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Infection with a male-killing *Spiroplasma* bacterium might drive morphological changes in female reproductive organs in the African monarch butterfly

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Background. Sexual selection and conflicts within and between sexes promote morphological diversity of reproductive traits within species. Variation in the morphology of diagnostic reproductive characters within species offer an excellent opportunity to study these evolutionary processes as drivers of species diversification. The African monarch, *Danaus chrysippus* (Linnaeus, 1758), is widespread across Africa. The species is polytypic, with the respective geographical ranges of the four colour morphs only overlapping in East Africa. Furthermore, some of the populations host an endosymbiotic bacterium, *Spiroplasma*, which induces son-killing and distorts the local host population sex-ratio, creating sexual conflicts.

Methods. We dissected females from Kenya, Rwanda and South Africa, and conducted microscopy imaging of their reproductive organs. We then characterized the effect of population, female body size, and female mating status, on the size and shape of different genitalia characters of the *D. chrysippus* female butterflies.

Results. We showed that although the general morphology of the organs is conserved, female genitalia vary in size and shape between and within populations. The small virgin females have the smallest organs, while the same organs were expanded in mated females. Females from highly female-biased populations, where the male-killing *Spiroplasma* is prevalent, also have a larger area of their corpus bursae covered with signa structures. These results suggest that male depletion due to the symbiont, might result in smaller spermatophores, and select for female genitalia features that optimize the digestion of small nutritious spermatophores.

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Abstract

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Keywords

Corpus bursa, signum, nuptial gift, sex-ratio distortion, bacterial symbiosis

Introduction

Reproductive organs, or genitalia, are under strong selection, which generally leads to considerable variation between species, but relative conservation of the traits within species (House et al. 2013; Langerhans et al. 2016). Consequently, many of these traits can offer diagnostic morphological characters that are of utmost importance for the systematic classification of fauna, but that may still present some variability due to stochasticity, plasticity, and diverse evolutionary processes, including sexual selection. For example, variations in body size often result in changes in the size of body structures and internal organs in insects (Polilov & Makarova 2017). Furthermore, although large specimens often hold large organs, the proportion of the changes might vary between organs and the species considered (Polilov & Makarova 2017). Similarly, sexual selection, male-male competition (ie. sperm competition) and/or male-female conflict (*ie*. divergent interests over fertilization) can induce changes in the morphology of genitals within species (Brennan & Prum 2015; Cordero 2005; Hosken et al. 2001). For example, in the taurus scarab, *Onthophagus taurus* (Schreber, 1759), morphological variation of the *endophallus* ~~*scerites*~~ was found to influence male success in populations where females mate multiple times (House & Simmons 2003; Simmons et al. 2009); while in gerrid water striders, male grasping structures have evolved as a response to male-male competition for female guarding until fertilization of the eggs (Arnqvist & Rowe 2002).

Maternally inherited bacterial symbionts, such as the bacterial taxa *Wolbachia* and *Spiroplasma*, are widespread in insects (Ferrari & Vavre 2011; Vancaester & Blaxter 2023). They owe their success to their ability to modify their host reproductive system towards their own successful transmission through the host generations. One of the phenotypes these bacteria can induce in their hosts is the selective death of the male offspring at early developmental stages. Male-killing symbionts have been reported in diverse insect species, including Diptera, Coleoptera and Lepidoptera (Dyson et al. 2002; Graham &

Wilson 2012; Hurst et al. 2000; v d Schulenburg et al. 2002), and their prevalence range from 5% to over 95% across the host populations (Charlat et al. 2009; Duplouy et al. 2010; Gordon et al. 2014). For instance, the blue moon butterfly, *Hypolimnas bolina* (Linneaus, 1758), and the African monarch butterfly, *Danaus chrysippus* (Linnaeus, 1758), can host a MK *Wolbachia* or *Spiroplasma* (Charlat et al. 2006; Dyson et al. 2002). In these insect hosts, the death of the sons of symbiont-infected mothers often leads to sex-ratio distortions (Charlat et al. 2005; Jiggins et al. 2000a; Jiggins et al. 2000b) that shape the ecology and evolution of the host species (Engelstädter et al. 2007; Engelstädter et al. 2004) by affecting host population size and increasing the risk of population extinction (Hurst & Jiggins 2000).

In Lepidoptera, and in some other insects, males produce spermatophores or 'mating gifts', which contain sperm and nutrients that are transferred to the female ovipore during copulation. Each spermatophore can make up to 13% of a male's body weight (Galicia et al. 2008), and their production by the male is costly and limited. The size of the spermatophore thus typically depends on resources acquired and depleted across the male lifespan (Duplouy & Hanski 2015; Duplouy et al. 2018; Kaitala & Wiklund 1994). As a result of the MK infection, the rare *H. bolina* males from the highly female-biased populations were described as resource depleted because they were in high mating demands (Charlat et al. 2007). Additionally, a high proportion of the local females were found unmated while the mated females laid few fertile eggs (Charlat et al. 2007) because the females had received a limited amount of sperm during copulation (Charlat et al. 2007). In this context, sexual conflicts may arise, as females race to evolve optimized reproductive organs that accommodate, digest, and convert spermatophores into resource towards their own fecundity.

We dissected specimens from several populations across the African range of *D. chrysippus*, including populations where the MK *Spiroplasma* symbiont was absent or present at high or low prevalence, to

unravel whether variation in the female reproductive organs is correlated with female size, population, mating status or local prevalence of the MK symbionts. We showed variations in several characters of the genitalia of mated versus virgin females, and in the context of local sex-ratio distortions due to the MK symbiont, and thus suggest that in addition to possible variations between populations and individuals due to stochasticity or plasticity, the size and shape of the female reproductive organs may differ due to selective pressures associated with changes in their males conditions.

