Morph frequencies, sex ratios and infections in Danaus chrysippus populations in Rwanda

Title: Morph frequencies, sex ratios and infections in *Danaus chrysippus* populations in Rwanda

Running title: *Danaus chrysippus* polymorphism in Rwanda

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ABSTRACT

The African Queen butterfly (*Danaus chrysippus*) is noted for its colour pattern polymorphism despite its aposematic and chemically defended lifestyle and for its infection with a male-killing endosymbiont (*Spiroplasma*). The polymorphism is largely restricted to east and central Africa where three different colour forms meet and interbreed in a large contact or hybrid zone. The primary objective of this study is to present recent data on the polymorphism, sex ratio, and the incidence of male-killing in *D. chrysippus* populations in Rwanda. We also report previously unpublished data from a random sample collected in 1914 in Bujumbura in Burundi and on the differential effects of weather on males and females in Ghana from 1972-1974. We sampled butterflies using butterfly nets and either marked, recorded and released then or kept specimens for subsequent examination. We used PCR/microscopy on the preserved specimens to detect *Spiroplasma* and the presence of a protozoan parasite (*Ophryocystis*). Our findings suggest that Rwanda lies close to the western edge of the joint distribution of both *Spiroplasma* and the polymorphism. Further biogeographical studies are recommended to test the hypothesis

that male killing is restricted to the contact zone in which polymorphism is commonly observed.

Keywords: African monarch, colour pattern polymorphism, hybrid zone, male-killing endosymbiont

1 INTRODUCTION

Microbial endosymbionts are common in insects and can have major impacts on host ecology and population dynamics. These impacts are particularly noticeable in the case of heritable male-killers, which cause the production of all-female broods and often lead to skewed sex ratios (Hurst and Jiggins 2000). The prevalence of male-killing endosymbionts can vary both between species and within species (in space and time) (Hurst and Jiggins 2000; Hassan, Idris and Majerus, 2012), highlighting a need to better understand this variation. We here report new data from Rwanda on colour pattern polymorphism, adult sex ratios, and the presence of a male-killing (MK) endosymbiont (*Spiroplasma*) in the butterfly *D. chrysippus* (L.). The data are of special interest because of the possibility that a causal connection exists between the polymorphism and the presence of the *Spiroplasma* (Gordon 1984).

Spiroplasma, a mollicute bacterium closely related to *Spiroplasma ixodetis* (Jiggins, 2000; Herren *et al*., 2007; Hassan, Idris & Majerus, 2012) is vertically transmitted through the maternal line of *D. chrysippus*. Where it is present, infected males die in the egg stage, resulting in populations with female-biased sex ratios. The extent of this bias reflects the frequency of females infected with *Spiroplasma* (Smith *et al*., 2019). Around Nairobi, Kenya, where this frequency is the highest yet recorded, males

make up less than 5% of the adult population at some times of the year (Smith *et al.*, 2019). Available data suggest that infection rates decline to the north, south, east and west from this central focus. Intriguingly, infection appears to be invariably associated with a fusion between the autosome (chr15) that carries a supergene for colour pattern and the female-determining W chromosome, forming a neoW sex chromosome (Smith, Gordon & Allen, 2010; Traut *et al*., 2017, Martin *et al*., 2020).

The Nairobi populations fall within a polymorphic contact zone between otherwise allopatric colour pattern forms. Since *D. chrysippus* is distasteful and aposematic (Rothschild et al., 1975; Boppré, 1979, 1981; Clarke, Clarke & Gordon, 1995), this polymorphism is anomalous (Fisher,1930; Ford, 1964; Turner, 1977). Over much of Africa, different colour forms are present in different regions of the continent (illustrations and distributions in Smith *et al*. 2019) as essentially monomorphic populations, though minor variation occurs, and rare vagrants from other ranges are found. The all-orange forms were called dorippus in Smith *et al*. 2019, but are now known as klugii (Vane-Wright, 2020). Within the polymorphic contact zone, various intermediates result from incomplete dominance and recombination as described by Smith *et al.*, (2019).

