

The Queen Butterfly, *Danaus Chrysippus* (L.) (Lepidoptera: Nymphalidae) at Khartoum, Sudan.

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ABSTRACT

Within the region of East and Central Africa, the aposematic butterfly *D. chrysippus* is characterized by unusual ecology, due to the co-occurrence of polymorphism, mimicry and sex ratio distortion. In this paper, we describe a survey conducted for *D. chrysippus* at Khartoum, based upon morphological and molecular investigations. The goal of the study was to determine whether Sudan is a part of the presumed hybrid zone of *D. chrysippus* in Africa, where the butterfly shows the unusual ecology. *D. chrysippus* was found to display considerable colour pattern polymorphism. Only one mimetic form, the batesian mimic, female *Hypolimnas misippus* was recorded. The population sex ratio of *D. chrysippus* did not show significant deviation from the 1:1 ratio. Molecular tests for the male-killing bacteria *Spiroplasma* yielded negative results. The study concluded that central Sudan belongs to the hybrid zone but lacks its characteristic ecology.

Keywords: Aposematic polymorphism; Mimicry; sex ratio distortion; male-killing; *Spiroplasma*; PCR

INTRODUCTION

Species that use aposematism as an anti-predator strategy are expected to be monomorphic for colour pattern, in order to facilitate avoidance learning by predators; if the aposematic prey is polymorphic, naïve predators will need to experience every colour form independently. On the other side, a naïve predator encountering any member of an aposematic monomorphic prey will subsequently avoid the entire species because all of them look perfectly alike. Thus, predation rates on monomorphic aposematic prey are substantially lower than these on polymorphic aposematic prey, and this is why natural selection favors aposematic monomorphism. Likewise, aposematic species in a Müllerian mimicry complex are expected to converge on a uniform colour pattern, so that the cost of predators learning is shared between members of the mimicry complex (Fisher, 1930; Ford, 1964; Matthews, 1977; Greenwood *et al.*, 1981). As a consequence, aposematic and mimetic polymorphism are rare phenomena and, wherever they occur, they represent cases of special interest, as they contradict expectations of evolutionary theory.

Early male-killing is a strategy of reproductive manipulation applied by some of the maternally-inherited endosymbionts of arthropods to bias the host sex ratio toward females, thus improving their vertical transmission down the female line. In this strategy, the endosymbiont kills male offspring of the infected female host during early development, but remains benign to female offspring, because they can transmit the male-killer to the next generation (O'Neill *et al.*, 1997; Majerus, 2003). A male-

killer will spread in its host populations only if the host life style permits the reallocation of resources that would otherwise be exploited by males toward their female siblings, to the degree of significantly improving the survival of females in the infected broods over females in the uninfected broods (Hurst, 1991). Resource reallocation occurs most commonly through reduced sibling competition and increased opportunities for sibling cannibalism. Thus, the distribution of male-killers in insect taxa is non-random with respect to ecology, since species in which females lay eggs in large, tight clutches are the most susceptible for invasion by male-killers (Hurst & Majerus, 1993).

The Queen butterfly, *Danaus chrysippus* is a nymphalid butterfly which belongs to the subfamily Danainae. It is widely distributed throughout the old world tropics and subtropics. It is found in all habitats including deserts and mountains. However, it is primarily an open country butterfly. In Africa, *D. chrysippus* is found in natural lowland biomes ranging from wooded savanna through grassland to semi-desert, closed-canopy forest, high mountains and sand deserts (Migdoll, 1988). The species is well adapted to human disturbance (Owen, 1970), since it is most abundant in human-made habitats such as farms, gardens, waste land and roadsides. *Danaus chrysippus* does not form discrete populations and the long-lived adults range widely in search of nectar, pyrrolizidine alkaloid sources, mates and food-plants. Moreover, it is a migratory butterfly which undergoes both short term and long term movements (Smith & Owen, 1997). *D. chrysippus* is involved in a mimicry complex in Africa, which includes Müllerian mimics (i.e. *Acraea encedon*, *A. encedana*) as well as Batesian mimics (e. g. *Hypolimnas misippus*) (Owen; 1970; Owen & Smith, 1993).

