

Is it just a coincidence that aposematic polymorphism and sex ratio distortion co-occur in a tropical butterfly?

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ABSTRACT

Background: The cosmopolitan butterfly *Danaus chrysippus* is polymorphic only within the geographic zone where it is infected by the male-killing bacterium *Spiroplasma*.

Hypothesis: The different colour forms of *D. chrysippus* represent incipient species that have undergone hybridization as a result of *Spiroplasma* invasion, because many females of the frequently infected forms are forced to mate with the males of the less infected forms.

Prediction: Some forms are more susceptible to *Spiroplasma* infection than others.

Times and places: Uganda and Sudan during 2005, 2006, and 2007.

Analytical method: We collected *D. chrysippus* butterflies in the wild. We recorded their colour pattern and sex. We then used polymerase chain reaction (PCR) to determine whether they were infected with *Spiroplasma*. We estimated the morph ratio, the sex ratio, and the prevalence of *Spiroplasma* in different populations and regions. The association between colour pattern and *Spiroplasma* infection was subjected to statistical analysis.

Conclusion: We found no significant difference in the sex ratio or the prevalence of *Spiroplasma* between different forms. Colour forms do not vary in their susceptibility to *Spiroplasma* infection.

Keywords: *Danaus chrysippus*, East Africa, hybrid zone, male-killing, polymerase chain reaction, *Spiroplasma*.

INTRODUCTION

Species that rely on aposematism as an anti-predator strategy are expected to be monomorphic for colour pattern, so as to maximize the efficiency of avoidance learning by naïve predators. The reason is that the predation effort required to develop the avoidance response by predators is proportional to the number of different colour forms that coexist in the

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prey population. Any genetic tendency for developing a novel colour pattern should be eliminated by positive frequency-dependent selection, as individuals with rare colour forms will suffer higher predation than those with the common colour form (Fisher, 1930; Ford, 1964; Matthews, 1977; Greenwood *et al.*, 1981). Thus, cases of aposematic polymorphism, wherever they occur, are considered to be of special interest, as they contradict conventional predictions of evolutionary theory.

Owing to their maternal inheritance, a wide variety of cytoplasmic endosymbionts of arthropods manipulate host reproduction in such a way as to bias the host sex ratio towards females. One strategy of achieving this end is through killing the male offspring of the infected mother host during their early development, so that resources that would otherwise be exploited by males are reallocated towards their female siblings (O'Neill *et al.*, 1997; Majerus, 2003). Early-acting male-killers spread in their host populations if the resource reallocation fitness advantage is of sufficient magnitude to compensate for the fitness cost of male death, the physiological cost of endosymbiont infection, as well as any loss of infection due to the imperfect vertical transmission of the male-killer (Hurst, 1991).

The African monarch butterfly *Danaus chrysippus* (L.) (Lepidoptera: Nymphalidae) is one of the most common and most widely distributed nymphalid butterflies throughout the Old World tropics and subtropics. It is an aposematic butterfly that is chemically defended, and characterized by bright and contrasting coloration (Reichstein *et al.*, 1968; Brower *et al.*, 1975, 1978; Rothschild *et al.*, 1975; Boppré, 1978). The ecology of *D. chrysippus* in East and Central Africa has been thoroughly investigated. Two phenomena have been reported that are not known to occur outside this geographic zone. First, wild populations show female-biased sex ratios, due to the production, by some females, of all-female broods (Owen and Chanter, 1968; Smith *et al.*, 1998). The cause of sex ratio distortion was found to be infection by a maternally inherited male-killing bacterium of the genus *Spiroplasma* (Jiggins *et al.*, 2000a). And second, the species shows spectacular colour pattern polymorphism in which four different colour forms (*chrysippus*, *dorippus*, *alcippus*, and *albinus*) coexist sympatrically, together with several intermediate forms (Owen and Chanter, 1968; Pierre, 1974; Rothschild *et al.*, 1975; Smith, 1980; Owen and Smith, 1993; Smith *et al.*, 1993; Owen *et al.*, 1994).

Both sex ratio distortion and colour polymorphism appear highly unusual when viewed in the light of the ecological background of *D. chrysippus*. The problem with male-killing is that *D. chrysippus* lacks the characteristic life-history traits that confer susceptibility to invasion by male-killers; females lay eggs singly on widely scattered larval food plants (various milkweeds), and the larvae feed singly. Antagonistic sibling interactions, and thus the advantage of resource reallocation necessary for the spread of male-killing endosymbionts, are not known to occur in this species (Mallet and Singer, 1987; Smith *et al.*, 1998; Mallet and Joron, 1999). As noted earlier, the problem with colour polymorphism is that it reduces the efficiency of avoidance learning by naïve predators, since they will need to experience every colour form independently, thus leading to higher rates of predation (Fisher, 1930; Ford, 1964). The ubiquity of hybrid forms in *D. chrysippus* further complicates the situation, as natural selection is expected to eliminate any genetic tendency for hybrid coloration in aposematic species (Smith, 1975a, 1980; Smith *et al.*, 1998; Lushai *et al.*, 2005).