Material and methods

Samples

The African monarch butterfly, *Danaus chrysippus* (Lepidoptera: Nymphalidae) is a species belonging to the family Danainae. It is one of the most common and widely distributed butterflies in Africa and Australasia (Hassan et al. 2012; Idris & Hassan 2012) and is increasingly common in southern Europe, especially during summer (Koren et al. 2019; Liu et al. 2022). The species is found in many different habitats, such as mountains and deserts, but primarily occurs in open landscapes such as around farmlands (Hassan et al. 2012). Caterpillars of *D. chrysippus* often feed on Asclepiadoideae plants, particularly on toxic milkweeds (*Asclepias*) (Robinson et al. 2010). Four subspecies of *D. chrysippus* live in separate areas of the overall African species range, but interbreed in one common central hybrid zone (Herren et al. 2007; Liu et al. 2022). In this hybrid region, the MK-symbiont is prevalent and the observed males are rare migrants from uninfected populations surrounding the hybrid zone (Martin et al. 2020; Ndatimana et al. 2022; Smith et al. 2016; Smith et al. 2019).

In this study, we included 67 female specimens from the hybrid zone collected in Rwanda (N=29) and from two localities in Kenya (N=38), and 21 female specimens from uninfected populations from three localities in South Africa (Table1). The Kenyan populations host a male-killing (MK) *Spiroplasma* symbiont and show high female-biased sex-ratio (Martin et al. 2020). In Rwanda, the MK-inducing

111 symbiont is less common and the sex-ratio distortion remains low but variable through/across the
112 year(s) in that region (Ndatimana et al. 2022). The populations from South Africa are not known to carry
113 the symbiont, and do not show any patterns of sex-ratio distortion (Hassan et al. 2012; Jiggins et al.
114 2000b).

115 All but a few specimens were collected as adults in the field and killed shortly after collection. The few
116 specimens collected as larvae in the field, originated from two populations (Rwanda and Kenya) and
117 were killed in the laboratory shortly after emerging from their pupae. All specimens were labelled and
118 stored individually in 90% ethanol in the freezer until further manipulated (Martin et al. 2020). Thorax
119 tissue from some of the specimens have been previously used for population genetics and genomics
120 research on the butterfly host (Martin et al. 2020), but all abdomens were intact and of good quality to
121 support the present research.
122
123 Samples were collected under the following permits: NACOSTI/P15/3290/3607;
124 NACOSTI/P15/2403/3602 (National Commission for Science and Technology, Kenya);
125 MINEDUC/S&T/459/2017 (Ministry of Education, Rwanda); MPB.5667 (Mpumalanga Tourism and Parks
126 Agency, South Africa); FAUNA 0615/202 (Department of Environment and Nature Conservation,
127 Northern Cape Province, South Africa).
128
129 Dissections
130 We prepared the genitalia of 51 specimens according to standard methods used in Lepidoptera
131 (Hardwick 1950; Robinson 1976). In brief: the abdomens were heat treated at 94 °C for about 10
132 minutes in 10% potassium hydroxide (KOH) to remove fat and other soft tissue. The remaining tissues
133 were cleaned in sterile water under the microscope with the help of small brushes. The abdomens were
134 then individually and carefully cut open laterally with small scissors and tweezers, starting from the base

of the abdomen until the seventh abdominal segment. We then detached the genitalia from the basal part of the abdomen by cutting and pulling apart the seventh and eight abdominal segments. The genitalia were then cleaned, coloured with Chlorazol Black, and transferred to 99 % ethanol to harden the remaining structures. The female organs targeted in this study were the corpus bursa, a bag-type organ that receive the male's contribution to reproduction during copulation, the so-called spermatophore or nuptial gift. All dissections were prepared at the Finnish Museum of Natural History in Helsinki (LUOMUS).

The abdomens of the remaining 35 samples, not treated with heat and KOH solution, were opened dry with the help of sterile toothpicks under the microscope to extract the undissolved spermatophores inside of the corpus bursae.

Imaging

The wings of all specimens were imaged using an Epson 10000XL flatbed scanner including a measuring ruler. We photographed each corpus bursa under four different angles (ventral, dorsal, left, and right sides) using the Leica LAS EZ software (version 3.4.0) with the same microscope magnification for each sample. We used the Fixator method as described in Wanke et al. (2019) to fix the female genitalia in the desired position on a petri dish using a nylon thread. The female genitalia and the spermatophores were individually photographed on the side of a graduated scale (millimetre paper or ruler).

Measurements of the wings, the genitalia and the spermatophores

All measurements were done using the software ImageJ version 1.53e (Collins 2007; Schneider et al. 2012). All images included a small piece of millimetre paper for scale.

To test whether the size of the females affected the size of their reproductive organs, we measured the length between forewing veins CuA1 and CuA2 (Figure 1d) as a proxi for the size of the butterflies. To

compare the size of each signum structure between individuals, or against the size of the corpus bursae, or that of the wings, we measured the total area of the corpus bursae (including the area of the appendix bursa), and the area of each signum (Figure 1a & b). To measure variation in the males' contribution to mating between populations, we measured the area of each spermatophore dissected out from the corpus bursae of the females (Figure 1c).

Mating status

Several specimens from two populations (Rwanda and Kenya) were collected as larvae in the field and remained virgin before being killed in the laboratory. We characterized the shape of the genitalia of those known virgin females, before suggesting whether the other females caught as adults were mated or not after visual comparisons. Unfortunately, the KOH treatment dissolved the spermatophore structures within the treated bursae. Thus, we could not directly test how many spermatophores were acquired by the mated females dissected with this method. Additionally, the dissection of the corpus bursae necessary to remove the stored spermatophore(s) led to the destruction of the corpus bursa which could therefore not be measured. Consequently, female genitalia measurements and spermatophore data came from different individuals.