The ecology of *D. chrysippus* within the zone of East and Central Africa has been thoroughly investigated. Two phenomena were observed within this region that are unknown to occur elsewhere, despite the universal distribution of the species: first, the queen butterfly shows spectacular colour polymorphism, in which four different colour forms may co-exist in the same population, together with several intermediate forms, thus representing an exceptional case of aposematic and mimetic polymorphism (e.g. Smith *et al.*, 1993; Owen & Smith, 1993; Owen *et al.*, 1994); and second, the queen butterfly shows female-biased population sex ratios, due to the production, by a considerable proportion of females, of all-female broods, because they are infected by a male-killing bacterium of the genus *Spiroplasma* (Owen & Chanter, 1968; Jiggins *et al.*, 2000a). The surprising aspect of male-killing in *D. chrysippus* is that host life history provides no resource reallocation fitness advantage for the male-killer, since eggs are laid singly on widely scattered larval food-plants (various milkweeds of the genus *Asclepias*) (Smith *et al.*, 1998). It is not obvious, then, how females gain from the death of their male siblings, and if they don't, how *Spiroplasma* is maintained in *D. chrysippus* despite its substantial fitness cost to infected females (since it involves the death of half their offspring).

The co-occurrence of two unusual phenomena within only a small fraction of the total geographic distribution of *D. chrysippus* might suggest the existence of a subtle link between them (Majerus, 2003). Supporting this view, aposematic polymorphism and male-killing were reported to co-occur in taxa as diverse as ladybird beetles (*Adalia decempunctata*, and *Harmonia axyridis*) (Brakefield, 1985; Majerus, 1998; Majerus & Roy, 2005) and Nymphalid butterflies (*A. encedon* and *A. encedana*) (Jiggins *et al.*, 1998; 2000b). Thus, the two phenomena are tightly associated not only geographically (as in *D. chrysippus* case), but also taxonomically, since insect species with aposematic polymorphism are more susceptible for invasion by male-killers.

A theory has been developed to explain the unusual ecology of *D. chrysippus* in which East and Central Africa is viewed as a hybrid zone (Smith *et al.*, 1997; Lushai *et al.*, 2003). According to this theory, *D. chrysippus* colour forms represent vicariant subspecies that arise through allopatry during the Pleistocene period, because the extensive forest cover of Africa during that period restricted butterfly dispersal (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). Subspecies of *D. chrysippus* have undergone a range expansion following habitat change. The entire region of East and Central Africa has become a hybrid zone, where different sub-species co-occur sympatrically (Smith *et al.*, 1998). Those sympatric sub-species are forced to hybridize, despite partial pre-zygotic reproductive isolation (which evolved earlier during the allopatric stage), as a consequence of female-biased population sex ratios caused by the male-killer invasion; some colour forms are more susceptible to infection by *Spiroplasma* than others, thus females belonging to susceptible colour forms tend to accept hybrid matings due to the rarity of males with their own colour form (Lushai *et al.*, 2003; 2005). The output would be an interruption of the speciation process and a restoration of the gene flow between subspecies/colour forms of *D. chrysippus*.

An alternative theory is that colour polymorphism in *D. chrysippus* evolved as adaptive response to extensive Batesian mimicry. Since batesian mimics are edible, they dilute the aposematic signal of their model; a naïve predator encountering an edible mimic will gain a rewarding predation experience and thus will learn to target the characteristic colour of the mimicry complex. Due to this ‘mimetic load’, batesian mimics should be much less abundant than their model; otherwise the aposematic signal will be maladaptive, reducing the fitness of the aposematic prey by attracting predators rather than deterring them. Under such conditions, any mutant individual which happens to develop a novel colour form will gain an immediate survival advantage, since predators attack common forms more than rare, unfamiliar forms. As a consequence, negative frequency-dependant selection, induced by mimics, will act to maintain polymorphism in the model species, thus counteracting the positive frequency-dependant selection, induced by predators, which pushes the species toward monomorphism (Owen, 1970; Allen, 1988; Smith *et al.*, 1993).

The goal of this paper is to investigate the ecology of *D. chrysippus* in Central Sudan, and to determine whether this region belongs to the zone of unusual ecology of East and Central Africa or to the zone of northern Africa, where the butterfly is monomorphic and shows equal sex ratio.

MATERIALS AND METHODS

Collection sites

D. chrysippus samples were collected at agricultural farms along the Southern Bank of the Blue Nile River, Khartoum (GPS reading: 15.612931 N, 32.540259 E), during four limited collections made at January 2005, April-June 2010, August 2011 and March 2012.

