MORPH RATIO DYNAMICS UNDER MALE-KILLER INVASION: THE CASE OF THE TROPICAL BUTTERFLY *ACRAEA ENCEDON* (LEPIDOPTERA: NYMPHALIDAE)

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Abstract - This study aimed to provide field-based assessment for the theoretical possibility that there is a relationship between colour polymorphism and male-killing in the butterfly *Acraea encedon*. In an extensive, three year study conducted in Uganda, the spatial variations and temporal changes in the ratios of different colour forms were observed. Moreover, the association between *Wolbachia* susceptibility and colour pattern was analyzed statistically. Two hypotheses were tested: first, morph ratio dynamics is a consequence of random extinction-colonization cycles, caused by *Wolbachia* spread, and second, particular colour forms are less susceptible to *Wolbachia* infection than others, implying the existence of colour form-specific resistance alleles. Overall, obtained data are consistent with the first hypothesis but not with the second, however, further research is needed before any firm conclusions can be made on the reality, scale and nature of the presumed association between polymorphism and male-killing in *A. encedon*.

Key words: Aposematic polymorphism, metapopulation dynamics, Müllerian mimicry; *Wolbachia*, female-biased sex ratio, PCR

INTRODUCTION

Acraea encedon is an Afro-tropical nymphalid butterfly, widely distributed across the tropics and subtropics of Africa south of the Sahara. The species prefers open country and is well adapted to human disturbance, achieving its highest densities in human-made habitats such as gardens and agricultural areas (Owen, 1970). *Acraea encedon* is an aposematic butterfly, which is involved in a mimicry complex in East and Central Africa with the Müllerian mimics *Acraea encedana* and *Danaus chrysippus*, together with several Batesian mimics, including *Hypolimnas misippus* (Smith *et al.*, 1998; Gordon & Smith, 1999). The biology of *A. encedon* is characterized by two distinct evolutionary dilemmas: aposematic polymorphism and female-biased sex ratios. Interestingly, these two unusual phenomena also occur in the other Müllerian mimics, *A. encedana and D. chrysippus* (e.g. Poulton, 1914; Owen & Chanter, 1968; Owen, 1970; Chanter & Owen, 1972; Owen & Smith, 1993; Owen *et al*., 1994; Smith *et al*., 1998; Jiggins *et al*., 1998, 2000a, 2000b). Indeed, such simultaneous occurrence of two "seemingly" unrelated phenomena within three sympatric, mimetic butterfly species represents a third evolutionary dilemma awaiting compelling explanation (Majerus, 2003).

Prey species in which chemical defence weaponry is accompanied with bright and contrasting coloration are termed aposematic (Poulton, 1890). Aposematism is an effective anti-predator strategy that works by developing learned avoidance response among experienced predators for the characteristic colour of the aposematic prey. Aposematic species are expected to be monomorphic for colour pattern, in order to maximize the efficiency of avoidance learning by predators, otherwise naïve predators will have to learn to avoid each colour form independently, thus leading to higher predation rate (Fisher, 1930; Ford, 1964; Matthews, 1977; Greenwood *et al*., 1981). *Acraea encedon*, being an aposematic butterfly belonging to the unpalatable Acraeidae family, is also extensively polymorphic, with eight distinct colour forms occurring sympatrically. The colour forms of *A. encedon* are: *encedon*, *daira*, *commixta*, *infuscata*, *encedon-daira*, *lycia*, suffused *lycia*, *sganzini* (Figure 1) (Owen *et al*., 1994).

