Male-killing in African butterflies

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Abstract

Female-biased sex ratios occur in many insect species as a consequence of infection by maternally-inherited male-killing bacterial endosymbionts. In this paper, we revise the research conducted on the phenomenon of male-killing in African nymphalid butterflies, with special focus on the cases of *Danaus chrysippus*, *Acraea encedon* and *Acraea encedana*. The evolution of male-killing in each case was addressed, together with the phylogeny of male-killers that were identified from this group. Moreover, the potential impacts that male-killers might impose on the evolution of their butterfly hosts were thoroughly investigated. In the end of this review, we present a number of unanswered questions to be targeted by future research work on the male-killing in these butterflies.

Introduction

In the vast majority of sexually reproducing organisms, parents invest equal resources on male and female offspring, thus maintaining a primary sex ratio of 1:1. Equal sex ratio is the evolutionary stable outcome of negative frequency-dependant selection favoring the rare sex.[1] However, many animal species are known to produce sex ratios that are biased toward one sex. Evolutionary speaking, unusual sex ratios fall into two distinct categories: adaptive and non-adaptive, from the point of view of the organism showing the sex ratio bias; natural selection may favor individuals that produce more progeny of one sex under special conditions such as to facilitate the inheritance of maternal social rank,[2,3] or due to local resource competition,[4] local mate competition and intracolony conflict over sex allocation,[5,6] on the other side, the sex ratio bias might be maladaptive to the organism showing the trait, nevertheless, it is maintained in the population as a result of manipulation by selfish genetic elements (SGE),[7] either within the host genome (*i.e.* sex chromosome meiotic drive)[8] or outside it (*i.e.* cytoplasmic endosymbionts),[9,10] in order to maximize their rate of transmission down the generations. In other words, the sex ratio bias might represent an *extended phenotype*; a trait expressed by the host but it is controlled by, and is beneficial to the parasite rather the host.[11]

A wide variety of cytoplasmic endosymbionts are known to perform sophisticated manipulations to the reproductive systems of arthropods, favoring their vertical transmission at the expense of host reproduction. The root of conflict between the *reproduction parasite* and its host is that the endosymbiont genes are passed only down the maternal line (from mother to offspring) while host genes are transferred through both maternal and paternal lines, thus males are *dead ends* from the endosymbiont genes point of view, while nuclear genes are indifferent with respect to whether they find themselves in a male or a female. There are four main strategies for reproductive manipulation: parthenogenesis induction, feminization, cytoplasmic incompatibility and male-killing (which can occur either early or late in development).[9,10,12-14] In the early-acting male-killing strategy, the selfish endosymbiont selectively kills the male-offspring of the infected females during their early development (usually before hatching). The principal condition under which male-killing is favored by natural selection is that males' death should improve the fitness of their female siblings that receive an identical copy of the male-killer from the infected mother. This occurs most commonly through reallocating valuable resources from dead males toward females in the brood either through reduced sibling competition or increased opportunities for cannibalism of unhatched eggs.[15,16] An alternative advantage for early male-killing is the avoidance of inbreeding depression which results from brother-sister mating.[10,17]

The phenomenon of early male-killing has been investigated mainly in one group of insects, the ladybird beetles (family: *coccinellidae*). Studies on ladybirds constitute the base of our current theory on male-killing.[10,18-21] The life history and the feeding style of aphidophagous ladybirds illustrate how male-killing endosymbionts might spread successfully despite their substantial fitness cost. In this group, eggs are generally laid in large clutches, while the major food source, aphids, is highly unpredictable, thus, cannibalizing the unhatched eggs provides immense survival advantage to the recently-hatched larvae, as it reduces the threat of starvation. Under such conditions, the male-killer-induced death of half the brood mates will contribute to the fitness of the other half, thus providing fitness compensation for an invading male-killer.[10] This is likely to be the reason of the unusual ubiquity and diversity of male-killing endosymbionts among ladybirds as well as the parallel infection of the same host species by multiple male-killers.[22]