Molecular work

All molecular work was done at the Molecular Ecology and Systematics lab at the University of Helsinki. We extracted the DNA from one abdominal section using a Qiagen DNeasy Blood & Tissue Extraction Kit (Cat. #69506, Qiagen, USA) for 67 specimens individually, while all other samples were screened for the infection for the purpose of an earlier study (Martin et al. 2020). The quality of the DNA extracts was tested by PCR through the amplification of the 5'-end region (~ 654 bp) of the *cytochrome oxidase I* (*COI*) mitochondrial gene using the primers LCO-1490/HCO-2198 (Folmer et al. 1994). To screen for

183 *Spiroplasma*, we amplified the *GDP Spiroplasma* gene, using the primer pair GDP1-F/GDPI-R (Martin et
184 al. 2020). Each PCR included a negative control (water) and a respective positive control (Deng et al.
185 2021).
186
187 Statistical analyses
188 All statistical analyses were performed in R version 4.1.3 (RCoreTeam 2020). The response variables
189 were individually checked for normality, and log-transformed prior analysis when appropriate (ie. for
190 largest spermatophore size, or signum area). We analysed signum area, and corpus bursa area using
191 ANOVAs with wing size, population, and female assigned mating status (mated vs virgin) as fixed factors.
192 We analysed wing size, wing size-corrected signum area, wing size-corrected corpus bursa area, wing
193 size-corrected signum to bursa area ratio, and spermatophore area using ANOVAs with population and
194 female assigned mating status (mated vs virgin) as fixed factors, followed by a Tukey's honest
195 significance test, and a Bonferroni adjustment for multiple testing. We used a Kruskal-Wallis test to
196 analyse the effect of population on the number of spermatophores found per female.
197
198 Data availability statement
199 All ecological data and images are available from Zenodo.org: doi: **10.5281/zenodo.7743561**
200
201 **Results**
202 Spermatophores
203 The spermatophores extracted from the corpus bursae varied in their colours, ranging from orange to
204 silky white; in their shapes, from spheres to flat shapeless shreds (Figure 3a); and in their size/surface
205 area, from 0.956mm^2 to 13.673mm^2 (Figure 3b). However, the size of the largest spermatophore per
206 mated female did not significantly differ between populations (ANOVA, df=2, p=0.18, Figure 3b).

207

208 The number of virgin females caught as adult in the wild was the highest in Kenya (56%), followed by the
209 Rwanda population (10%), while all females from South Africa were mated (Figure 3c). The mated
210 females had acquired between 1 and 6 spermatophores, with an average of 1.95 spermatophores per
211 dissected mated female, or 1.54 spermatophores per dissected female (including all field collected adult
212 females, Table 1). The spermatophore count per female was not significantly different between
213 populations (Kruskal-Wallis H=3.605, df=2, p=0.17; Figure 3c, Table 1).

214

215 Wing size

216 The distance between forewing veins CuA1 and CuA2 (Figure 1) ranged from 3.504mm up to 5.867mm
217 in length. Wing length did not significantly vary between populations (ANOVA, df=2, p=0.88), nor
218 between females of different infection status (ANOVA, df=1, p=0.47, Figure 2).

219

220 Infection status

221 In total, 37 specimens were found infected with *Spiroplasma*, including seven from Rwanda
222 (*Spiroplasma* prevalence=24%) and 30 from Kenya (prevalence=79%). All specimens from South Africa
223 were uninfected.

224

225 Female reproductive organs

226 We showed that the corpus bursa of *D. chrysippus* female butterflies was often topped by an appendix
227 bursa, and had two signa, one on the ventral side and the second on the dorsal side. The signa were
228 darker than the rest of the corpus bursa due to rows of sclerotized spike-like structures across their
229 surfaces (Figure 4). The shape and size of the corpus bursae, the appendix bursae and the signa visually
230 varied between our specimens.

231 There was no size difference between the ventral and dorsal signa of a specimen (Supplementary Fig.
232 S1). Thus, for simplicity, we only used the values from the ventral signum for the following analyses.
233 Additionally, although larger females showed slightly larger corpus bursae, the difference was not
234 statistically significant (ANOVA, *df*=1, *p*=0.15); however larger females showed significantly larger signa
235 (ANOVA, *df*=1, *p*=0.04, data not shown). To take the size of the female into account, we used wing size-
236 corrected organ areas for all analyses described below.
237
238 a Virgin versus mated females
239 We had specimens collected as larvae from two populations (Rwanda and Kenya), which provided adult
240 virgin specimens. The corpus bursae and associated signa and appendix bursae from these specimens
241 were compact, folded, and highly wrinkled (Figure 5). Based on these observations, we suggested that
242 field collected specimens with expended corpus bursae of orange colour, and signa of beaver-tail shape,
243 were mated females (Figure 5); while others showing clear, compact, folded, and wrinkled organs were
244 virgin.

245 Using only field collected adult females, we found that the corpus bursa area and the signum area were
246 smaller in virgin compared to mated females (Tukey test, $p<0.01$, and $p<0.01$, respectively,
247 Supplementary Fig. S1). Additionally, the ratio between the signum area and the corpus bursa area was
248 larger in the virgin females in all populations (Tukey test, $p<0.01$, Figure 6). There was no significant
249 interaction between female mating status and population on neither trait ($p>0.05$).
250
251 b Population comparison
252 Using only field collected adult females, we showed that the surface area of the corpus bursae (including
253 the appendix bursae) varied between 4.10mm^2 and 12.69mm^2, while the surface area of the signa varied
254 between 1.24mm^2 and 4.03mm^2 (Supplementary Fig. S1). Females from Rwanda showed the largest

corpus bursae (Rwanda vs Kenya, Tukey test, $p<0.01$; vs South Africa, Tukey test, $p<0.01$); and females from South Africa showed larger corpus bursae than females from Kenya (Tukey test, $p=0.015$; Supplementary Fig. S1A). Females from Rwanda also showed larger signa than females from Kenya (Tukey test, $p<0.01$, Supplementary Fig. S1B). A small ratio between the signum area and the corpus bursa area would mean that the signa cover a small surface of the bursa, while a large ratio would mean that the signa cover a large surface of the bursa. The ratio between signum area and corpus bursa area was significantly smaller in females from Rwanda compared to females from Kenya (Tukey test, $p<0.01$), but not when compared to females from South Africa (Tukey test, $p=0.12$, Figure 6). The ratio between signum area and corpus bursa area was also significantly smaller in females from South Africa compared to females from Kenya (Tukey test, $p<0.01$, Figure 6).