Our new findings broadly support an earlier prediction that male-killing is confined within this contact zone (Gordon 1984), as would be expected if there was a causal connection between MK and the polymorphism. We discuss them with reference to previously reported data and a random sample of *chrysippus* in the Tervuren Museum in Belgium (described here for the first time) that was collected in 1914 in Bujumbura in Burundi. The sex ratios in this sample and the new ones from Rwanda are based on free flying adult butterflies. We agree with Idris and Hassan

(2015) that adult sex ratios are not a reliable indicator of the presence or absence of male killing and we use old unpublished data from Ghana (where male killing is absent), to show that this is partly because of weather conditions. To determine whether or not *Spiroplasma* occurs in *D. chrysippus* in Rwanda, we conducted PCR tests for its presence. Last, we report the presence of a protozoan parasite (*Ophryocystis*) that could play a role in the dynamics of *Spiroplasma* infection and male killing.

2 MATERIALS AND METHODS

2.1. Study areas and sampling procedures

In Rwanda, the present study was conducted in two sites namely Huye, in and around the Ruhande Arboretum (-2.613, 29.756, 1638-1737 m.a.sl) and the Nyamata area (-2.154, 30.076, 1368 m.a.sl) (Figure 1). The Ruhande Arboretum (RA) is approximately 200 ha in area in the Southern part of Rwanda (Tuyizere *et al.,* 2018). RA contains around 207 native and exotic tree species and provides habitats to a variety of animals including arthropods such as butterflies (Nsabimana *et al.,* 2013). The surroundings of Ruhande Arboretum are mainly composed of deforested land and marshland. The Nyamata site is marshland and Acacia savanna dominated by the invasive *Lantana camara,* an attractive nectar resource for African Queens. No larval food plants or other preferred plants were recorded at either site. Nyamata is located in the Bugesera District of Rwanda.

At Huye, butterfly samples were collected from March to May 2018. The existing trails in the Arboretum were used and sampling was performed three times a week; all collections were done between 9:00 am and 4:30 pm. Observed individuals along the trails were caught using a butterfly net and stored in envelopes. The collected specimens from

Huye were placed in plastic envelopes and preserved at -70° in the University of Rwanda Biotechnology Complex Laboratory for further analysis and educational purposes. The sex and colour pattern of each specimen (klugii, alcippus, orientis, chrysippus) were subsequently recorded. Recombinant and heterozygous forms (Smith *et al*. 2019) were classed as intermediates.

At Nyamata, we collected three sets of data between 2017 and 2019. Two of these data sets (December, 2017 to January, 2018; August-September, 2019) were from daily mark-recapture-release exercises. We captured butterflies from between 10:00 am and 01:00 pm, and marked (using permanent marker pens and individually coded spots on the hindwings) and released them to their natural habitat after recording their sex and colour pattern as for Huye. The third set of data from Nyamata came from butterflies collected and retained on 1-2 days each month, mainly in the morning between July, 2017 and August, 2018. The sex and colour patterns were recorded following the same procedure used for the Huye samples. No records of daily weather conditions were kept, although sampling on rainy and cold days was avoided because of low butterfly activity.

To better understand the possible influence of weather on observed sex ratios in our data, we reanalyzed sampling data collected for a previous study in Ghana (Gordon 1984), where *Spiroplasma* infection is believed to be absent. Sampling was conducted on the Cape Coast University Campus (5.07, -07.27, 15-20 m.a.sl) between 10.00 and 14.00 hours once a week from August 1974 to August 1976 (Gordon, 1984). At the time of collection, the campus comprised a variety of habitats

ranging through small patches of sacred forests through cassava, maize and vegetable farms and fallow land to the lawns, gardens, roads and car parks of the academic and administrative staff residential areas. The original vegetation (at one time, forest) had been drastically altered by human activities but still supported a large and vigorous population (since much diminished) of *D. chrysippus* and other open-country butterflies. Average rainfall was 1,193 mm p. a. during the study period, with four seasons (hot dry, major rains, cold dry and minor rains). Temperatures ranged from 21.8 °C (August) to 30.7 °C (April), conditions which were very close to those observed at Huye and Nyamata in Rwanda: 20-30 °C (Haggag *et al.,* 2016). No voucher specimens were retained: butterflies were captured, marked and released after recording their sex and their colour pattern. Field notes recorded the daily weather conditions as one of four possibilities: hot sunny, cool cloudy, sunny intervals, or partly rainy.