Recent surveys reveal that male-killing is much more common in nature than what previously thought,[23,24] thus questioning the generality of theoretical conclusions based on studies of only one group. Nymphalid butterflies represent an ideal *model system* to test the general applicability of the current male-killing theory. The family Nymphalidae is a notable host spot for male-killers, as they occur in at least three unrelated members (*i.e.* *Danaus*, *Acraea* and *Hypolimnas*), and they are invaded by at least two distinct male-killing endosymbionts (*i.e.* *Wolbachia* and *Spiroplasma*).[25-28] Moreover, nymphalids differ from coccinellids in two fundamental aspects of the male-killing phenomenon: first, the life history seems to be less permissive to the male killers' invasion, and second, male-killer prevalences in wild populations are considerably higher.[10,19,29] Interestingly, these two aspects seem to contradict each other, since conventional models of male-killing predict that lower fitness compensation (the logical output of a life history that doesn't favor male-killer's invasion) should result in lower equilibrium prevalences of male-killers.[15] Thus, careful comparative analysis of male-killing patterns in the two taxa can yield valuable insights on the mechanisms, the ecology and the evolution of male-killing that are likely to improve our current theories on the subject.

In this paper, we provide a critical review for the incidences of male-killing in African butterflies. Cases of male-killing in this group were briefly described. The evolution of male-

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killing in each case was investigated, together with the phylogeny of the bacterial endosymbionts that cause male-killing to occur. The special focus of this review was on the impact of male-killers on the biology of their butterfly hosts; we have investigated a wide range of the evolutionary consequences of male-killing, including both the well-supported links (*i.e.* with sex role reversal) and the highly speculative potential links (*i.e.* with colour pattern polymorphism). In the end of this review, we summarized the findings of past research and provided a set of challenging questions to be targeted by future research.

Susceptible species

Danaus chrysippus

The African monarch butterfly *Danaus chrysippus* (L.) (Lepidoptera: Nymphalidae) is a cosmopolitan butterfly, widely distributed across the old world tropics and subtropics. Throughout most of its geographic range, the monarch butterfly shows normal sex ratio; however, within East and central Africa, female-biased sex ratios were observed in wild populations. Breeding experiments showed that the reason is the production, by a considerable proportion of females, of all-female broods, due to the death of male offspring as embryos or early instar larvae.[30] The reduced hatch rates in SR strains (*i.e.* those produce the female-biased sex ratio) have led to the suspicion that all-female broods are caused by a male-killer. Antibiotic treatment was found to cure the SR trait and led to the production of equal sex ratio,[26] implying that the male-killer is a bacterium. Molecular analysis revealed the identity of the male-killing bacteria to be a *Spiroplasma*. Jiggins *et al*.[26] found the prevalence of *Spiroplasma* to vary between 0 and 60% in East African populations. Small female samples from outside East Africa were negative for the bacterium.

The evolutionary forces that maintain male-killing in *Danaus chrysippus* remain poorly understood to date. Studies on other insect taxa,[20] as well as theoretical models,[15] both agree that large clutch size and antagonistic sibling interactions are critical prerequisites for the development of the male-killing trait, otherwise a male-killer will be eliminated by natural selection, soon after invading the species, as a result of the substantial fitness cost which it imposes on infected females.[10,15] Earlier, the possibility that a male-killer is the causal factor for sex ratio distortion in *D. chrysippus* was initially ruled out,[30] because the species is not a likely host for a male-killer; females *D. chrysippus* lay eggs singly on widely-scattered larval food plants (milkweeds of the genus *Asclepias*), where the larvae feed and develop without interacting with its siblings. Moreover, the highly dispersive behaviour of adults makes a high incidence of inbreeding unlikely.[30-32] It is not obvious, given a life history without sibling interactions, how the death of infected males might contribute to the fitness of their female siblings, yet such fitness contribution *should* take place somehow, according to our current theory of male-killing.