Discussion

According to our knowledge, we provided the first images and study of the female reproductive organs of the African Monarch *D. chrysippus*. Consistent with early schematic drawings by Mal et al. (2015), female *D. chrysippus* butterflies have two signa. Each signum is of similar size, of a beaver-tail shape, and cover on average 32% of each side of the corpus bursa, but do not extend into the appendix bursa.

We demonstrated that in *D. chrysippus* the size of the corpus bursa, that of the signa, and their ratio, varied with the mating status of the females regardless of their population of origin. In each population, the virgin females showed smaller organs, while mated females showed expanded organs. Comparative illustrations of virgin versus mated female genitalia are scarce in insects and other arthropods (Mouginot et al. 2015; Sihvonen & Mikkola 2002), but there is evidence for the female organs to vary in their shape, size and possible functionality after mating. For example, in seed beetles (Coleoptera: Bruchidae), male genitalia are armed with sclerotized spikes that serve as anchors to the female during

copulation ~~but~~ that cause scar-tissues to be observed in mated females only (Crudgington & Siva-Jothy 2000; Edvardsson & Tregenza 2005). Similarly, in the orb-weaving spider, *Larinia jeskovi* (Marusik, 1987), the male removes a coupling device (i.e. scapus) from the female external genitalia after copulation, inhibiting the possibility for the female to remate (Mouginot et al. 2015). In *D. chrysippus*, we suggest that mated females showed larger organs because they were filled up with one to several spermatophores from their mate(s). Unfortunately, the KOH treatment for the fixation of the female organs destroyed the spermatophores within the bursae in *D. chrysippus*. This has challenged our ability to obtain both the data on males' contribution and female genitalia traits from the same individuals as it does not allow (I) to determine with certainty which females were mated or not before dissection, although lab-reared virgin individuals were informative, (II) to determine how many times each female had mated before dissection, and (III) to evaluate the size and composition of the male's contribution to mating in each population.

The signum structures coupled with muscles associated with the corpus bursa (Allman 1930) have been described as '*lamina dentata*' (Petersen, 1904), a structure possibly involved in the digestion, by grating, of the nutrient-rich surface of the spermatophores after copulations (Cordero 2005; Galicia et al. 2008; Xochipiltecatl et al. 2021). If this is true, we expected that natural selection will act on the female genitalia in response to sexual conflict. In polyandrous butterfly species, such as *D. chrysippus*, we expect males to transfer large spermatophores that can act as mating plugs in the receiving females (McNamara et al. 2009; Wedell 1993). In response, females might evolve organs that efficiently digest each nuptial gift to allow for multiple mating and the avoidance of the fertilization of all the eggs by a unique genitor. Additionally, as the nutrients received from the males can be upcycled towards the production of eggs, or of better-quality eggs (Wedell & Karlsson 2003), females might evolve organs that

also optimise the intake from the nuptial gift (Meslin et al. 2017), especially in population where males transfer small spermatophores.

We found that the surface area of the female corpus bursa, that of the signa, and their ratio, were different between populations of *D. chrysippus*. Unfortunately, although we showed that spermatophores vary in size between *D. chrysippus* specimens, the size variation between populations was not significant and could not be linked to variation in the local prevalence of the MK-Spiroplasma symbiont between populations, maybe due to our sample size being too small. The study in the blue-moon butterfly by Charlat et al. (2007) thus remains the only known example of male butterflies becoming resource depleted because of a prevalent MK-symbiont. Nonetheless, in the *D. chrysippus* *Spiroplasma*-free populations from South Africa with no sex-ratio distortion, the ratio between signum and bursa area is intermediate. In contrast, in the Kenyan populations, where we expected smaller spermatophores due to male resource depletion and local female-biased sex-ratio due to high prevalence of the MK-*Spiroplasma* symbiont, females presented a larger area of their small corpus bursae being covered with signa structures. Such larger signa structures would likely more efficiently mechanically digest the spermatophores the females received from their local mates. Clearly, additional studies in other Lepidoptera are needed to test whether male resource depletion is common in MK-infected species and whether changes in spermatophore size between populations can indeed drive local changes in the female reproductive organs.

The location of the genes responsible for the female reproductive characters will likely influence the possibility that changes in those traits might be driven by selection (Charlesworth et al. 1987; Rice 1984). In Spiroplasma-infected *D. chrysippus*, the W female chromosome is fused to an autosome, and is called the neo-W chromosome (Smith et al. 2016; Smith et al. 2019). If the genes coding for the signa and/or

bursa were to be located on the neo-W, there could be a strong role for selection in the low sex ratio Kenyan populations, since this chromosome is matrilineally inherited and is found in up to 95% of all females (Martin et al. 2020). However, if these genes were to be located on any other chromosome, the dilution effect of incoming genes (50%) from immigrant males that are likely to have come from high sex ratio populations, together with their subsequent elimination with dead males in each generation, may considerably impede such selection.