Male-killing is a strategy of reproductive manipulation adopted by some of the maternally-inherited endosymbionts of arthropods to alter the host sex ratio in the direction that favours their own transmission (O'Neill *et al*., 1997; Majerus, 2003). A male-killer spreads in the host population because of the fitness advantage gained by infected females due to the death of half of their brood mates, either through reduced competition or increased cannibalism, termed resource reallocation fitness advantage (Hurst, 1991; Hurst & Majerus, 1993). Most male-killing endosymbionts occur at low prevalences in natural populations (Majerus, 2003) and there is sound theoretical reasons why this is so: effective, unsuppressed male-killers would spread to fixation, drived by the fitness advantage of infected females over uninfected ones, ultimately leading to the extinction of the entire host species when all females become infected (Hamilton, 1967). *Acraea encedon* is infected by a male-killing bacterium of the genus *Wolbachia*, which has led to extremely female-biased population sex ratios. Females were found to comprise up to 86% in Ugandan populations of *A. encedon*, with 97% of females, in some populations, bearing the male-killing *Wolbachia*. Moreover, studies suggest that *Wolbachia* has a near perfect vertical transmission in the wild, and it is neither suppressed in infected males nor it imposes any fitness cost on infected females (Jiggins *et al*., 1998, 2002).

How can *A. encedon* persist in the evolutionary time despite the immense cost of *Wolbachia*? Theoretically speaking, there are two ways out of this paradox: host metapopulation dynamics and *Wolbachia* suppression genes. Considering the first possibility, infection with male-killers might have led to substantial enhancement in the rate of population extinction. Habitat batches that were emptied following extinction are then recolonized by migrants from nearby populations, thus, the whole metapopulation dynamics of the species will be accelerated following invasion by the male-killer. Theoretical

modelling (Heuch, 1978) suggests that this process may slow down the spread of the male-killer and thus contribute to the maintenance of the species in the evolutionary time, as the male-killer's impact would be "absorbed" at the population level rather at the species level.

The second possibility stems from the fact that the act of male-killing imposes substantial fitness cost on the host genome. Thus, natural selection will favour the spread of nuclear genes that suppress the male-killing phenotype or interfere with the vertical transmission of the male-killing endosymbiont (O'Neill *et al*., 1997; Majerus, 2003). Male-killing suppressors that rescue infected males have been reported from ladybird beetles (Majerus & Majerus, 2010). In the nymphalid butterfly *D. chrysippus* (which is infected with a male-killing *Spiroplasma*), a unique system of colour form-limited resistance genes was hypothesized to exist, since certain colour forms were shown to be less susceptible to infection with the male-killing *Spiroplasma* than others (Smith *et al*., 1998; Herren *et al*., 2007). The interaction between colour polymorphism and male-killing is now widely accepted to be the major force driving the evolution of *D. chrysippus* species complex (Smith *et al*., 1997, 1998; Lushai *et al*., 2003a, 2005).

In this paper, we present descriptive field data on the spatial and temporal variations in the frequency of different colour forms of *A. encedon* in Uganda, gathered from the field during a period of three years (2005-2007). Moreover, we provide a statistical analysis for the relationship between colour polymorphism and *Wolbachia* prevalence. Our study was designed to test two hypotheses:

1. Colour polymorphism in *A.encedon* is maintained, despite positive frequency-dependent selection by predators, as a consequence of repeated episodes of population extinction and colonization, induced by *Wolbachia* spread. Those cycles will lead to continuous random fluctuations in the morph ratio, preventing the population from responding adaptively to selective forces favouring monomorphism.

2. There is a genetic variation within *A. encedon* populations with respect to *Wolbachia* susceptibility, and *Wolbachia* resistance alleles are linked to specific colour patterns, as is the case in *D. chrysippus*.

MATERIALS AND METHODS

Collection of samples

Between 2005 and 2007, samples of *A. encedon* were collected from a variety of sites in Uganda. The sites within the Kampala-Entebbe region visited in this study were studied previously by Owen & Chanter (1968), Owen & Smith (1993), Owen *et al.* (1994), and Jiggins *et al*. (1998). Four sites: Mabira Forest, Malabigambo, Mpanga Forest and Kibale National Forest Reserve, are located outside the Kampala-Entebbe zone, and had not been sampled by previous researchers. These sites were included in the temporal analysis within the current study, as well as in the bacterial preference analysis.