The key for solving this paradox might lay in another piece of research in *D. chrysippus*; surveying *Spiroplasma* infection in the wild populations of *D. chrysippus* revealed that bacterial prevalences exhibit consistent spatial variations and temporal changes.[26,33,34] The extensive population dynamics experienced by the male-killing *Spiroplasma* is likely to underlay the substantial difference in the overall *Spiroplasma* prevalence at Uganda estimated by Jiggins *et al.*[26] and Hassan *et al.* (40% and 24.9%, respectively). It has been suggested[34] that the male-killer dynamics reflect fundamental differences in the equilibrium prevalence of *Spiroplasma* between populations, maintained by natural selection.[34] According to this view, resource reallocation (*i.e.* the fitness advantage gained by infected females due to the death of their male siblings) does occur in *D. chrysippus*, not as a consequence of a genetically-determined life history trait, but, rather, as a response to an environmental limiting factor, which is the density of larval food-plants, the milkweeds; lower milkweed densities can enforce a modification of the innate oviposition behaviour so that females lay multiple eggs on a single plant. Another possibility is that unrelated eggs may be laid and develop on the same food-plant if, coincidentally, a single plant is visited by multiple females. The frequency of such an occasion will rise as the density of food plants in the habitat drops. In this case, male death will improve the transmission of the non-identical *Spiroplasma* strain infecting the non-sibling female eggs residing at the food-plant. If enough genetic homogeneity exists between *Spiroplasma* strains in wild *D. chrysippus* populations, such non-sibling interactions can provide some fitness advantage for invading *Spiroplasma*. Indeed, both mechanisms can reinforce each other, leading to the maintenance of the male-killer in the host species but with prevalences that show high variability over space and time, following the spatial and temporal variability in the abundance of milkweeds.

Acraea encedon and *A. encedana*

Acraea encedon and its sibling species *A. encedana* are Afro-tropical nymphalid butterflies that belong to the sub-family Heliconiinae. They are common in the tropics and sub-tropics of Africa south of the Sahara.[35] Wild populations of both species show female-biased sex ratios due to the occurrence of all-female broods.[36-40] Breeding data showed that the causal factor is maternally-inherited and can be treated by antibiotics.[25] Molecular analysis in *Acraea encedon* and *Acraea encedana* then revealed the presence of a bacterium which belongs to the genus *Wolbachia* in females producing all-female broods.[41,27]

The problem with male-killing in the two *Acraea* species lies not in the original evolution of the trait (as is the case in *D. chrysippus*), but, rather, in the stability of the symbiotic association between the host and the male-killer through the evolutionary time.[42] Prevalences reported from the wild populations of both *A. encedon* and *A. encedana* were extremely high; studies showed that females comprise up to 86% in Ugandan populations of *A. encedon* with 97% of the females in some of these populations infected with the male-killing *Wolbachia*.[25,43] Jiggins *et al*.[27] found that 79% of the populations of *A. encedana* were females, with 95% of these females infected with *Wolbachia*. Based on studies of wild populations in Uganda, Jiggins *et al*.[42] concluded that the *Wolbachia* has near-perfect vertical transmission in infected females; moreover, there is no suppression of the male-killing phenotype in infected males and no physiological cost of bearing *Wolbachia* on infected females.

Male-killers typically occur at low prevalences in nature,[10] and there are sound reasons why this is so; theoretical models for the population dynamics of male-killing suggest that perfectly-transmitted, unsuppressed male-killers will spread to fixation, driving the entire host species toward extinction due to the lack of males, together with the efficient male-killer itself.[5,15,44] As a consequence, selection acting at the species level should eliminate those host-endosymbiont associations in which the prevalences are exceedingly high. The question is why this scenario did not apply to the sibling *Acraea* species? How they manage to avoid extinction? It is possible that resilience to male-killers invasion is inherent in the metapopulation structure of *Acraea* butterflies.[45-47] To begin with, *Wolbachia* prevalences were found to vary between different regions in Africa as well as between different populations of the same region. In a thorough investigation for the dynamics of *Wolbachia* infection in Uganda, Hassan *et al.*[48,49] found an overall prevalence of 44.7% in *A. encedon* and 79.1 % in *Acraea encedana*; considerably lower estimates than these of Jiggins *et al.*[42,43] More importantly, re-investigating the same habitat patches studied by Jiggins *et al.* revealed consistently lower prevalences than that previously reported. This pattern can be explained by considering the male-killer's impact on host metapopulations; populations with extremely high prevalence that were observed by Jiggins

were extinct due to the lack of males, and the habitat patches were then recolonized by new immigrants from nearby populations, with initially low bacterial prevalences. According to a theoretical model,[45] if recurrent cycles of population extinction and recolonization take place in *Acraea encedon* and *A. encedana* as a response to high *Wolbachia* prevalences, the resulting enhancement in the natural metapopulation dynamics of the host species would lead to *absorbing* the impact of the male-killer at the population level rather than the species level and the extinction fate would thus be avoided.