Morphological traits such as the count and shape of the signa and the smoothness or complexity of their surface can provide taxon-specific diagnostic characters as they vary enormously among Lepidoptera species (Scoble 1995). The signa have for example been described as smooth, or ornamented with micro-protuberances of different ornamented shapes (ie. spikes, teeth, spines, horns, bands, patches, or plates, Galicia et al. 2008). We showed that both signa are covered with spike-like sclerotized structures of similar size, which give the signa their darker colour compared to the rest of the bursa. In comparison, the Monarch butterfly, *D. plexippus* (Linnaeus, 1758), has a large, pear-shaped corpus bursa with two large signa, each covered with bands of heavily chitinized micro-protuberances pointed in opposite directions from the median (Rogers & Wells 1984; Urquhart 1960), while Mal et al. (2015) only described the bursa in the striped tiger butterfly, *D. genutia* (Cramer, 1779), as a large balloon-shaped bag with two rod-life sclerotized signa. The female organs thus seem similar between these three *Danaus* butterfly species, however in general the bi-signate condition is uncommon in Lepidoptera. For example, in geometrid moths, the two signa character is rare (Murillo-Ramos et al. 2021 and references therein). However, the lack of extensive morphological revision describing the female genitalia of Nymphalidae or Danainae butterflies, where *D. chrysippus* is classified, does not currently allow the use of these morphological results in a wider evolutionary framework for these butterflies, contrasting for instance with the work done in Tortricidae (Lincango et al. 2013). Furthermore, although it has been suggested

from few other Lepidoptera species (Xochipiltecatl et al. 2021), whether and how the digestion of the spermatophore occurs, and whether the signa and bursa are indeed involved in the mechanical digestion of the spermatophore in *D. chrysippus* remains to be fully experimentally tested.

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Author contributions

AD and PS designed the study; SHM, IJG, JM and AD collected the data; JM and AD analyzed the data; All authors discussed the results, wrote, and commented on the manuscript.

Competing interests

The authors declare no competing interests.

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Table 1(on next page)

Table 1: Number of female specimens dissected from each population that were either treated with KOH for morphometric measurements of the female genitalia, or untreated for collecting their spermatophores.

Sample size includes specimens collect in the field both as adults or larvae. 'NA': Not applicable

1

Population	*Spiroplasma* prevalence	Sex-ratio	Number of samples (N=)	Dissection	
				Genitalia	Spermatophore
Kenya	High	Female biased (Martin et al. 2020; Smith et al. 2019a)	38	28	10
Rwanda	Low	Variable (Gordon et al. In Press)	28	10	18
South Africa	Null	Unbiased	21	12	9
Total of all populations:			**87**	**50**	**37**

2

3

Table 2(on next page)

Table 2: Number of mated and virgin females from each population, with the average number of spermatophore per mated female, and average spermatophore count per population, excluding specimens collected as larvae.

'NA': Not applicable. Numbers in parenthesis indicate the number of field-collected larvae reared to adulthood in the lab. One dissected specimen from Rwanda was excluded from the total number of dissected specimens due to hard unidentified material in the female tissues.

1

Population	Life stage at collection	Mated females	Virgin females	Dissected females	Spermatophore count	Average spermatophore count per mated female	Population average spermatophore count
Kenya	Adult Larva	4 NA	5 1	9 1	11 NA	2.75 NA	1.22 NA
Rwanda	Adult Larva	9 NA	1 7	10 7	17 NA	1.89 NA	1.7 NA
South Africa	Adult	9	0	9	15	1.67	1.67
Total		**22**	**6 (+8)**	**28 (+8)**	**43**	**1.95**	**1.54**

2
3
4

Figure 1

Figure 1: Four types of measurements.

(a) the surface area of the corpus bursa including the appendix bursa, (b) the surface area of the signum, (c) the surface area of spermatophores, and (d) the absolute vein length between forewing veins CuA1 and CuA2 as a proxi for wing size.

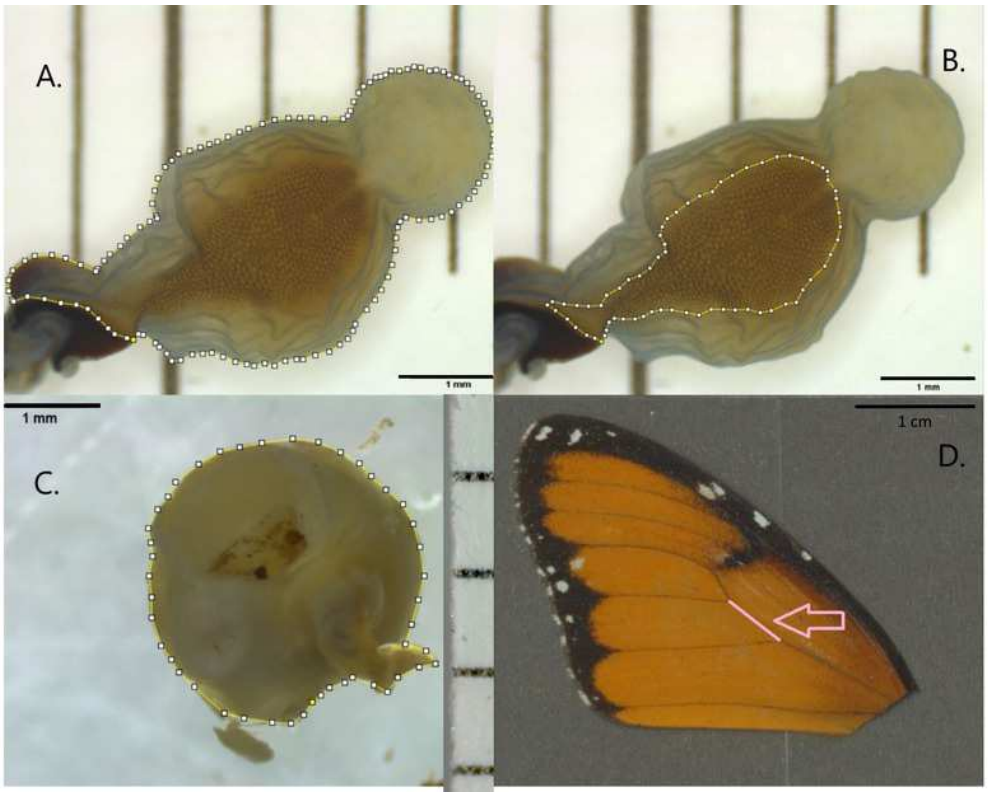


Figure 2

Figure 2: Female wing size (measured as the distance between forewing veins CuA1 and CuA2) at each population, and between specimens infected (dashed-lines) or not (full-lines) by a male-killing *Spiroplasma*.