Seasons in Uganda

Uganda falls within the equatorial zone, which lacks a distinct winter season, but experiences cool nights during the rainy seasons. Two shifts from wet to dry conditions occur in Uganda per annually. The main annual dry season extends from November through to March (referred to here as 'Dry A'). This is followed by a short rainy period from April to June (referred to as 'Wet A'). July is generally dry (referred to as 'Dry B'), and the period from August through to October comprises the long rainy season (referred to as 'Wet B'). This general seasonal pattern applies to all sites visited, although average annual rainfall does vary between sites. Seasons' data were obtained from the Uganda Meteorological Department.

Molecular assays

All females *A. encedon* were tested for the presence of the male-killing *Wolbachia*. DNA was extracted from the preserved abdomens using the Wizard® Genomic DNA Purification Kit. The resulting DNA was checked specifically for the presence of the *Wolbachia* previously reported by Hurst *et al.* (1999), using the *Wolbachia*-specific primers *wsp*81F and *wsp*691R (Zhou *et al.*, 1998). General insect primers, C1-J-1751f and C1-N-2191r (Simon *et al.*, 1994), were used with all the DNA samples to check for the success of the extractions. All the PCR premixes were cross-linked using a UV light illuminator to destroy any contaminant DNA.

Table 1: Number of colour forms of *Acraea encedon* collected in Uganda (2005-2007).

Site	*commixta*	*daira*	*encedon*	*encedon-daira*	*infuscata*	*lycia*	*sganzini*	suffused *lycia*	Total
Entebbe Bot. Gardens	3	82	232	63	48	2	2	4	**436**
Gangu	0	0	1	1	0	0	1	0	**3**
Kagolomolo	1	38	180	32	42	3	2	2	**300**
Kajjansi	0	20	83	12	5	2	0	0	**122**
Kawanda	0	1	1	0	0	0	0	0	**2**
Kazi	1	14	40	16	6	0	0	1	**78**
Kisubi Forest	0	0	5	1	0	0	0	0	**6**
Lubya	0	2	5	2	1	0	0	0	**10**
Makerere	2	46	126	27	19	2	0	0	**222**
Malabigambo	0	3	36	0	0	0	0	0	**39**
Nalugala	0	0	1	0	0	0	0	0	**1**
Ziika Forest	0	0	2	0	0	0	0	0	**2**
Total	**7**	**206**	**712**	**154**	**121**	**9**	**5**	**7**	1221

Table 2: Comparison of the frequencies (%) of colour forms in *Acraea encedon* from five sites within the Kampala-Entebbe region (after Owen *et al.*, 1994).

Colour forms	Entebbe		Kagolomolo		Kazi		Makerere	
	64-66	05-07	64-66	05-07	64-66	05-07	64-66	05-07
commixta	2.7	0.7	4.8	0.3	2.8	1.3	3.1	0.9
Daira	15.1	18.8	28.6	12.7	15.1	17.9	9.4	20.4
Encedon	43.8	53.2	34.9	60.0	49.8	51.3	43.8	56.9
encedon-daira	0.0	14.4	0.0	10.6	0.7	20.5	3.1	12.4
Infuscata	2.7	11.0	6.3	14.0	4.1	7.7	3.1	8.5
Others	35.7	1.9	25.4	2.4	27.5	1.3	37.5	0.9
Sample size	**73**	**436**	**63**	**300**	**1059**	**78**	**32**	**222**

Morphological investigations

The sex and the colour form pattern of each *A. encedon* specimen was described and recorded, following the descriptions of Owen *et al.* (1994). Specimens that tested positive for male-killer's presence were plotted against the respective colour form to check for any association between particular colour forms and male-killer presence or absence.

Statistical analysis

The statistical methods used in the analysis of the data were performed using the data analysis package in Microsoft® Excel 2007 and the statistical package Cytel StatXact® 8 for Windows. The data were tested using the Chi-squared test of heterogeneity (χ^2). The Chi-squared test was performed first on all the data involved, and whenever heterogeneity was found, specific comparisons between every two samples were done using (2 x 2 χ^2) tables. The level of significance used was typically ($P < 0.05$). However, when repeated comparisons were made, the Bonferroni correction ($\alpha\beta$) was used. The level of significance was determined using the formula (P/n), where $P = 0.05$ and n was the number of comparisons performed. The Fisher-Freeman-Halton Test, also known as Fisher's Exact Test, was used instead of the heterogeneity χ^2 test, to check for variation between samples when the expected values were less than five.