In order to examine the effect of sunshine on the activity levels of males and females in the Ghana samples, the weekly data were scanned for examples in which consistent and obvious fluctuations in weather conditions occurred between consecutive samples, thus minimizing any possible seasonal influences other than daily weather variations. Specifically, cases were identified in which two continuously hot sunny days were separated (in the records) by a continuously cool cloudy day or one such hot sunny day was sandwiched between two cool cloudy days. Five such sets of data were located as unambiguous examples and the numbers of captured males and females were examined for each data set. These records were obtained as part of a general study of the biology

of *D. chrysippus* in Ghana. They are included here because of their retrospective relevance for the interpretation of adult sex ratios.

Additional data from a random sample collected on one day (29th September) in 1914 at Bujumbura in Burundi were recorded from specimens preserved at the Tervuren Africa Museum, Belgium.

2.2. Laboratory processes

We analyzed 181 queen butterflies sampled at Huye, and 10 butterflies that had been captured at Nyamata, in the Biotechnology Laboratory Complex of the University of Rwanda. For detection of *Spiroplasma* infection, we used PCR following Martin *et al*. (2020). For detection of infection by the protozoan *Ophryocystis*, we used microscopic analysis of scales scraped from the thorax area to identify the spores, which have a characteristic shape and colour (Altizer and Oberhauser 1999). We also tested PCR-based detection of *Ophryocystis* on the 10 specimens from Nyamata. For PCR, tDNA was extracted from thorax and abdomen tissue using Smart Extract Protocol (Eurogentec, Belgium) following manufacturer instructions, and PCR was performed using OneTaq Master Mix (New England Biolabs, USA). For *Spiroplasma*, the primer pair F: 5'-GAAAATTTGCCAAGCAGTA-3' and R: 5'-AACTACGGAAATTGAAGGA-3' that amplifies 500bp of the *Spiroplasma* GDP1 gene was used (Martin *et al.*, 2020). For *Ophryocystis*, the primer pair F: 5'-GGAAAGGTTTTCTTTGAAAGCTC-3' and R: 5'-TGCCACATACACTTTCAGTCG-3' was used. The 25 µL PCR reaction contained 12.5 µL one Taq Mix, 1 µL each primer (0.4µM final concentration), 2 µL DNA and the volume was brought to 25 µL with nuclease free water. PCR conditions were as follows: predenaturation at 94°C for 30 sec, followed by 30 cycles of

denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec and elongation at 72°C for 30 sec and final elongation at 72°C for 5 min.

PCR amplification was checked by loading 15 µL of PCR products in 2% (w/v) agarose gel stained with Gel red in 1X Tris-acetate-EDTA (TAE) buffer for 30 min at 200 V and the PCR products were visualized on a UV gel documentation system.

2.3. Data Analysis

We performed Chi-square to test for differences in *D. chrysippus* abundances in terms of sex and morph by site and across the three sites (Huye, Nyamata and Bujumbura) and to test for the effect of weather conditions (cool cloudy and hot sunny) on the numbers of males and females captured during the earlier study in Ghana.

3 RESULTS

The present study reports findings on a total of 3,044 butterflies; 187 were from Huye and 850 from Nyamata, collected between 2017 and 2019; 82 from Bujumbura collected in 1914; and 1,925 from Ghana collected between 1974 and 1976. We present the proportions of males and females (Figure 2) and those of the major identified colour forms (klugii, chrysippus, orientis and alcippus, plus intermediate recombinants and heterozygotes) (Figure 3) across the Rwandan and Burundian sites (Huye, Nyamata and Bujumbura). We also report previously unpublished data on the differential effect of weather conditions on the activity of males and females in Ghana where all butterflies were form alcippus.