Other Acraea species

Male-killing has been implicated in two other *Acraea* species: *Acraea eponina* and *A. stoikensis*. In *A. eponina*, breeding experiments showed that some females produce all-female progeny. Egg hatch rates in the SR broods were lower than in normal broods, suggesting that the sex ratio distortion is due to a male-killer infection. Antibiotic treatment reveals the male-killing agent to be a bacterium. Molecular analysis then detects *Wolbachia* in infected strains. Unlike *A. encedon* and *A. encedana*, *Wolbachia* prevalence in *A. eponina* is low, and the hatch rate in infected broods is higher than 50%.[10,23,50] In *Acraea stoikensis*, surveying wild populations in Uganda showed that populations are severely female-biased. Molecular investigation revealed the presence of high prevalent *Wolbachia* that is confined to females (Hassan SSM., unpublished data). The co-occurrence of female-bias and *Wolbachia* invasion strongly supports the male-killing scenario; however, breeding experiments are needed to confirm the male-killing in *A. stoikensis*. The ubiquity of male-killers in *Acraea* has been assessed by surveying *Wolbachia* infections in wild samples of different species. In an intensive survey, Jiggins *et al*.[23] estimated that 17% (n=24) of *Acraea* species are infected with *Wolbachia*. Hassan (unpublished data) reported that 21% (n=34) of *Acraea* species in his collection were infected. These percentages should be treated with considerable caution, however, as they may represent overestimation for the occurrence of male-killing; *Wolbachia* exploits a wide range of other adaptive strategies, ranging from cytoplasmic incompatibility to beneficial endosymbiosis.[14,51,52] Discerning between these possibilities cannot be made by molecular screening but through careful breeding experiments. On the other side, current methodologies may underestimate the ubiquity of male-killers, since molecular screening typically targets one genus of cytoplasmic endosymbionts, *Wolbachia*, and the number of samples screened is small. Low prevalence infections, as well as infections with *Spiroplasma*, *Rickettsia* and other endosymbionts will be missed, thus leading to lower overall estimations.[23,24]

Male-killers

To date, male-killers belonging to two bacterial groups were identified from African butterflies: *Wolbachia* and *Spiroplasma*; both are well-known manipulators of arthropod reproduction and were reported to cause male-killing in other insect species.[13,14,52,53] The male-killing bacteria *Spiroplasma* belong to two distinct groups; those of group II that cause male-killing in some *Drosophila* species,[54] and those of group VI that cause male-killing in some ladybird beetles.[22] The male-killing *Spiroplasma* in *D. chrysippus* belongs to group VI.[26] It is not obvious whether the *Spiroplasma* strains in coccinellids and that in *D. chrysippus*, represent two independent evolutions of male-killing, or has there been horizontal transmission between the hosts, either directly or through some intermediate vector? However, given the difference in sex chromosome systems (*i.e*. ZW female heterogametic in butterflies and XY male heterogametic in ladybirds) as well as the difference in life history (*i.e.* the lack of strong resource reallocation fitness advantage *D. chrysippus*), the independent evolution possibility seem more likely. Male-killing in *A. encedon* and *A. encedana* is caused by *Wolbachia*.[25,27,41] Two different *Wolbachia* strains were reported in *A. encedon*. Interestingly, the two male-killing strains occur sympatrically in the Tanzanian populations but not in the Ugandan populations of *A. encedon*, where only one strain was recorded.[55] The male-killing *Wolbachia* in *A. encedana* is identical to one of the two strains of *A. encedon*. No evidence for male-killer polymorphism was obtained from *A. encedana*.[27,55] We believe the possibility that the two sibling species inherit this *Wolbachia* strain from their common ancestor is more likely than the alternative possibility of horizontal transmission after speciation has already taken place. The reason is that speciation will be extremely unstable and liable to interruption through extensive hybrid matings if males are abundant in one species and rare in the other. In *A. stoikensis*, phylogenetic analysis revealed that the detected *Wolbachia* is distantly related to the *Wolbachia* strains found in *Acraea encedon* and *A. encedana* thus, male-killing seems to evolve independently in this species (Hassan SSM. *Unpublished data*).