RESULTS

Morph ratio dynamics

Spatial variations in the morph ratio

Table 1 shows the colour form frequencies of *A. encedon* butterflies collected in Uganda during the period of 2005-2007 at each of the different sites sampled. Collections were highly heterogeneous (Fisher's exact test = 131.9, d.f. = 77, $P < 0.001$), contrary to the findings made by Owen *et al.* (1994) on the homogeneity of the 1964-1966 collections of *A. encedon* obtained from the different sites. Form *encedon* had the highest frequency in the current sample (58.3%).

The sample of *A. encedon* from Malabigambo represents a new record for *A. encedon* from this site. Only two forms, *daira* and *encedon*, were recorded at Malabigambo.

Temporal changes in the morph ratio

Annual changes at individual sites

Four of the sites previously sampled in 1964-1966 had sufficient numbers in the current study (>10) to allow frequency comparison of the different colour forms of *A. encedon* over time (Table 2). The samples of the three less common forms *lycia*, *suffused lycia* and *sganzini* were summed together, under 'others'.

The samples from Entebbe Botanical Gardens, Kagolomolo, Kazi and Makerere, were heterogeneous (Fisher's exact Test = 91.21; 62.68; 93.63; 45.52 respectively, d.f. = 5, $P < 0.001$). At Entebbe, there was no significant difference between the frequencies of the three forms, *commixta*, *daira* and *encedon* recorded in the 1964-1966 collection and the current study (Fisher's exact Test = 2.75; 0.49; 2.19 respectively, d.f. = 1, $P > 0.05$). The forms *encedon-daira* and *infuscata* were significantly more frequent in the current study than in the 1964-1966 collection (Fisher's exact Test = 17.17; 5.28 respectively, d.f. = 1, $P < 0.05$). The frequency of the less common colour forms (others) was significantly lower in the current study than in the 1964-1966 collection (Fisher's exact Test = 74.88, d.f = 1, $P < 0.001$).

At Kagolomolo, the frequencies of the forms *commixta*, *daira* and the less common forms scored in the current study were significantly lower than the frequencies scored in 1964-1966 (Fisher's exact Test = 6.9; 9.12; 33.81 respectively, d.f. = 1, $P < 0.01$). The frequencies of f. *encedon* and f. *encedon-daira* increased significantly in the current study (Fisher's exact Test = 13.16; 9.5 respectively, d.f. = 1, $P < 0.01$). Although the frequency of f. *infuscata* recorded in the current study is higher than that recorded in 1964-1966, the difference is not significant (Fisher's exact Test = 2.69, d.f. = 1, $P > 0.05$).

At Kazi, the frequencies of the forms *commixta*, *daira*, *encedon*, and *infuscata* in the current study did not differ significantly from the frequencies recorded in the 1964-1966 collection (Fisher's exact Test = 0.27; 0.56; 0.07; 2.45 respectively, d.f. = 1, $P > 0.05$). The frequency of f. *encedon-daira* in the current study increased significantly compared to that of the 1964-1966 collection (Fisher's exact Test = 62.61, d.f. = 1, $P < 0.001$). A lower frequency of the less common forms was recorded in the current sample than the 1964-1966 collection (Fisher's exact Test = 37.17, d.f. = 1, $P < 0.001$).

At Makerere, no significant differences were detected

between the frequencies scored in the current study compared to the 1964-1966 collection in any of the different colour patterns ($P > 0.05$), except for the frequency of the less common forms, which significantly declined in the current study (Fisher's exact Test = 43.03, d.f. = 1, $P < 0.001$). The decline in the frequencies of the less common forms, *lycia*, suffused *lycia* and *sganzini*, persisted at the four sites.