3.1. Sex ratios and morph frequencies

Sex ratios were significantly male biased at both Huye ($\chi^2 = 27.66$, df = 1, N = 187, male = 143, female = 44, $p < 0.001$) and Nyamata ($\chi^2 = 4.78$, df = 1,

N = 850, male = 470, female = 380, $p = 0.03$), but not different from 1:1 at Bujumbura ($\chi^2 = 1.20$, df = 1, N = 82, male = 34, female = 48, $p = 0.20$) where females were in excess. They were significantly different between the three sites ($\chi^2 = 37.89$, df = 2, N = 1,119, $p < 0.001$) (Figure 2).

The data on polymorphism show significant differences across the study sites ($\chi^2 = 420.19$, df = 8, N = 1,119, $p < 0.001$). Form orientis was the most frequent morph in the Huye and Nyamata sites with proportions of 0.70 and 0.60, respectively, while form klugii was the most frequent in Bujumbura study site with proportion of 0.52. Form alcippus was only recorded in Nyamata (Figure 3).

The overall proportion of males in the Ghanaian sample (N = 1925) was 0.59, showing a significant excess of males ($\chi^2 = 57.60$, df = 1, $p < 0.001$). There were significant changes ($\chi^2 = 84.02$, df = 24, $p < 0.001$) in monthly sex ratios, ranging from a minimum of 0.24 in the cool cloudy month of September 1974 (average min-max temperatures 24-30 °C, with exceptionally high rainfall in that year) to a maximum of 0.73 in November 1975 (22-27.5 °C, the month with the highest average number of sunshine hours /day) (Hayward & Addae, 1978; Gordon, 1982). We observed a strong effect of weather conditions on sex ratios and butterfly activity. Figure 4 shows that the average proportion of males was significantly higher ($\chi^2 = 17.65$, df = 1, $p < 0.001$) on hot (0.65) than on cool days (0.32). In nine of the ten successive days of contrasting weather, the direction of change in sex ratios was consistent with this result: proportions of males decreased as the weather turned from hot to cool and increased when it went from cool to hot.

3.2. Infections of *Spiroplasma* and *Ophryocystis*

The analysis of 181 and 10 *D. chrysippus* samples at Huye and Nyamata, respectively by PCR revealed the absence of *Spiroplasma* among all tested butterflies collected at Huye (139 male and 42 female), and its presence in one female out of seven females and three males from Nyamata that were tested (Table 1). The microscopic analysis revealed that one female butterfly from Huye was infected with *Ophryocystis* (Table 1). Although no *Ophryocystis* infection was detected in the 10 Nyamata specimens tested using microscopy, our *Ophryocystis* PCR detected three positives (two female and one male). Further work is needed to confirm the accuracy of this PCR.

4 DISCUSSION

If we are to understand ecological and evolutionary processes, we need to track them in space and time. This need is obvious in the case of *Danaus chrysippus*, where the joint concurrence of male killing due to infection with *Spiroplasma* and the presence of a neoW chromosome appears to be restricted to a large contact zone in east and central Africa. Genomic and biogeographic data (Martin *et al*., 2020) suggests that this neoW-MK complex first arose just once around 2,200 years ago, putatively near Nairobi, and has since spread in a selective sweep over much of east Africa. Its ecology and evolution are deeply entwined. It may have spread because of a tri-trophic interaction involving the butterfly, its natural enemies and its hostplant (Gordon, Ireri & Smith, 2014b), and it is sustained by migration. The neoW-MK complex restricts gene flow between populations and may be involved in an early speciation event (Gordon, Ireri & Smith, 2014a).

Adult samples from three of the four sites (Huye, Nyamata and Cape Coast) reported here showed significant excesses of males. There was no

significantly unequal sex ratio in the Bujumbura collection. However, it is clear that data based on wild adult captures (or sightings) should be interpreted with caution (Idris and Hassan 2015). Males are commonly over-represented in adult butterfly samples because they are more active, especially on hot and sunny days. The reverse can also occur when cool cloudy conditions reduce male activity, lowering the proportion of males to as low as 0.24 (Godon, 1982). Such conditions could have prevailed on the 29th September 1914 when the Bujumbura collection was obtained. It follows that we cannot conclude that the female excess in the Bujumbura sample (Figure 2) is evidence for male killing in 1914. As shown here, changing weather conditions can cause adult fluctuations in sex ratios from one day to the next. The changes in the proportions of males recorded in Ghana suggest that cool weather reduces activity levels of females and males by 30% and 80% respectively (Gordon, 1982). It is also possible that the time of day differentially affects their activity levels but no records were kept of the time each specimen was captured.