A presumed adaptive link between male-killing and colour polymorphism has been incorporated within the 'hybrid zone' hypothesis for the evolution of colour polymorphism in *D. chrysippus*. According to this view, colour forms of *D. chrysippus* represent vicariant sub-species that arose through allopatric sub-speciation rather than being sympatric forms in a polymorphic species (e.g. Lushai *et al.*, 2003, 2005; Smith *et al.*, 2010). During the Pleistocene period,

forests may have acted as a barrier to gene flow by restricting the dispersal of butterflies between the different geographic zones of Africa (Moreau, 1963). When the climate changed during the mid-Holocene, forests were replaced by open savannah habitats that permit the dispersal of butterflies (Roberts, 1989). The different sub-species of *D. chrysippus* have undergone a range expansion following the climatic and faunal change. The entire region of East and Central Africa has become a hybrid zone for *D. chrysippus*, where sub-species co-occur sympatrically. Those sympatric sub-species are then driven to hybridization despite partial, pre-zygotic reproductive isolation as a consequence of female-biased population sex ratios caused by the male-killer invasion; because some colour forms are more susceptible to invasion by the male-killing *Spiroplasma* than others, females belonging to susceptible colour forms are forced to accept hybrid matings due to the rarity of males with their own colour pattern (Lushai *et al.*, 2003, 2005; Smith *et al.*, 2010). The disappearance of the geographic barrier and the ubiquity of hybrid matings saw a restoration of the gene flow between the once-isolated sub-species of *D. chrysippus* and, subsequently, the speciation process was interrupted.

Thus, this hypothesis is based on two independent assumptions: first, that East and Central Africa represent a hybrid zone for *D. chrysippus*; and second, that heterogeneous sex ratios occur within the different colour forms of the butterfly throughout this presumed hybrid zone (Smith, 1980; Smith *et al.*, 1993, 1997, 1998). Evidence supporting the first assumption includes the finding of assortative mating between colour forms of *D. chrysippus* (Smith, 1973, 1975a, 1980; Smith *et al.*, 1998) as well as the morph ratio clines shown by the geographic distribution of colour forms in Africa (Smith *et al.*, 1997). Evidence supporting the second assumption includes the general taxonomic association between aposematic polymorphism and male-killing in insect taxa (Majerus, 2003), as well as the geographic association observed between the two phenomena, since *D. chrysippus* from outside the region of East and Central Africa are neither polymorphic nor female-biased. In further support of this assumption is the finding of Smith (1975b) that in wild samples of *D. chrysippus*, females predominated among *f. chrysippus*, while a 1:1 sex ratio was observed in *f. dorippus*.

On the other hand, the critical assumption of this hypothesis (i.e. colour forms are incipient species in a hybrid zone) remains highly controversial. The morph ratio variation between the different parts of Africa may simply represent spatial variation in aposematic colour patterns, a phenomenon seen in other butterfly taxa [e.g. *Heliconius* (Joron *et al.*, 1999)]. Moreover, hybrid zones should be much smaller than the zone of allopatry; however, the polymorphism in *D. chrysippus* prevails across an area as large as East and Central Africa (Smith *et al.*, 1997). In addition, hybridization between the different colour forms takes place frequently in the wild (e.g. Smith, 1975a; Gordon, 1984), with no direct indication of reproductive isolation.

Alternative theories have also been suggested to explain the anomalous colour polymorphism in *D. chrysippus*; for example, polymorphism may have developed as a response to Batesian mimics such as *Hypolimnas misippus*. Due to their edibility, Batesian mimics exert a diluting influence on the aposematic signal of their model species because naïve predators that have experienced the mimic will subsequently target the model rather than avoid it (Owen, 1970). The geographic distribution of *D. chrysippus* Batesian mimics in Africa seems to support this view, since polymorphism is extensive where Batesian mimicry is most developed (i.e. in East Africa). In West Africa, where few mimetic forms are observed, *D. chrysippus* is monomorphic (Edmunds, 1969; Gordon, 1987).

The goal of this study was to provide a field-based assessment of the ‘hybrid zone’ hypothesis. Two specific predictions of this hypothesis were tested against field data:

1. The sex ratio of *D. chrysippus* and the prevalence of *Spiroplasma* vary significantly between the different colour forms.
2. Colour polymorphism and hybrid mating develop only in regions where the population sex ratio is female-biased.