Annual changes at all sites

Table 3 compares the overall frequencies of the different colour forms collected from the Kampala-Entebbe area during the current study with previous reports. The three collections (1964-1966, 1991, 2005-2007) are highly heterogeneous (χ^2 = 773.3, d.f. = 10, $P < 0.001$). Temporal changes in the frequencies of the different colour patterns have been found between the three collections: 1964-1966, 1991, 2005-2007. Form *commixta* declined gradually from 2.9% in 1964-1966, with the frequency scored in the current study significantly lower than the frequencies recorded both in 1964-1966 and 1991 ($P < 0.01$). The frequency of f. *daira* increased significantly from 21.2% in 1964-1966 to 47.7% in 1991. The frequency found in this study, had declined from 1991 and was significantly lower than the frequencies in both 1964-1966 and 1991 ($P < 0.01$). The frequencies of the forms *encedon* and *infuscata* showed an opposite pattern, where, although the frequencies scored in 1991 declined from those scored in 1964-1966, the difference was not significant ($P > 0.05$). The frequencies of both forms increased again in the current study, with the current frequencies significantly higher than those recorded in the two previous studies ($P < 0.001$). The frequency of f. *encedon-daira* in the current study is significantly higher than the frequencies in the two previous studies ($P < 0.001$). The frequency of the less common forms in the current study did not differ from that scored in 1991; both being significantly lower than the frequency recorded in 1964-1966 collection ($P < 0.001$).

Seasonal changes

Figure 2 shows the change in the collected numbers of each of the colour forms during the different seasons of the study period. Colour pattern frequencies were found to show considerable seasonal fluctuations, but without any consistent pattern. Although the total number of the forms collected during the wet seasons is higher than those collected in the dry seasons, this difference is not statistically significant (Fisher-Freeman-Halton Test = 6.16, d.f. = 5, $P > 0.05$).

The association between colour polymorphism and male-killing

The sex ratio among different colour forms

The proportion of "females: males" collected from the three forms: *daira*, *encedon* and *encedon-daira*, did not differ from the overall ratio of "females: males" in the total sample (684: 537) ($\alpha\beta = 0.006$; χ^2 = 0.04; 1.77; 1.4; respectively, d.f. = 1, $P > 0.006$). The ratio of "females: males" of f. *infuscata* was significantly biased towards males compared to the ratio of the total sample ($\alpha\beta = 0.006$; $\chi^2 = 36.58$, d.f. = 1, $P < 0.006$). Similar morph ratios of females and males were recorded in the other four forms, *commixta*, *lycia*, *sganzini*, and suffused *lycia* ($\alpha\beta = 0.006$; Fisher-Freeman-Halton Test = 0.07; 0.38; 1.01; 0.61 respectively, d.f. = 1, $P > 0.006$) (Table 4).

Table 3: Comparison of the overall frequency of colour forms in *Acraea encedon* from the Kampala-Entebbe area (1964-1966 and 1991 data cited from Owen *et al.* (1994)).

Colour forms	1964-1966		1991		2005-2007	
	N	%	N	%	N	%
commixta	102	2.9	6	2.7	7	0.6
daira	750	21.2	105	47.7	203	17.2
encedon	1603	45.2	96	43.6	676	57.2
encedon-daira	50	1.4	3	1.4	154	13.0
infuscata	152	4.3	7	3.2	121	10.2
Others	887	25.0	3	1.4	21	1.8
Totals	**3544**		**220**		**1182**	

Table 4: Morph ratios of *Acraea encedon* between sexes in Uganda (2005-2007).

Morph	N	Females	Males	% Males
commixta	7	4	3	42.9
daira	206	117	89	43.3
encedon	712	421	291	40.9
encedon-daira	154	94	60	39.0
infuscata	121	33	88	72.7
lycia	9	6	3	33.3
sganzini	5	4	1	20.0
suffused *lycia*	7	5	2	28.6
Totals	**1221**	**684**	**537**	**44.0**

Table 5: Prevalence of *Wolbachia* in the colour forms of *Acraea encedon* collected in Uganda (2005-2007).