The boxes represent the interquartile range of the data, and the heavy horizontal lines represent median values, whiskers give the 95% lower and upper percentiles.

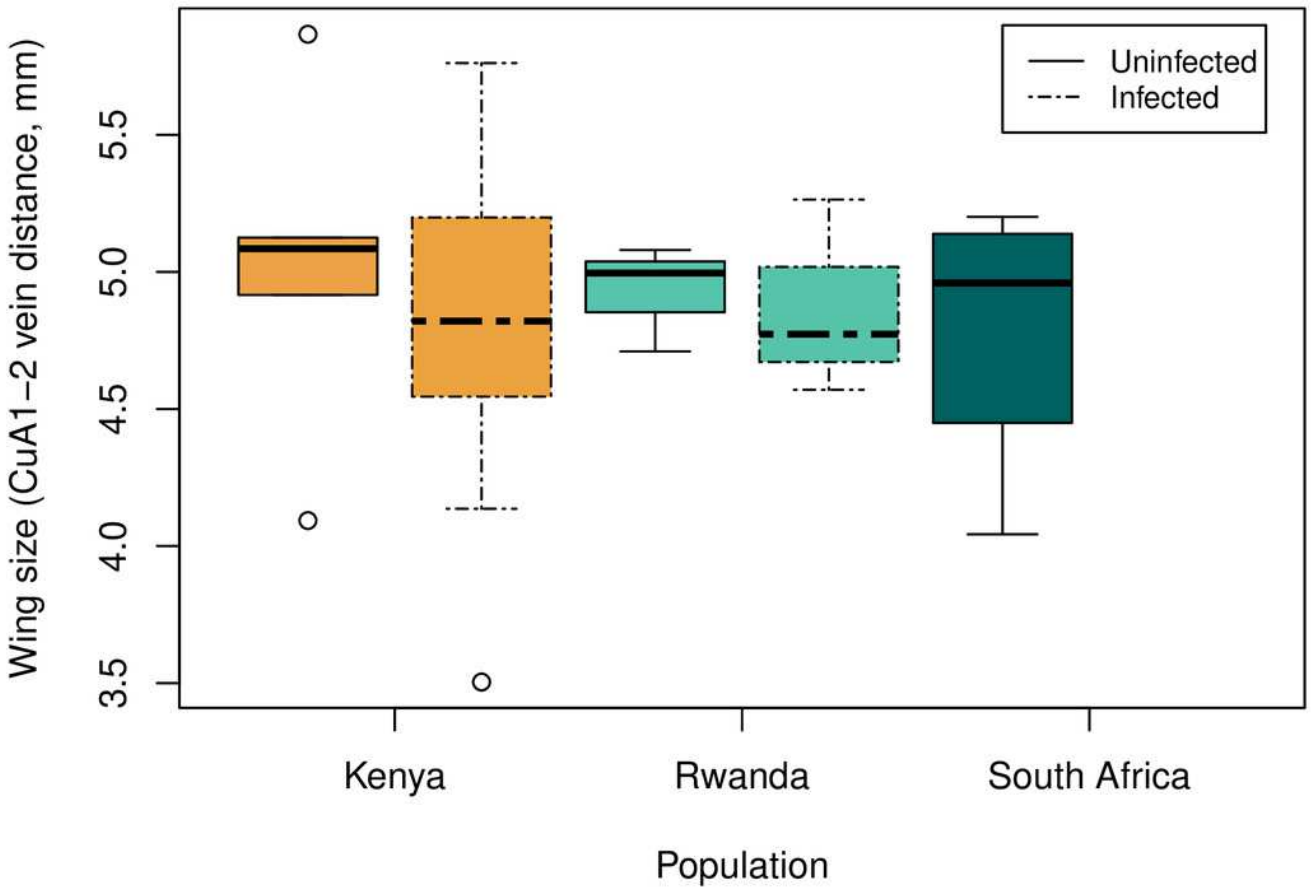
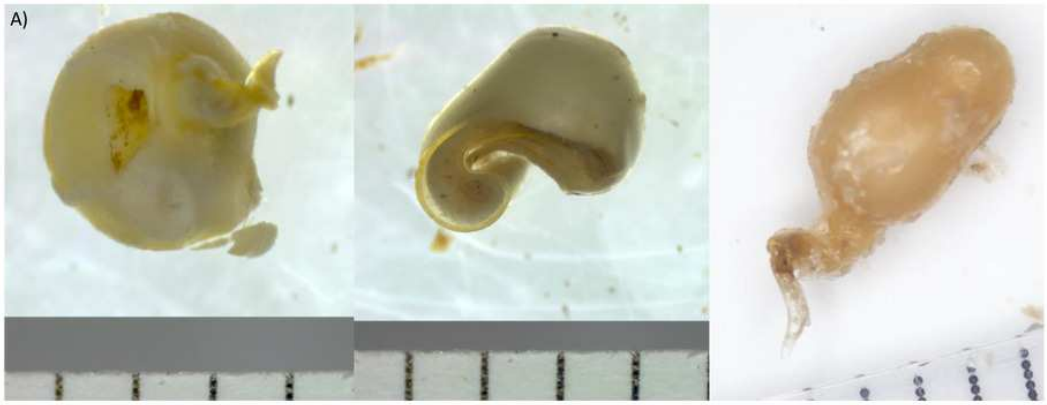