While the estimates of the effects of weather could potentially be used to “correct” observed sex ratios in cool and hot weather in order to detect biases arising from male-killing, the results might be misleading. Sex ratios are influenced by too many other factors, such as the nearby presence or absence of larval foodplants or pyrrolizidine alkaloid sources, and the sampling location in relation to the differential dispersal and migration tendencies of males and females (Smith *et al.*, 2019). The presence or absence of *Spiroplasma* (and by inference of male-killing) can therefore only be reliably demonstrated by molecular screening of large samples of females. Where this is not possible, more laborious methods, such as breeding from wild females, or the collection of wild-laid eggs

and the monitoring of hatch rates, are needed to determine with certainty whether male killing is present. The PCR tests showed that *Spiroplasma* is present in Rwanda but at low frequencies; only one out of 49 females was infected.

If we assume that the Huye sample is representative of current populations at Bujumbura (the sites are separated by only 100km, a distance well within routine dispersal distances of *D. chrysippus* and a short distance in the context of the size of the overall contact zone), then the data suggest major changes in the last 100 years. At Huye and Nyamata (2017-2019), the rank order of the first three colour pattern morphs is orientis > chrysippus > klugii; in the 1914 Bujumbura sample it is klugii > orientis > chrysippus (Figure 3). It therefore appears that *klugii* has greatly declined in frequency in this region in the last hundred years. A similar decline has been recorded in Uganda (Smith *et al*., 1993, Table 4 and Figure 2), suggesting that it may apply generally to areas west of the Rift. We now know that high frequencies of the neoW-MK complex to the east would completely block the transmission of the all-orange forewing genes (Martin *et al*., 2020). If these high MK frequencies are relatively recent, this could explain the decline in *klugii* in the region since 1914.

We also report the infection by a protozoan parasite related to *Ophryocystis electroscirrha* in *D. chrysippus* in Rwanda. This had previously been discovered in two populations in Kenya (Watamu amd Kitengela) by Laemmermann (2018) Infection by *Ophryocystis* in North American *D. plexippus* is associated with reduced fitness (Altizer and Oberhauser 1999) and altered egg laying behaviour in a form of trans-generational medication (Lefèvre *et al.*, 2010). Our finding of a related parasite in *D. chrysippus* highlights a need to better understand its distribution and effect on the butterfly, as well as any possible interaction with

Spiroplasma. The latter is strictly maternally inherited (Martin *et al.*, 2020), whereas *Ophryocystis* is transmitted through ingestion of spores (Leong *et al.*, 1997), and is therefore capable of horizontal transmission. The rapid spread of the neoW-MK complex implies some fitness benefit to *Spiroplasma*-infected females, either as an indirect consequence of male killing (Gordon, Ireri & Smith, 2014b) or a direct effect such as boosted immunity (Hurst *et al.*, 2010). Whether this benefit is influenced by the presence of *Ophryocystsis* or other pathogens or paraistoids remains to be investigated.

5 CONCLUSION

We present evidence that morph frequencies in Rwanda and Burundi may have changed over the last hundred years, and that adult sex ratios need to be interpreted with caution because they are so strongly affected by weather conditions. Our most important finding is that Nyamata and Huye lie on the south western limits of the range of *Spiroplasma* (10% in Nyamata, 0% at Huye) and form klugii (2% at Nyamata, 1% at Huye). These estimates provide baselines for any future changes and are directly relevant to the prediction that male killing will be found to be limited to the hybrid zone. Further studies are required at other similar sites on the boundaries of this zone. In particular we need data from DRC, South Sudan, Ethiopia, Somalia, Zambia and Mozambique. A citizen science project would be ideal for addressing this gap in our knowledge.