The evolutionary consequences of male-killing

Extinction

In theory, invasion by a perfectly-transmitted male-killer may drive infected populations,[45] or even the entire host species toward extinction.[5,15,44,51] It is theoretically possible that species extinctions have played a major role in maintaining male-killers at low prevalence in insect natural populations. Based on the assumption that the long-term probability of extinction increases following invasion by efficient male-killer, it is expected that species selection should eliminate high prevalent male-killers, together with their susceptible hosts. On the contrary, the observed pattern of low prevalences in insect species may be a product of individual selection rather than species selection; genes for male-killers suppression will be automatically favored in populations with high prevalent infection, since it would double the egg hatch rate and facilitate the production of the rare sex (*i.e*. males). As a consequence, the spread of resistance genes can contain the male-killer before reaching prevalences that threaten the host species with extinction. Thus, whether species extinction is a common evolutionary fate of species invaded with male-killers is a question which depends on the performance of the host side in the evolutionary arm race between the host and the selfish endosymbiont. To date, the subject is a matter of pure speculation, as no data exists regarding the ubiquity of extinction and resistance as fates of species infected with male-killing endosymbionts. Studies on the population dynamics of male-killers as well as male-killing resistance (or the absence of it) in *A. encedon* and *A. encedana* can provide valuable information on the relative importance of adaptive (*i.e*. male-killer suppression) versus non-adaptive mechanisms (*i.e*. population/species extinctions) in controlling the spread of male-killing endosymbionts.

Resistance to male-killing

Invasion by male-killers imposes substantial cost on host genome, as it leads to the death of half the offspring of infected females, and prevents the production of the rare sex.[10,15,16] Thus, selection on nuclear genes will favor the spread of alleles that interfere with the replication or the vertical transmission of the male-killing endosymbiont or suppress the expression of the male-killing phenotype in infected males.[10] Male-killing resistance genes have been reported from ladybird beetles as well as the nymphalid butterfly *Hypolimnas bolina*,[19,56-58] however, they were found to be absent in other infected species such as *Drosophila innubila*.[59] There is no conclusive evidence demonstrating the occurrence or the absence of genetic resistance in any African butterfly. However, field observations as well as breeding data on *D. chrysippus* suggest that different colour patterns vary in the sex ratio and *Spiroplasma* prevalence, implying the existence of colour form-limited resistance

alleles in this species.[30,33] Efforts toward identifying male-killing resistance in *A. encedon* and *A. encedana* were not successful.[42] Curiously, selection for resistance is expected to be more intense in *Acraea* species, since they show considerably higher male-killer prevalences than that of *D. chrysippus*. So, why *Acraea* didn't evolve resistance to *Wolbachia*? An intriguing possibility is that current prevalences may be the consequence rather than the cause of evolution of the male-killing resistance; initial invasion by a male-killer may result in extremely high prevalences, seriously affecting the recruitment rate of the host. Selection on the host species will gradually accumulate resistance against the endosymbiont, leading to declining prevalences.[10] *D. chrysippus* might have been invaded earlier than *Acraea*, and thus had much more time to evolve resistance.

Sex role reversal

Because males invest less in the production of gametes, they have greater reproductive potential than females and thus they gain from extra matings. On the other side, female reproduction is intrinsically limited by the maximum number of eggs they can produce (and, in species with parental care, the number of offspring they can raise successfully during each season), rather than by mating frequency. Thus, sexual differences in the size of reproductive cells are the root of male-female asymmetry which characterizes the mating systems of most animals.[60-63] If the population sex ratio is moderately biased toward females, no net impact on the population reproductive rate will result, since sperms far outnumber eggs, and a considerable minority of males can easily fertilize all females in the population. However, under extreme sex ratio distortion, population reproduction may become sperm-limited. Under these conditions, normal sex roles are expected to undergo full reversal, with females competing for access to males and males choosing between females.[61,64-66]

Elegantly confirming theoretical expectations, behavioural observations on wild populations of *A. encedon* and *A. encedana* have reported a case of sex role reversal. Butterflies were found to aggregate at resource-free landmarks (such as hilltops), where the sex ratio is highly biased compared to food-plant sites and most females are virgins. These observations have led to the conclusion that the resource-free sites are *female lekks* that are visited by virgin females in order to mate.[10,43,51] Lekking behaviour occurs widely among animal taxa, including many insects, but males, rather than females are the sex usually undergoing the lekking habit.[67] It is likely that female swarming at landmarks was evolved from an ancestral, male swarming habit which is common in *Acraea* species not infected with male-killers.[68]

