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Frequency-dependent flight activity in the colour polymorphic wood tiger moth

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Abstract Predators efficiently learn to avoid one type of warning signal rather than several, making colour polymorphisms unexpected. Aposematic wood tiger moth males *Parasemia plantaginis* have either white or yellow hindwing coloration across Europe. Previous studies indicate that yellow males are better defended from predators, while white males have a positively frequency-dependent mating advantage. However, the potential frequency-dependent behavioural differences in flight between the morphs, as well as the role of male-male interactions in inducing flying activity, have not been previously considered. We ran an outdoor cage experiment where proportions of both male morphs were manipulated to test whether flying activity was frequency-dependent and differed between morphs. The white morph was significantly more active than the yellow one across all treatments, and sustained activity for longer. Overall activity for both morphs was considerably lower in the yellow-biased environment, suggesting that higher proportions of yellow males in a population may lead to overall reduced flying activity. The activity of the yellow morph also followed a steeper, narrower curve than that of the white morph during peak female calling activity. We suggest that white males, with their presumably less costly defences, have more resources to invest in flight for predator escape and finding mates. Yellow males, which are better protected but less sexually selected, may instead compensate their lower flight activity by 'flying smart' during the peak female-calling periods. Thus, both morphs may be able to behaviourally balance the trade-off between warning signal selection and sexual selection. Our results emphasize the greater need to investigate animal behaviour and colour polymorphisms in natural or semi-natural environments [*Current Zoology* 61 (4): 765–772, 2015].

Keywords Frequency-dependent selection, Flight, Colour polymorphism, Sexual selection, Aposematism

Animals employ a variety of strategies to deter predators (Endler, 1986). Aposematic animals advertise their inedibility through conspicuous colours, odours or sounds, which predators associate with chemical or physical secondary defences and subsequently learn to avoid (Poulton, 1890; Cott, 1940; Ruxton et al., 2004; Rojas et al., 2015). The effectiveness of these warning signals relies on their abundance (Lindström et al., 2001), as predators need a threshold number of encounters with defended prey in order to create an aversion (Endler and Rojas, 2009). Several studies show that predators are better at learning one type of warning signal than several and, thus, signal polymorphisms are not expected (i.e. positive frequency-dependent survival selection) (Endler, 1988; Joron and Mallet, 1998; Endler and Mappes, 2004). Surprisingly, however, several taxa show intra-specific variation in warning signals (Myers and Daly, 1983; O'Donald and Majerus, 1984; Brakefield, 1985; Ueno et al., 1998; Mallet and Joron, 1999; Borer et al., 2010; Rojas and Endler, 2013; Hegna et al., 2015).

Several findings have been proposed to explain colour polymorphisms in aposematic species, including: a combination of negative and positive frequency-dependent selection (Thompson, 1984); trade-offs between natural and sexual selection (Nokelainen et al., 2012; Crothers and Cummings, 2013; Cummings and Crothers, 2013); spatio-temporal variation in selection (Endler and Rojas, 2009; Valkonen et al., 2012; Galarza et al., 2014; Mappes et al., 2014; Nokelainen et al., 2014; Rojas et al., 2014b); an association between behaviour (or other fitness-related traits) and signal type (Nokelainen et al., 2013; Rojas et al., 2014a); relaxation of selection towards warning signals (Amézquita et al., 2013; Richards-Zawacki et al., 2013); or non-adaptive forces such as hybridization or drift (Gray and McKinnon, 2007; Medina et al., 2013). Thus, it seems likely that several non-mutually exclusive mechanisms are responsible for the maintenance of warning signal variation, despite the expected strength of positive frequency-

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dependent selection.

The wood tiger moth is a diurnal aposematic arctiid with a wide distribution across the Holarctic region, where it shows an extensive amount of warning signal variation (Hegna et al., 2015). Thus, it provides a unique possibility to study how selection operates on phenotypes at different locations. In Europe, males can have either yellow or white hind wings with varying degrees of melanisation (Fig. 2) (Hegna et al., 2013; Nokelainen et al., 2014). Previous studies have shown that yellow individuals are less attacked by predators (Nokelainen et al., 2012), have more effective (Rojas et al., unpublished), yet costly (Suisto et al. unpublished; Nokelainen et al., 2012) chemical defences, and are able to warm-up considerably faster than white males (Hegna et al., 2013). On the other hand, studies done in the laboratory have shown that white moths generally have better mating success than yellow males under similar frequencies, especially when mating occurs at a cost (Nokelainen et al., 2012). Surprisingly, however, a recent study in semi-natural conditions indicated that mating success is positively frequency-dependent for the white morph, such that white males are more likely to mate when common (Gordon et al., 2015). Taken together these results suggest that opposing natural and sexual selection favour both morphs, which makes their sympatric co-existence not easy to explain.