Morph	Number of females tested	Positives	Negatives	*Wolbachia* prevalence (%)
commixta	4	1	3	25.0
daira	113	48	65	42.5
encedon	402	183	219	45.5
encedon-daira	93	44	49	47.3
infuscata	31	9	22	29.0
lycia	6	1	5	16.7
sganzini	4	2	2	50.0
suffused *lycia*	5	1	4	20.0

Wolbachia's prevalence among different colour forms

Table 5 shows the number of females from each of the eight colour morphs that were tested for *Wolbachia*. The number of tested females from the forms *commixta*, *lycia*, *sganzini* and suffused *lycia* were too small for statistical analysis, but for the other four forms there was no correlation observed between the bacterial presence and any of the colour forms; they were all homogeneous ($\chi^2 = 1.07$, d.f. = 3, $P > 0.05$).

DISCUSSION

For aposematic species involved in Müllerian mimicry, colour pattern is a trait under strong selection, which acts to promote morphological convergence, both within and between the mimetic species (Owen, 1970). In the mimicry complex

Figure 1: The colour forms of *Acraea encedon*: a) form *encedon*; b) form *daira*; c) form *commixta*; d) form *infuscata*; e) form *encedon-daira*; f) form *lycia*; g) form suffused *lycia*; h) form *sganzini*.