Figure 3

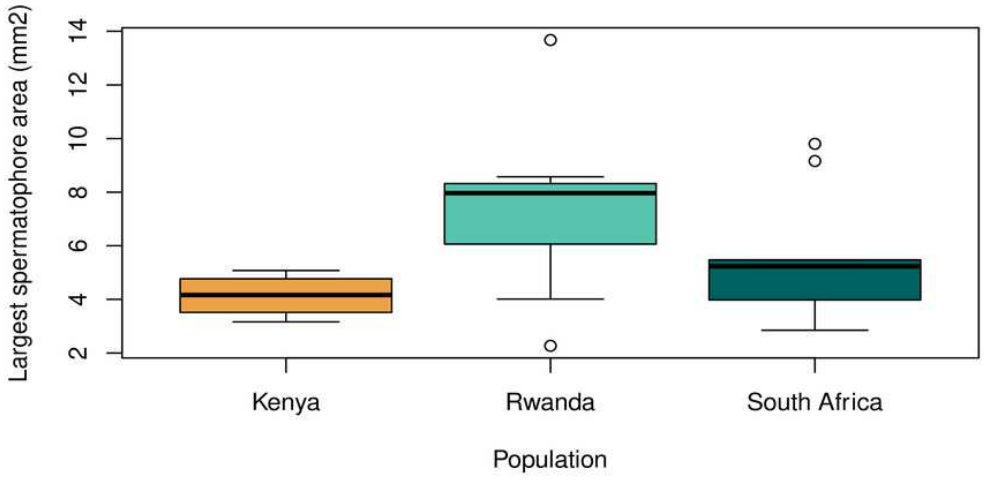
Figure 3: Spermatophores of various colours, shapes and sizes ~~(a)~~.

(b). Surface area (mm^2) of the largest spermatophore dissected from each mated female in each population, and (c) spermatophore count per female in each population (data includes mated and virgin females). The boxes represent the interquartile range of the data, and the heavy horizontal lines represent median values, whiskers give the 95% lower and upper percentiles. Coloured circles show the small sample repartition for each population

A)



B)


Largest spermatophore area (mm2)
2
4
6
8
10
12
14
Kenya
Rwanda
South Africa
Population

C)

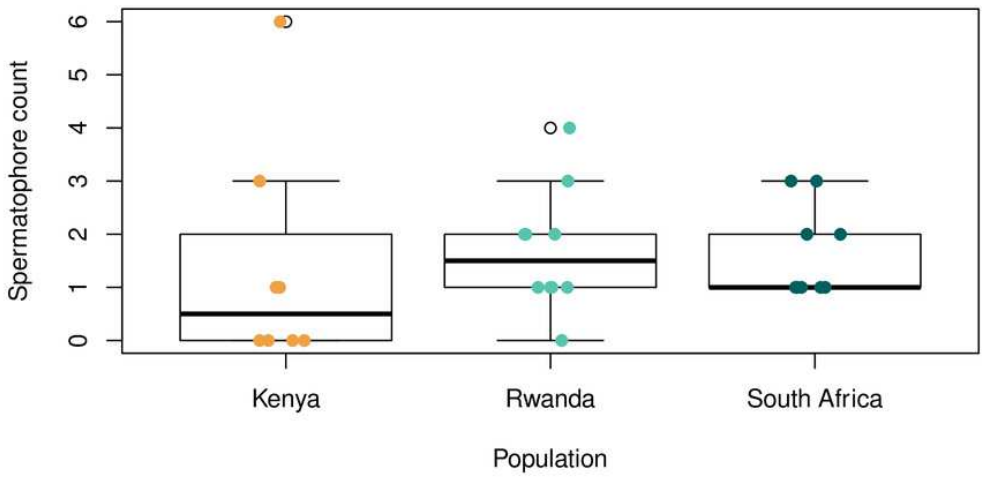

Spermatophore count
0
1
2
3
4
5
6
Kenya
Rwanda
South Africa
Population

Figure 4

Figure 4: The reproductive organs of female *Danaus chrysippus* butterfly, with the corpus bursa and signa (a and b), a closer-up of the sclerotized spike-like structures of the signa (c), and appendix bursa (d).

The ventral view (a) is a composite of two pictures, resulting from the removal of ventral sclerites, which were obstructing the underlying structures. The lateral view (b) has the ventral sclerites still in place. See Figure 5 for variation of structures. Pictures (c) and (d) are not in scale relative to pictures (a) and (b).