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* (which might be the species itself or a mimic of it). Non mimetic forms of *D. chrysippus* mimics were also targeted during collection. Specimens obtained were killed by exerting sufficient pressure to the thorax. In the case of 2005 *D. chrysippus*

samples, 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 and the colour pattern of each *D. chrysippus* specimen were 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). Colour patterns were identified following the descriptions of Owen *et al.* (1994). Specimens with intermediate morphology were recorded and then excluded from the morph ratio (which contains only the four major colour forms). Females *H. misippus* were identified and distinguished from *D. chrysippus* using slight differences in wing morphology.

Molecular investigations

Only 2005 samples were subjected to the molecular investigation for the male-killing *Spiroplasma*. All females from this collection were tested. In addition, ten males were also 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

Analysis of data was performed using the Chi-squared test of heterogeneity (χ^2). A result was taken to be significant if $P < 0.05$.

RESULTS

During the current study, a total of 318 samples of *D. chrysippus* were obtained from Khartoum, from which 101 samples collected at 2005, 100 samples at 2010, 77 samples at 2011 and 40 samples at 2012

Sex ratio

The total sex ratio of samples collected during the four years did not differ significantly from 50% (% males= 53.4; $\chi^2 = 0.46$; d.f. = 1, $P > 0.05$) (see Table 1 for the sex ratio of each of the four collection).

Table 1: Sex ratios of *D. chrysippus* samples collected from the wild during four different years at Khartoum, Sudan.

Year	males	Females	% males	N
2005	53	48	52.5	101
2010	67	33	67	100
2011	33	44	42.8	77
2012	17	23	55	40
Total	170	148	53.4	318

***Spiroplasma* infection**

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

Polymorphism

Considerable colour polymorphism has been recorded at Khartoum (Table 2). Dominant colour forms were found to be *F. alcippus* and *F. chrysippus*, while *F. dorippus* and *F. albinus* were rarely encountered in the field. The general morph ratio in Khartoum was *alcippus* 73.5%: *chrysippus* 25.2%: *dorippus* 1%: *albinus* 0.3%.

Table 2: Morph ratios of *D. chrysippus* samples collected from the wild during four different years at Khartoum, Sudan (samples with intermediate morphology are excluded).

Year	<i>alcippus</i>	<i>chrysippus</i>	<i>Dorippus</i>	<i>albinus</i>	N
2005	71	21	2	1	95
2010	68	25	0	0	93
2011	56	17	0	0	73
2012	24	12	1	0	37
Total	219	75	3	1	298

Hybrid mating

Hybrid mating occurs at Khartoum; twenty specimens (6.3 % of the total collection) were found to bear an intermediate morphology between *F. alcippus* and *F. chrysippus* (i.e. reduced white colour on the hind wing), indicating their hybrid origin.

Mimicry

Only one batesian mimic, the female diadem butterfly *Hypolimnas misippus* was found at Khartoum. Twelve samples of *H. misippus* were collected from which seven were females. All obtained *H. misippus* samples belong to 2011 collection. Collections of 2005, 2010 and 2012 were made in the same location at the Blue Nile River Bank and yet no *H. misippus* was found. All samples belong to the typical form *H. misippus misippus*, which mimics the form *chrysippus* of *D. chrysippus*.

DISCUSSION

During the current study, male-killing in *D. chrysippus* was assessed directly by molecular tests for *Spiroplasma* infection and indirectly by field estimations of host population sex ratio. All samples of the 2005 collection that were tested for the male-killing *Spiroplasma* were negative. The general sex ratio of *D. chrysippus* did not deviate significantly from the 50%. Moreover, three of the four collections were, in fact, slightly male-biased, thus excluding any potential for *Spiroplasma* affecting the sex ratio. A conclusion can be drawn from current data that *Spiroplasma* is absent from Khartoum. However, because of the small scale of the current study, the possibility that *Spiroplasma* is present but at very low prevalence cannot be ruled out.

