overall ratio of males to females in the collection was 52.6% that did not differ significantly from the 50% ratio ($\chi 2 = 0.27$, d.f. =1, $P > 0.05$).

The Spiroplasma prevalence

A total of 426 *D. chrysippus* females were tested for *Spiroplasma*, from which 106 were positive (i.e. *Spiroplasma* prevalence = 24.9%).

Discussion

In this study, we provide a critical assessment for the two methodologies commonly used to estimate the population sex ratios of butterfly species invaded by bacterial male-killers: the field-based and the lab-based methods. The accuracy of each method was tested against field and molecular data regarding *Danaus chrysippus* populations in Uganda and Sudan.

The simplest way to estimate the sex ratio of a wild butterfly population is to collect samples from that population, calculate the ratio of males to females in the collection and then generalize this ratio to the whole wild population. The underlying assumption of this practice is that wild collection of butterflies is random with respect to sex, that is, the ratio of males to females in the collection is a good approximation of that ratio in the population from which the collection was made. However, a major difficulty with this assumption is that the activity patterns of butterflies vary with sex (Gilchrist, 1990); generally speaking, males are more active than females and thus are more easily detected by human collectors. As a consequence, the probability of collecting males tends to be higher than that probability for females, thus leading to artificially-higher representation of males in wild collections (i.e. male-bias).

The data on the sex ratios and the *Spiroplasma* prevalences in *D. chrysippus* that are recorded during this study supports the view that the wild collection is inherently subjected to male-bias. In Khartoum, the survey of *Spiroplasma* prevalence did not detect any bacterial infection among *D. chrysippus* females, which implies that the wild population sex ratio is 1:1. The sex ratio of the 2005 collection was slightly higher than the 50% (i.e. more males are collected than females), but the difference was not statistically significant. In the 2010 collection, however, the male-bias was extremely significant. It is very unlikely that the recorded male-bias underlies a real feature of Khartoum population, since we know of no natural mechanism that can bias the sex ratio of butterflies in the male direction; rather, it appears that this bias is artificial, representing a property of the collection rather than the wild population itself. These observations lead to two conclusions: first, estimating the population sex ratio directly from the field is inherently males biased, and second, field estimations of the population sex ratio varies considerably between different collections even when they are conducted at the same site, during the same hours of the day, by the same collector and using the same sampling methodology (as is the case in the 2005 and 2010 collections at Khartoum).

In Uganda, The overall population sex ratio obtained after three years of sampling (2005-2007) was 52.6%. This ratio does not differ significantly from 50%, but it is slightly male-biased. Despite this, the molecular screening of females for the *Spiroplasma* bacterium has yielded a considerable prevalence estimation of 24.9%, which implies that nearly a quarter of the females in Uganda have no male siblings. It is strongly expected that a population with such level of infection would show female-biased sex ratio in a random, unbiased collection. The discrepancy between the prevalence data and the sex ratio data could only be explained if we assume that the collection of *D. chrysippus* was artificially biased toward males. If this is the case, the female-bias in the wild population and the male-bias in the collection method would cancel each other, leading to equal or nearly-equal field estimation for the population sex ratio.

The bacterial prevalences provide an indirect route to estimate the population sex ratio; since infected females have no male siblings while uninfected females do have, then the ratio of infected to uninfected females contains information on the relative abundance of males compared to females in the population and hence the population sex ratio. Although this method is based primarily on the wild collection of butterflies, only collected females are screened for bacterial infection. Thus, the inherent male bias in the wild collection has virtually no influence on the accuracy of the bacterial prevalence method. Importantly, there is no reason to expect infected and uninfected females to differ in the probability of being captured in the field, since infection with the male-killing bacteria does not exert any physiological load on females (Jiggins *et al*., 2000a; 2002). It follows, then, that this method is neither biased toward infected nor uninfected females and thus it represents their actual ratio in the wild population.

The general conclusion that could be drawn from the present study is that the investigation of the prevalences of the male-killing bacteria is the most accurate approach for estimating the population sex ratio in butterflies infected with male-killing bacteria; the direct method of counting the ratio of males to females in a field collection is biased since males are more active and easily recognizable than females and thus are more frequently collected. The formal confirmation or rejection of this conclusion requires further work that includes *A. encedon* and *A. encedana* in addition to *D. chrysippus*. The data on sex ratios and bacterial prevalences should be compared over larger spatial and temporal scales to detect potential similarities or discrepancies between the estimations of the two methods.

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