In highly female-biased populations where females aggregate to maximize mating opportunities, males are expected to be choosy, potentially through developing a preference for uninfected females. Mating with uninfected females is adaptive because it doubles the number of progeny per each mating and leads to the production of the rare, valuable males. Majerus speculated that male mate choice is the underlying mechanism which prevents *Wolbachia* from reaching fixation in *A. encedon* and *A. encedana*, thus rescuing the species from extinction.[10] Theoretical modeling demonstrated that the evolution of male mate choice can maintain stable polymorphism of both infected and uninfected females in the host population.[69] However, field studies on *A. encedon* did not find any evidence that males mate assortatively, since mating frequency did not differ between infected and uninfected females.[42] The idea that *Acraea* males can choose adaptively is extremely attractive on theoretical grounds and thus it deserves further field assessment conducted on a larger scale before being ruled out.

Color pattern polymorphism

The hidden link between aposematic polymorphism and male-killing

Natural selection acting on aposematic prey is theoretically expected to promote monomorphism, so as to maximize avoidance learning by predators,[1,70-72] however, contrary to expectations; colour polymorphism has evolved in few aposematic species, including the three nymphalid butterflies (*Acraea encedon*, *Acraea encedana* and *Danaus chrysippus*),[10,40] and the ladybird beetles of the family coccinellidae *Adalia decempunctata*, and *Harmonia axyridis*.[73-75] interestingly, the screening of all these organisms for male-killers yielded positive results.[25-27,41,42] Is it just a coincidence?

The taxonomic association between male-killing and colour polymorphism has led to the speculation that a subtle link might exist between these seemingly-independent phenomena.[10] One possibility is that the ubiquity of male-killers in aposematic species is higher than in non-aposematic species because the two traits require similar life-history conditions to evolve. It has been suggested that the initial evolution of both aposematism and male-killing might require the act of kin selection, thus, species with strong sibling interactions have higher than average probability of developing both traits.[10] However, this presumed association is based only on anecdotal observations and there is a considerable doubt that any real pattern exists; in a thorough investigation for the prevalence of *Wolbachia* among Ugandan butterflies, no statistical association has emerged between *Wolbachia* susceptibility and aposematism,[76] thus suggesting that the taxonomic distribution of the two traits (male-killing and aposematism) might be independent from each other. Further research is surly needed to judge the reality and the precise nature of this presumed link.

The hidden link between Mimetic polymorphism and male-killing

A further reason that makes colour polymorphism in *D. chrysippus*, *A. encedon* and *A. encedana* highly unusual is that the three species are members of a mimicry complex in East and Central Africa.[40,77-79] Prey species involved in Müllerian mimicry are expected to be monomorphic for colour pattern in order to promote morphological convergence and, consequently, predator avoidance. As a result, mimetic polymorphism, in which there are multiple colour forms within each species, resembling parallel colour forms in the mimetic species, forces naïve predators to experience every colour category independently, thus leading to higher predation rates on the mimicry complex.[77] We have suggested a selective scenario, in which colour polymorphism is maintained in the mimicry complex through the repeated extinction episodes experienced by the sympatric populations of the three species as a consequence of male-killers' spread.[80]

In aposematic, mimetic species there are two selective forces acting to sustain monomorphism, and both are mediated by predation pressure; first, selection toward within-species convergence, favoring the most abundant form in the species, and second, selection toward between-species convergence, favoring the most abundant form in the mimicry complex. It is easy to see that the output of such selective system is colour monomorphism, as long as within-species and between-species selections are acting harmoniously for long time. Invasion by highly efficient male-killer is likely to have fundamental impact on the host species meta-population dynamics.[45-47] The enhanced rate by which population extinctions and recolonizations take place implies that colour form frequencies, accumulated in each region after long selective process, would be regularly destroyed following every extinction event, and will be replaced by a random combination of allelic frequencies following every colonization event. The impact of this cycle on the selective system favoring colour monomorphism is immense; imagine two sympatric populations of *A. encedon* and *A. encedana*, both undergoing cyclic extinctions at variable rates; assume that, initially, both within species and between-species selective pressures are favoring the same colour pattern. However, when extinction strikes one population, it will cause three disturbing effects: first, the outcome of past selection will be destroyed; second, the direction of within-population

selection will change, favoring the most abundant form in the migrant population and third, the direction of selection imposed on the sympatric population of the mimetic species will also change, again favoring the most abundant form in the migrant population. Thus, selection toward monomorphism will be continuously randomized by the extinction-recolonization cycles, not only within the species, but also in other members of the mimicry complex. To conclude, in a system of polymorphic mimetic species subjected to repeated population extinctions and recolonizations, selection will continue to favor the state of monomorphism; however, no single colour pattern will be favored for a period long enough to actually promote monomorphism.[80]