CONFLICT OF INTEREST

The authors declare that they have no financial interests or personal relationships which may have inappropriately influenced them in authoring this paper.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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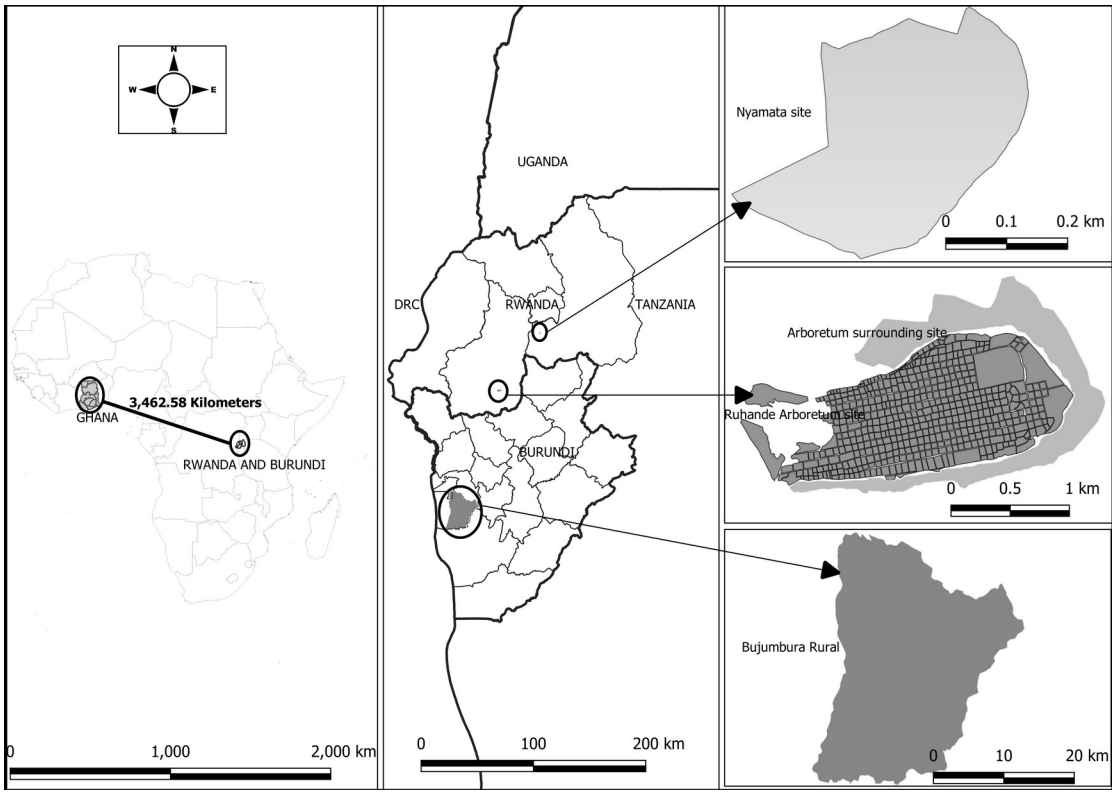
504 Table 1. Abundances (% in parenthesis) of *D. chrysippus* (L.) from PCR
505 analyses for *Spiroplasma ixodetis* and microscopy for *Ophryocystis*
506 *elektroscirrha* across the two study sites in Rwanda

Sites	Totals	Male	Female	*Spiroplasma* +		*Ophryocystis* +	
				Male	Female	Male	Female
Huye	181 (100)	139 (76.8)	42 (23.2)	0 (0)	0 (0)	1 (0.7)	0 (0)
Nyamata	10 (100)	3 (30)	7 (70)	0 (0)	1 (14.3)	1 (33.3)	2 (28.6)

507


N
W
E
S
3,462.58 Kilometers
GHANA
RWANDA AND BURUNDI
0
1,000
2,000 km
UGANDA
DRC
RWANDA
TANZANIA
BURUNDI
0
100
200 km
Nyamata site
0
0.1
0.2 km
Arboretum surrounding site
Ruhande Arboretum site
0
0.5
1 km
Bujumbura Rural
0
10
20 km