MATERIALS AND METHODS

Collection sites

Between March 2005 and May 2007, specimens of *D. chrysippus* were collected from 15 sites in Uganda. The Sudanese samples were collected along the Nile River Bank, Khartoum during three successive visits in 2005, 2010, and 2011.

Collection of samples

Adult *D. chrysippus* were collected from the wild using a standard butterfly net. The specimens collected were killed by exerting sufficient pressure to the thorax. Fine spring entomological scissors, thoroughly sterilized with absolute ethanol, were 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. The remaining body parts of each butterfly were kept in a labelled glassine envelope for later identification.

Morphological investigations

The sex and the colour pattern of each *D. chrysippus* specimen was described and recorded based on their external morphology. Investigation of sex was done according to the pattern of spots on the butterfly hind wings. Colour patterns were identified following the descriptions of Owen *et al.* (1994).

Molecular investigations

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.

The association between polymorphism and male-killing

The sex ratio (i.e. the percentage of males) within each colour pattern was compared statistically with that of other colour forms as well as with the total sex ratio. The prevalence of *Spiroplasma* (i.e. the percentage of females infected) within each colour pattern was also compared with that of other forms and with the total prevalence.

Statistical analysis

Data analysis was performed using the chi-squared test of heterogeneity (χ^2). When repeated comparisons were made, the Bonferroni correction ($\alpha\beta$) was used. The level of significance was determined using the formula (P/n), where $P = 0.05$ and n is the number of comparisons performed.

RESULTS

The sex ratio among different colour forms in Uganda

The sex ratio within each of the four colour forms was calculated for all the 2005–2007 Ugandan samples summed (*albinus* 75%, *alcippus* 54.5%, *chrysippus* 47.9%, and *dorippus* 63.1%) (Table 1). The sex ratios of the colour forms were heterogeneous ($\chi^2 = 13.91$, d.f. = 3, $P < 0.01$). The sex ratio of the three forms *alcippus*, *chrysippus*, and *dorippus* did not differ significantly from that of the total sample (52.6%) ($\alpha\beta = 0.01$; $\chi^2 = 0.4$, 2.77, and 2.66 respectively, d.f. = 1, $P > 0.01$). The sex ratio of the form *albinus* was significantly higher than that of the total collection ($\alpha\beta = 0.01$; $\chi^2 = 6.21$, d.f. = 1, $P < 0.01$).

Prevalence of male-killing *Spiroplasma* among different colour forms in Uganda

Table 2 shows the prevalence of the male-killing *Spiroplasma* for each of the four colour forms. No specific preference of the bacterial invasion for any particular colour form was detected, as prevalences within each colour pattern were all statistically homogeneous (*albinus* 25%, *alcippus* 26.4%, *chrysippus* 23%, and *dorippus* 31.8%) ($\chi^2 = 1.23$, d.f. = 3, $P > 0.05$). Moreover, those colour form-specific prevalences did not show significant deviation from the total prevalence in Uganda (24.9%) (*albinus* $\chi^2 = 0.0005$, *alcippus* $\chi^2 = 0.12$, *chrysippus* $\chi^2 = 0.19$, and *dorippus* $\chi^2 = 2.55$; all $P > 0.05$, d.f. = 1).

Table 1. Morph-specific sex ratios in Uganda (2005–2007)

Morph	<i>N</i>	Females	Males	% Males
<i>albinus</i>	32	8	24	75.0
<i>alcippus</i>	411	187	224	54.5
<i>chrysippus</i>	455	237	218	47.9
<i>dorippus</i>	65	24	41	63.1
Total	963	456	507	52.6

Table 2. Prevalence of *Spiroplasma* in the colour forms of *Danaus chrysippus* collected in Uganda (2005–2007)

Morph	Number of females tested	Positives	Negatives	Prevalence of <i>Spiroplasma</i> (%)
<i>albinus</i>	8	2	6	25.0
<i>alcippus</i>	174	46	128	26.4
<i>chrysippus</i>	222	51	171	23.0
<i>dorippus</i>	22	7	15	31.8

Table 3. Colour morphs of *Danaus chrysippus* collected at Khartoum in three different years

Year	<i>alcippus</i>	<i>chrysippus</i>	<i>dorippus</i>	<i>albinus</i>	Total
2005	71	21	2	1	95
2010	73	27	0	0	100
2011	58	19	0	0	77
Total	202	67	2	1	272

Table 4. Comparison of colour form frequencies between the Sudanese sample and the Ugandan sample

Sample	<i>alcippus</i> (%)	<i>chrysippus</i> (%)	<i>dorippus</i> (%)	<i>albinus</i> (%)	Sample size
Sudan	74.3	24.6	0.7	0.4	272
Uganda	42.9	47.0	6.7	3.4	869

The morph ratio in Sudan and Uganda

Table 3 compares the morph ratios of the three collections made at Khartoum during 2005, 2010, and 2011. Morph ratios were found to be homogeneous ($\chi^2 = 6.56$, d.f. = 6, $P > 0.05$).