Hybrid mating and male-killing

Invasion by a male-killer, if occurs in a lineage which recently undergone, or is currently undergoing speciation, might have fundamental consequences on the reproductive isolation which defines the species boundaries. If a considerable proportion of females in the infected species are virgins, and if the sex ratio in the sibling species (or the partially isolated subspecies) is normal, then it may pay females to mate with males that belong to the other species and produce less fit progeny, because the only available alternative is to stay virgin and not to have progeny at all; the sex ratio bias would thus select against the assortative mating induced by reinforcement,[81] leading to an interruption or a reversal in the ongoing speciation process. Interestingly, this mechanism is far more powerful in female-biased rather than male-biased populations, because mating selectivity is usually enforced by females, as they are the sex which invests more in offspring.[61]

Unlike the cases of *A. encedon* and *A. encedana*, the link between colour polymorphism and male-killing is widely accepted and well supported by evidence; It has been suggested that the muddled taxonomy of *D. chrysippus* is a product of extensive hybrid mating between incipient species that is induced by *Spiroplasma* invasion.[82,83] In Africa, four major colour forms exist for this species: *Chrysippus*, *Alcippus*, *Dorippus* and *Albinus*, which are vicariant across most of the continent, however, within a particular zone in East and Central Africa, all the colour forms co-occur sympatrically and mate extensively with each other, producing several hybrid forms.[40,78,84-87] Phylogenetic analysis revealed substantial genetic divergence between colour forms.[88] Furthermore, experimental data suggests the existence of partial assortative mating between colour forms.[87,89] These findings have led to the speculation that colour forms of *D. chrysippus* are incipient species that arised through past geographic isolation and were forced to hybridize, despite partial pre-zygotic reproductive isolation, following infection with the male-killing *Spiroplasma.*[30,83,88,89] Hybrid mating might be driven by variations in the abundance of males between colour forms; supporting this, Smith have found that in wild samples of *D. chrysippus*,[90] females predominated among f. *chrysippus*, while a 1:1 sex ratio was observed in f. *dorippus*. However, later research has failed to detect any association between colour pattern and susceptibility to *Spiroplasma* invasion; no significant differences were found between *Spiroplasma* prevalences specific to each colour form.[76]

In the wild populations of *A. stoikensis* where the sex ratio is severely female-biased, hybrid matings were found to take place with the related species *Acraea alicia*. The direction of inter-species matings were as expected, with females *A. stoikensis* mating with males *A. Alicia* (Hassan SSM, unpublished data). To our knowledge, no previous research has investigated the potential interrupting influence of the extreme female-bias on the recent speciation of *A. encedon* and *A. encedana*. Since the two sibling species are sympatric, it is possible that, in populations where sex ratio distortion is very high, mating across the species barriers is adopted as an alternative female reproductive strategy. We strongly recommend future research to assess this stimulating possibility.

Prospects

The theory of endosymbiont-induced male-killing predicts that both the taxonomic distribution of male-killers and their equilibrium prevalences within host species are determined by the life history of potential hosts. According to this view, there is a simple relationship between the magnitude of the fitness advantage of infected females over uninfected ones due to resource reallocation and the prevalence of the male-killer among female hosts; if the species life history provides little or no opportunities for resource reallocation, any male-killing endosymbiont that happens to invade this species would soon be eliminated by natural selection. On the other side, if male death provides considerable survival advantage to its female siblings, the invading endosymbiont will spread in the host population, achieving a prevalence that is dependent on the magnitude of the fitness advantage gained by female siblings. If a perfectly-transmitted male-killer gained sufficient resource reallocation fitness advantage from the host lifestyle, it will spread to fixation, thus leading to host extinction as the sex ratio approaches 0:1. Data obtained from nymphalid butterflies do not fit very well within this theoretical frame. In *Danaus chrysippus*, the *Spiroplasma* infection has spread to considerable level despite the lack of obvious fitness advantage for infected females. In *Acraea encedon* and A. *encedana*, both species have persisted in the evolutionary time despite infection with efficient *Wolbachia* with near-perfect vertical transmission. Thus, it appears that life history is not a good predictor of male-killing patterns in this insect group; other aspects of host ecology, such as female egg-laying behaviour in the case of *D. chrysippus* and the metapopulation dynamics in the case of *Acraea* species seem to have deep influence on the output of the host-endosymbiont interaction.