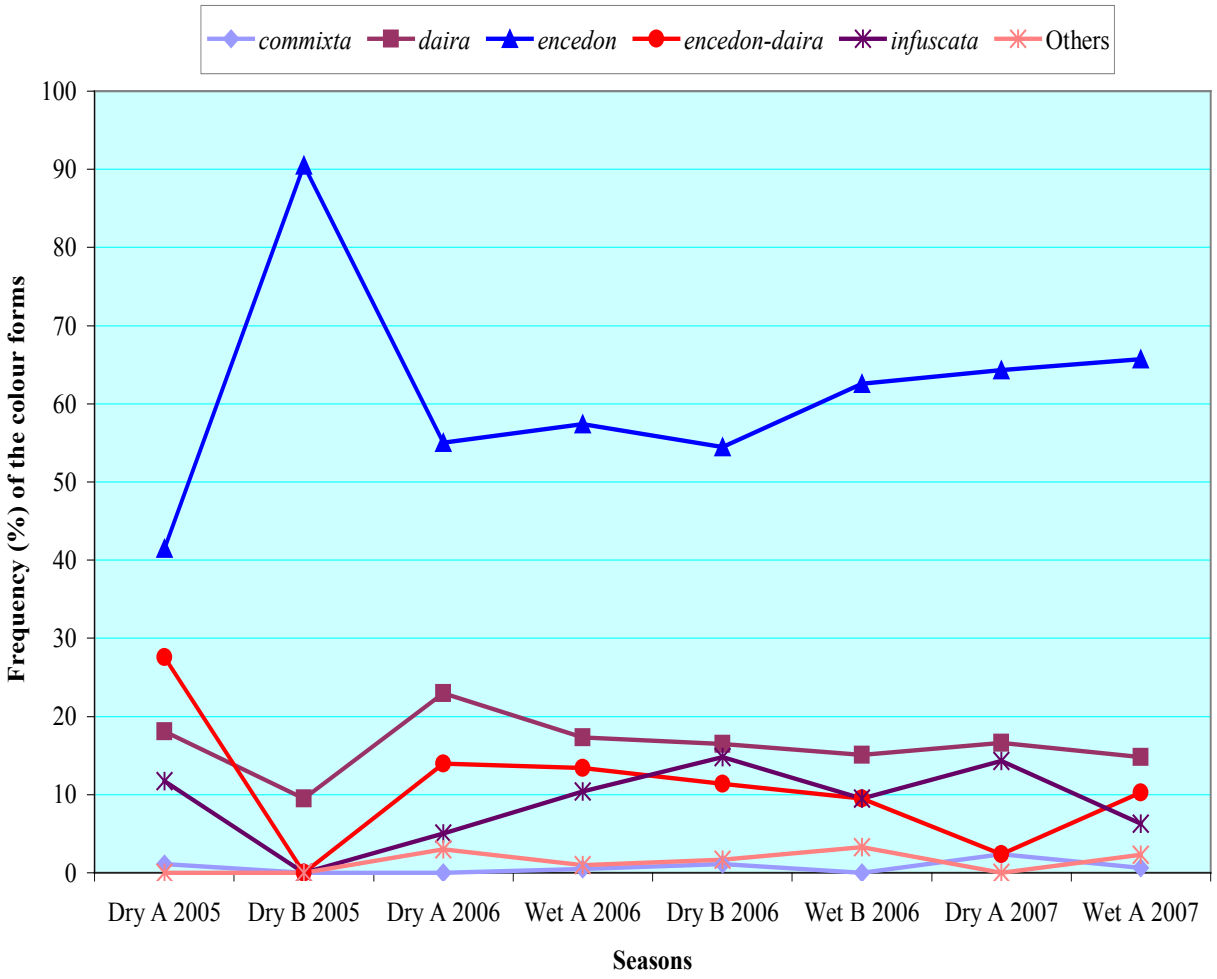

Figure 2: Frequencies (%) of each of the colour forms of *Acraea encedon* collected in Uganda, during the different seasons (2005-2007).

that involves *A. encedon*, *A. encedana* and *D. chrysippus*, all species are extensively polymorphic, with distinct colour forms, each of them resembling parallel colour forms in the two other species. Despite the substantial complication posed by the existence of genuine polymorphism in the mimicry complex, the direction of natural selection acting on the system is expected to remain the same as in any Müllerian mimicry complex, favouring colour convergence (Owen & Smith, 1993; Owen *et al*., 1994). However, one fundamental difference is that selection is expected to show strong spatial variation; the reason is that, in polymorphic species, initially slight variations in colour frequencies between different sites will be magnified by positive frequency-dependent selection, and thus the particular colour form that is favoured by selection in each site will vary, depending on which one was initially more frequent. The expected outcome of this selective process would be monomorphism on the local scale, with every population maintaining a single colour pattern, but polymorphism on the large scale, with different populations adopting different colour patterns. This is not the case in *A. encedon*, and neither is the case in *A. encedana* and *D. chrysippus*, which raises the question "why"?

Demographic turnover is likely to have deep influence in the polymorphic structure in *A. encedon*; migrational movements have already been found to cause substantial morph ratio fluctuation in *D. chrysippus* (Smith & Owen, 1997; Lushai *et al*., 2003b). We suggest that one mechanism by which within-population polymorphism is maintained in *A. encedon* is through extinction-recolonization cycles driven by *Wolbachia* invasion (Heuch, 1978). Our argument is based on one critical assumption: there is no functional association of any kind between colour pattern and male-killing susceptibility. If this condition is fulfilled, it is easy to see that population extinctions will lead to random shifts in the morph ratio, in which the local optimal ratio, gradually accumulated by the act of natural selection through many generations, is continuously destroyed when the population undergoes extinction, and is replaced by a new combination of colour frequencies representing the morph

ratio of the new colonizers of the habitat batch. Importantly, the geographic variation in dominant colour forms (discussed above) implies that the new morph ratio will be different from the morph ratio of the extinct population. Since the outcome of selection will be eliminated every time the population undergoes extinction, the adaptive state of colour monomorphism cannot be reached. The maintenance of polymorphism in *A. encedon* can thus be explained by the simple fact that species with enhanced metapopulation dynamics are resistant to site-specific natural selection, however, this hypothesis does not explain the initial evolution of the state of colour polymorphism, but assumes it.

Considering a scenario in which the two mimetic species, *A. encedon* and *A. ence*dana, are both subjected to repeated population extinctions and recolonizations (it is safe to exclude *D. chrysippus* here, as it is doubtful if *Spiroplasma* wild prevalences are high enough to affect the population dynamics of the host (Jiggins *et al*., 2000; Herren *et al*., 2007; Hassan *et al*., 2012). Since selection on a Müllerian complex acts to maximize convergence between members, the adaptive colour pattern that is selected for in a population of one species is determined by the morph ratio in the sympatric population of the other species. When extinction and replacement take place in the population of that mimic (e.g. *A. encedana*), the favourable selected-for colour pattern in *A. encedon* population would change accordingly, and randomly, depending on the morph ratio of the new *A. encedana* colonizers. As a consequence, predator-driven selection toward monomorphism is repeatedly randomized by cyclic population extinctions and recolonizations not only within the species (since it changes the outcome of selection) but also outside the species boundaries (since it changes the direction of selection). Polymorphism in *D. chrysippus* can thus be maintained even if the species itself does not undergo extinction-recolonization cycles, through the continuous fluctuations in the direction of selection imposed on *D. chrysippus* populations by its Müllerian mimics *A. encedon* and *A. encedana*.