Regarding colour pattern, *D. chrysippus* was found to show considerable polymorphism within the region of Khartoum. *Alcippus* was the most common form, while *chrysippus* was fairly abundant. On the other side, *Dorippus* and *Albinus* were very rarely encountered. The occurrence of colour polymorphism at Khartoum is an interesting result, since it contradicts an expectation of the hybrid zone theory that sex ratio distortion is a prerequisite for the development of colour polymorphism. Similar results were also reported from Oman (Jiggins *et al.*, 2000a), and the island of Saõ

Vicente, Cape Verde Islands (Lushai *et al.*, 2003) where *D. chrysippus* is polymorphic in the absence of *Spiroplasma*.

The distribution of *D. chrysippus* colour forms in Africa was thoroughly studied since Owen & Chanter (1968). *F. dorippus* occurs mainly in East Africa together with *F. albinus*. *F. alcippus* is most common in West Africa. *F. chrysippus* occurs over most of the continent except West Africa and is the only form found in Madagascar. In the hybrid zone of East and Central Africa, (e.g. Uganda, Tanzania), all forms co-occur sympatrically (Smith *et al.*, 1997). The four colour forms of *D. chrysippus* were reported at Khartoum, indicating that Central Sudan is a part of the hybrid zone. Hybrid mating between *F. alcippus* and *F. chrysippus* and the production of intermediate colour forms further support this conclusion. The morph ratio at Khartoum (*alcippus* 73.5%: *chrysippus* 25.2%: *dorippus* 1%: *albinus* 0.3%) differs significantly from the morph ratio recorded at Uganda (*alcippus* 42.9%: *chrysippus* 47%: *dorippus* 6.7%: *albinus* 3.4%) (Hassan *et al.*, in prep.), as well as from the morph ratio recorded at Tanzania (*alcippus*: 1.3% *chrysippus*: 42.7% *dorippus*: 55.3%: *albinus*: 0.7%) ($\chi^2 = 21.4$, d. f. = 3, $P < 0.001$) (Smith, 1973) ($\chi^2 > 16.27$, d. f. = 3, $P < 0.001$), thus supporting Smith *et al.* (1997) conclusion that the hybrid zone shows heterogeneous sex ratios.

Despite having several mimics in Africa, Müllerian and Batesian, *D. chrysippus* has only one mimetic species in Khartoum, the batesian mimic female *H. misippus*. Interestingly, although *H. misippus* is a polymorphic butterfly, with colour forms that show impressive resemblance to the four colour forms of *D. chrysippus* (Owen, 1970), only one form, *misippus*, was recorded during this study, which is the mimic of *F. chrysippus*. Most notable is the absence of *F. alcippoides*, although its model, *F. alcippus*, is the most abundant form of *D. chrysippus* at Khartoum. According to the mimetic load theory, the subtle factor underlying the geographic variation in colour polymorphism throughout Africa is the impact of batesian mimics; in East and Central Africa, *D. chrysippus* is a model for a variety of Batesian mimics, including some of the female-limited forms of *Papilio dardanus* (Papilionidae), both forms of *Pseudacraea poggei* (Nymphalidae) and the female forms of *Hypolimnas misippus* (Owen, 1970). But in West Africa, where the butterfly is monomorphic, *D. chrysippus* is mimicked only by *F. alcippoides* of *H. misippus*, which, however, is rare, and in Ghana it comprises only 2-3% of females (Edmunds, 1969). It seems therefore that polymorphism in *D. chrysippus* occurs where aposematism is diluted by batesian mimics, as in East and Central Africa, but in areas where batesian mimics are few, as in West Africa, polymorphism does not develop (Owen, 1970; Smith, 1976). Data obtained from Khartoum contradicts this theory as well, since the pattern of mimicry was similar to that of West Africa, while the pattern of polymorphism was similar to that of East and Central Africa, indicating that the lower mimetic load (only one mimic was observed) did not prevent *D. chrysippus* from showing considerable colour polymorphism (all the four forms were observed).

Future research on *D. chrysippus* at Sudan should adopt larger scale of sampling than the current study, so that conclusions can be made on more robust basis. Spatial variations as well as temporal changes in *Spiroplasma* prevalence, Sex ratio, morph ratio and mimics density should be investigated. Moreover, the cardenolides content (i.e. pyrrolizidine alkaloids) of Sudanese samples should be analyzed and compared with that of West and East/Central African populations, since it was suggested to vary geographically and to affect the intensity of Batesian mimicry (Brower *et al.*, 1975; 1978; Rothschild *et al.*, 1975).

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