Table 4 compares the overall morph ratio of the Ugandan collection (*albinus* 75%, *alcippus* 54.5%, *chrysippus* 47.9%, and *dorippus* 63.1%) with that of the Sudanese collection (*albinus* 0.4%, *alcippus* 74.3%, *chrysippus* 24.6%, and *dorippus* 0.7%). The Ugandan collection was significantly more 'polymorphic' than the Sudanese one ($\chi^2 = 22.65$, d.f. = 3, $P < 0.001$), with the more abundant forms relatively less common (*alcippus* + *chrysippus* = 89.9% and 98.9%, respectively) and the less abundant forms relatively more common (*dorippus* + *albinus* = 10.1% and 1.1%, respectively).

The sex ratio in Sudan

The sex ratio of the Sudanese collection did not show significant deviation from 50% (% males = 55.1, $\chi^2 = 1.04$, d.f. = 1, $P > 0.05$, $N = 272$).

Prevalence of the male-killer in Sudan

None of the females tested from the Sudanese collection were found to be positive for *Spiroplasma* infection ($N = 48$).

DISCUSSION

We tested the hypothesis in the field that sex ratio distortion is the underlying factor in maintaining colour pattern polymorphism in *D. chrysippus*. The data obtained are at odds with the expectations of the ‘hybrid zone’ hypothesis. First, neither the population sex ratio of *D. chrysippus* nor the prevalence of *Spiroplasma* recorded for different colour forms showed consistent deviation from the mean values. The only contrary result, i.e. deviation of the *albinus* sex ratio towards males, is not very strongly significant and is likely to be a chance occurrence. Second, *D. chrysippus* was found to show considerable colour polymorphism in Khartoum, although both the investigation for population sex ratio and the molecular tests for bacterial infection suggest a *Spiroplasma*-free zone at Khartoum. Similar results were previously recorded from Oman (Jiggins *et al.*, 2000a) and the island of Saõ Vicente, Cape Verde Islands (Lushai *et al.*, 2003) where *D. chrysippus* is polymorphic and the sex ratio is normal. Taken together, these results suggest that colour polymorphism did not develop specifically as a response to the female-biased sex ratios caused by the male-killing act of *Spiroplasma*.

It is important to note, however, that our data do not formally falsify the prediction that susceptibility to the male-killer varies between colour forms; previous studies on *D. chrysippus* have indeed reported a statistical association between the colour pattern on the one hand and sex ratio distortion and/or *Spiroplasma* prevalence on the other (Smith *et al.*, 1993, 1997; Herren *et al.*, 2007). Even if this prediction (i.e. susceptibility to the male-killer varies between colour forms) is ruled out, there is still a weak version of the ‘hybrid zone’ hypothesis that does require variable susceptibility. According to this version, hybrid mating develops as a response to the bias in the general sex ratio, rather than form-specific sex ratios. This is possible if the population sex ratio is extremely female-biased so that finding a mate is a difficult task for females of any colour form, even without a colour form preference. Under such conditions, females that mate only with males carrying the same colour form will die frequently without leaving an offspring, while females that mate indiscriminately will achieve greater reproductive success, as they have more potential mates in the population than selective females. Natural selection would thus counteract the incomplete pre-zygotic reproductive isolation. The major difficulty with this view is that it requires a markedly female-biased sex ratio to the extent of seriously reducing the population recruitment rate. This might be the case in other nymphalid butterflies such as *Acraea encedon* and its sibling *A. encedana* (Jiggins *et al.*, 1998, 2000b, 2002), but has yet to be noted in *D. chrysippus* (Jiggins *et al.*, 2000a; Hassan *et al.*, 2012).

We believe that the maintenance of colour pattern polymorphism in the African populations of *D. chrysippus* is a complex process that involves multiple factors, including both male-killer invasions and Batesian mimetic load. Thus, we recommend that future research on *D. chrysippus* adopt the approach of recording all potentially relevant factors from the field rather than focusing on a single factor as we did in the current study. The application of this pluralistic approach would improve our understanding of polymorphism in *D. chrysippus*, since it would enable the quantitative assessment of the relative

contribution of each factor. Moreover, future surveys should use larger sample sizes as well as wider spatial and temporal sampling scales. Although this might be difficult, the long-term monitoring of individual populations at specific locations through routine surveys of sex ratio, *Spiroplasma* prevalence, and mimetic load would yield valuable data to allow us to test the predictions of the different hypotheses on *D. chrysippus* polymorphism.

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