The theoretical scenario outlined above predicts that the morph ratio should vary randomly over space and time. During this study, the spatial variations and temporal changes in the morph ratio of *A. encedon* were assessed in Uganda. Overall, the study demonstrated that colour form frequencies vary over both spatial (among sites) and temporal scales (through years). Importantly, although morph ratios recorded during different seasons were variable, there was no significant difference in the morph ratio correlating with season. Moreover, comparing morph ratios between the three collections (1964-1966, 1991, and 2005-2007) revealed extensive variations that lack any consistent pattern. The importance of this data is that it excludes two alternative possibilities; the first is that polymorphism in *A. encedon* is not under Hardy-Weinberg equilibrium and that natural selection is currently acting on the morph ratios promoting monomorphism. Similar argument was once suggested for polymorphism in *D. chrysippus* (Smith *et al*., 1993). However, if evolution is taking place in the polymorphic system of *A. encedon*, then a comparison between morph ratios obtained during 1964-1966, 1991 and 2005-2007, should reveal neat, consistent trend in which abundant forms increase in abundance while rare forms become even more rare. The second scenario, which is also ruled out, is that the colour form performs a functional role related to climatic adaptation, possibly through affecting thermoregulation, and polymorphism is maintained because some forms are favoured during wet conditions, while others are favoured in dry conditions, again, a similar hypothesis was suggested for *D. chrysippus* (Smith, 1980). The lack of consistent seasonal pattern in the morph ratio dynamics contradicts this hypothesis.

Do extinction-recolonization cycles really take place in *A. encedon* and *A. encedana*? In a survey for the population dynamics of the male-killing *Wolbachia* in the wild populations of the two species in Uganda (Hassan *et al*. in prep.), we found a particular pattern which supports the 'population cycles' idea. When the extremely female-biased wild populations studied by Jiggins et al. (2002, 2000b) were reinvestigated, these populations, almost invariably, showed lower *Wolbachia* prevalences, and in some cases the prevalence decline was substantial. Since the fitness advantage of infected females does not allow selection to reduce the *Wolbachia* prevalence directly, the obvious interpretation for the observed pattern is that the old, heavily infected populations reported by Jiggins were extinct due to the spread of the male-killer and the subsequent lack of males and were then replaced by new migrant populations with lower *Wolbachia* prevalences.

The second finding of the current study is that there is no statistical association between colour pattern and susceptibility to *Wolbachia* infection. This result confirms the critical assumption on which the '*Wolbachia*-maintains-polymorphism' idea was based. If certain colour patterns are more resistant to *Wolbachia* than others, population extinctions will impose directional selection rather than random drift, increasing the frequency of resistant colour forms. The reason is that populations that happen to be dominated by resistant forms will suffer lower extinction rate than populations dominated by susceptible forms. On the other side, the second hypothesis tested in this study, that '*A. encedon* has colour-linked suppression alleles' is not supported by the data.

It is important, however, to note that results presented in the current study do not formally prove or exclude any of the theoretical scenarios that were discussed. For example, the spatial and temporal variations in the morph ratio can also be interpreted through the mimetic load hypothesis, which is based on the diluting effect of Batesian mimics on the aposematic signal (Owen, 1970). Moreover, *A. encedon* gene pool may contain alleles for male-killer suppression, but they are not linked to colour pattern alleles. Further research is definitely needed before any judgment can be made on the presumed associations between colour polymorphism and male-killing in *Acraea* butterflies.

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