As revised above, many studies have addressed the various aspects of male-killing in African butterflies; however, beyond the demonstration of the causative role of bacterial endosymbionts in developing the female-biased sex ratios, most of the challenging questions regarding the causes and consequences of this phenomenon remains unanswered; for example, what determines the substantial variation in male-killer prevalences among insect tax, such as that between coccinellid ladybirds and *Acraea* butterflies? There are three potential answers: maybe there is a subtle aspect of the ecology or the life history of *Acraea* which promotes susceptibility to male-killing. Another possibility is that prevalence is a property of the male-killer rather than the host, and the *Wolbachia* strains infecting *Acraea* happen to be highly efficient. The third possibility is that the prevalence in the host species is inversely proportional to the time passed since initial invasion with the male-killer. Thus, current low prevalences in coccinellids are the output of long evolutionary arm race in which the host species accumulated resistance against the male-killer, while *Acraea* are invaded much more recently and thus they are yet to develop the male-killing resistance.

What is the fitness advantage of *Spiroplasma* for infected *D. chrysippus* females? As we discussed above, the source of fitness compensation in this species might be an aspect of the environment rather than a genetically-determined life-history trait. Field studies on *D. chrysippus* should assess this possibility through careful recording of relevant environmental parameters, especially milkweed abundance, together with the data on population sex ratios and bacterial prevalences. The hypothesis of *environmentally-dependent resource reallocation* expects the existence of a statistical correlation between the density of milkweeds and *Spiroplasma* prevalence in the area. The ecology, behaviour and life history of *D. chrysippus* should be further investigated in the wild, with special emphasis on comparing populations from inside and outside the *Spiroplasma* zone, in order to understand why

Spiroplasma invasion is highly specific with respect to geography.

What defines the host species response to invasion by an efficient male-killer? As outlined above, response in the ecological time scale is non-adaptive and may involve cyclic population extinctions. In the evolutionary time scale, the host species may simply become extinct, or it may avoid this fate through evolving wide range of adaptive responses, both physiological (*i.e.* male-killer suppression) and behavioural (*i.e.* hybrid mating, female lekking, male mate choice). With the exception of female lekking, evidence is lacking that any of these theoretical possibilities actually take place in African butterflies. Further research is needed in order to delineate the scale of host adaptive responses in the evolutionary arm race with the male-killer.

Is there a fundamental difference regarding the mechanism of male-killing between butterflies and beetles which parallels the fundamental difference in their sex determination system (ZW *vs* XY sex chromosomes)? Unlike the ecology and the evolution of male-killing phenomenon, the detailed physiology by which male-killing takes place in insect remains poorly known; to our knowledge, no study have addressed this subject in butterflies. Understanding male-killing at the cellular level would shed the light on two important aspects of endosymbiont-host interaction: how and when, during the embryonic development, an endosymbiont can detect the host sex? And is it easier to manipulate the sex determination system, thus leading to feminization or parthenogenesis, or to interfere with the entire embryonic development process, thus causing male death? Answering the first question will explain variations in the efficiency of male-killers' vertical transmission as well as incidences of incomplete expression of the male-killing phenotype, while the answer to the second question would explain the distribution of male-killing among insect taxa, versus other routes of reproductive manipulation.

Is it true that extinction is a frequent evolutionary fate for species invaded by sex ratio distorters? Attempts should be made to develop sophisticated, more realistic theoretical models that describe the population dynamics of efficient male-killers. If it turns out that the answer is yes to the above question, then it fellows that group selection was a major force underlying the current abundance and efficiency of male-killers in nature. Demonstration of the importance of species selection in this case would contribute significantly to the long-lasting debate in evolutionary biology regarding the importance of selective forces acting above the individual level.

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