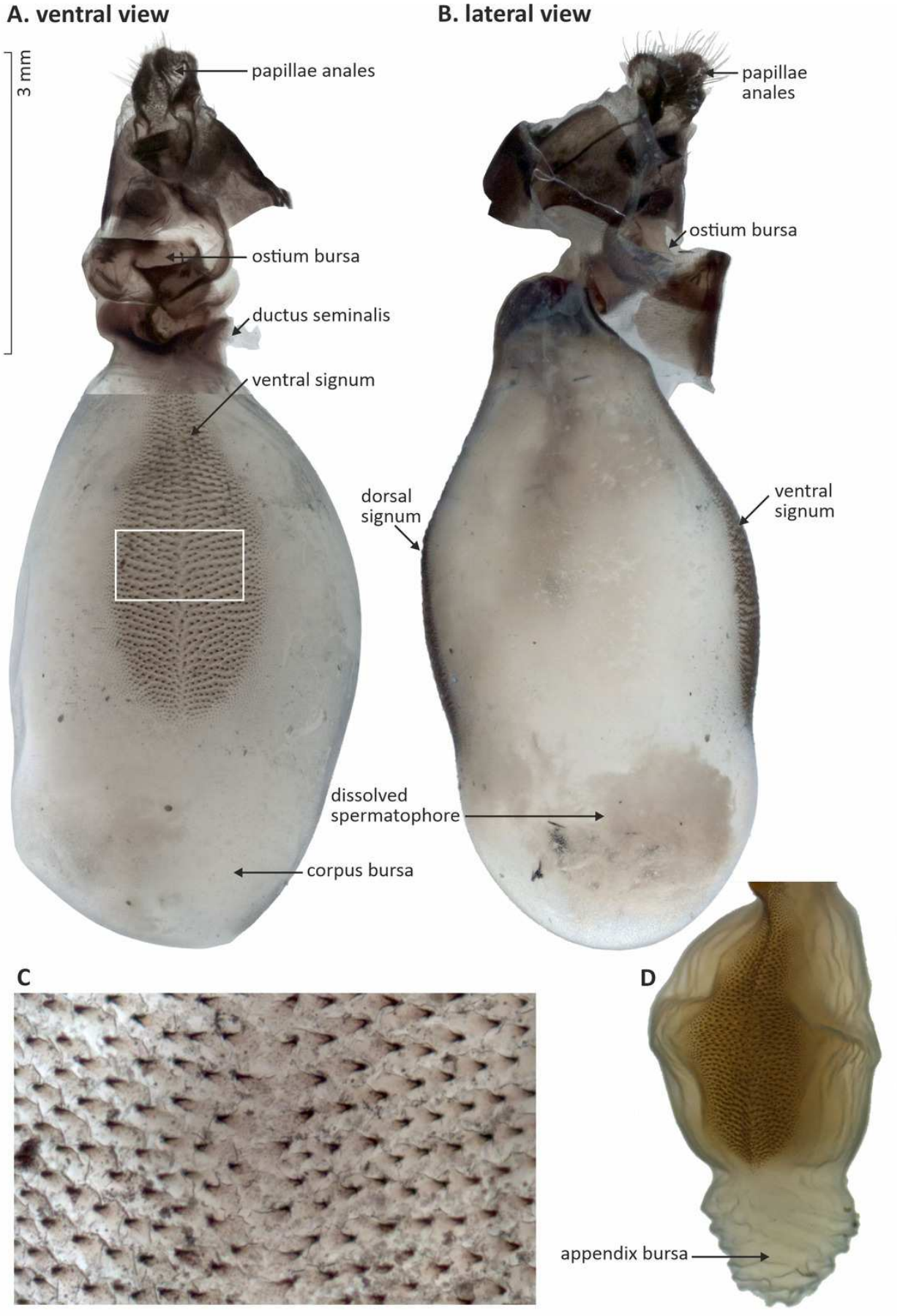

A. ventral view
B. lateral view
3 mm
papillae anales
ostium bursa
ductus seminalis
ventral signum
dorsal signum
dissolved spermatophore
corpus bursa
papillae anales
ostium bursa
ventral signum
C
D
appendix bursa

Figure 5

Figure 5: Reproductive organs of a virgin specimen (from Kenya), and three possibly mated specimens (from Kenya, Rwanda and South Africa), from left to right.

Virgin females showed a folded corpus bursa (a) and a very wrinkly appendix bursa (b), while we suggest that mated females show expanded corpus bursae (c) and appendix bursae (d) filled with spermatophore structure(s) (not visible on the images here). Sample IDs: JM stands for the initials of the first author's name, followed by a unique sample number.

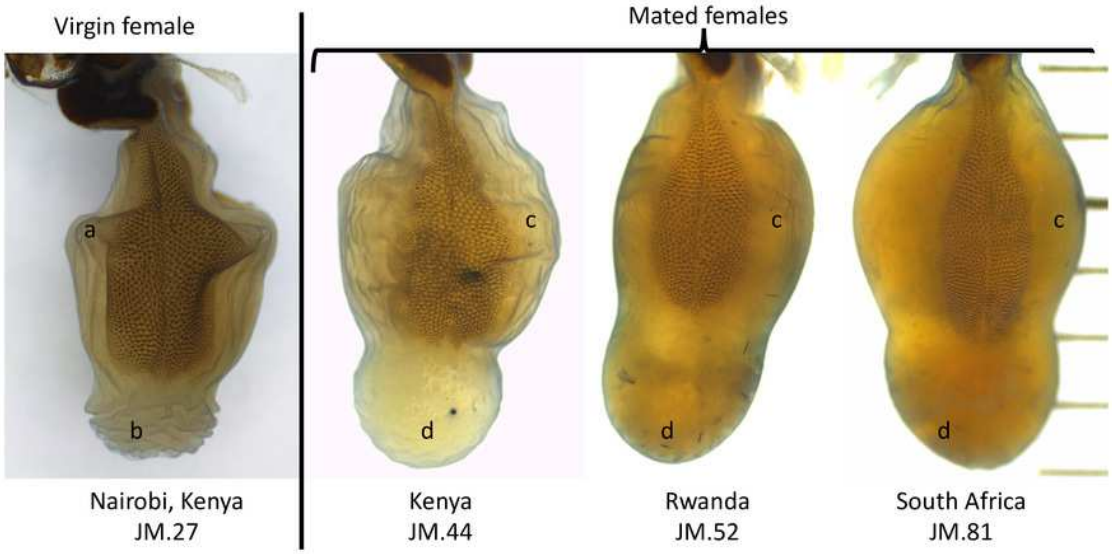


Figure 6

Figure 6: Variations in the ratio between signum and bursa area between virgin versus mated females from three populations across *Danaus chrysippus* natural range.

The boxes represent the interquartile range of the data, and the heavy horizontal lines represent median values, whiskers give the 95% lower and upper percentiles.