Local polymorphism is, against theoretical predictions, common in nature. With the exception of monomorphic populations in Scotland (yellow) and Estonia (white) (Hegna et al., 2015), most populations of the wood tiger moth are polymorphic, with frequencies varying both temporally and geographically (Galarza et al., 2014; Nokelainen et al., 2014). Although variable selection driven by differences in predator community structure (Nokelainen et al., 2014) and dispersal between sub-populations (Galarza et al., 2014) are likely to play an important role, it is possible that morph-related differences in unmeasured fitness-related traits help maintain polymorphism in this system. One of such unmeasured traits may be related to differences in behaviour between the morphs.

An intrinsic limitation of previous studies is that they were performed on dead moths or dummies where the frequencies of both morphs were the same (predation experiments; Nokelainen et al., 2012; Nokelainen et al., 2014), or in laboratory settings with limited possibility for flying and displaying (mating trials; Nokelainen et al., 2012). Thus, it is possible that male behavioural mechanisms that may compensate for the colour preference of predators or females had been missed. Moreover, it is likely that at least some of these mechanisms are frequency-dependent. One such mechanism, which we here address, is flying activity. For example, when more common, individuals of the white morph may be able to compensate for a lower warning signal and weaker chemical defences by relying more on active flight to escape and find mates. Alternatively, a higher activity of individuals of the yellow morph, aided by better thermoregulation abilities, could compensate for a lower female preference. It is also possible that male behaviour depends on the other males' activity, which could explain the recently found positively frequency dependent mating success (Gordon et al., 2015). Here we use a semi-natural enclosure experiment to investigate whether frequency-dependent flying activity could be a behavioural mechanism enabling the widespread co-existence of white and yellow individuals by balancing the colour preferences of predators and females.

1 Materials and Methods

1.1 Study species

Wood tiger moth *Parasemia plantaginis* males present a discrete polymorphism in the coloration of their hind wings, which can be either yellow or white with varying degrees of melanisation (Fig. 1) (Nokelainen et al., 2012; Hegna et al., 2013). Females, on the other hand, exhibit continuous variation in their hind wing colour, ranging from yellow to red (Lindstedt et al., 2011), and a similar melanisation pattern as seen in males. Both sexes are considered to be aposematic, as their coloration is conspicuous against their natural resting backgrounds (Lindstedt et al., 2011; Nokelainen et al., 2012), and their chemical secretions make them distasteful for bird predators (Nokelainen et al., 2012).

As capital breeders (Tammaru and Haukioja, 1996), wood tiger moths do not feed in their adult stage and have therefore a short lifespan. They are polyphagous,

Fig. 1 The two male morphs of the wood tiger moth, white (left) and yellow (right). Photo: B. Rojas.

have one generation per year, and overwinter at the larval stage (Lindstedt et al., 2010). Under laboratory conditions, they are easy to breed and can be kept on a diet consisting mostly of dandelion (*Taraxacum sp.*) leaves. The individuals used in the present study were obtained from a laboratory wood tiger moth stock (F6-generation) reared at the University of Jyväskylä, Finland.

1.2 Enclosure experiment

In order to test for variation in flying activities under different morph frequencies we ran an enclosure experiment in semi-natural conditions at Konnevesi Research Station (University of Jyväskylä, Finland) between July 4th and July 24th, 2012. A large outdoor enclosure measuring 20 m × 30 m × 3 m (length × width × height) was divided into six compartments, each approximately 6m x 10m. The bottom of each compartment was open to the natural foliage, while the top and two sides were open to natural light through a white mesh. The remaining two sides were covered with a plastic green tarp that blocked visual contact between compartments.

We ran ten trials, each with three treatments: a balanced-ratio treatment, with equal numbers of yellow and white individuals (12 of each colour); a white-biased treatment (16 white, 8 yellow); and a yellow-biased treatment (16 yellow, 8 white). In order to resemble a natural situation, we had in each enclosure five females, which were tethered to a Styrofoam box so they could not escape, but could call the males via pheromones thus motivating them to fly. In each trial run we therefore had a total of 15 females and 72 males. Before each run, three of the six available cages were randomly assigned a specific treatment.

Once tethered to their cages, females were allowed to acclimatise for approximately one hour. Males were released into each compartment at about 16:00h, based on previous information about their mating times in both laboratory and field conditions (S. Gordon, pers. observ.). Following release, males were watched during the whole afternoon and night, and collected the next day at approximately 8:00h. One observer in each cage performed scans on the flying activity, counting the number of fliers of each colour morph every 15 minutes. Preliminary analyses on the cycles of activity in each of the compartments revealed that most of the flying activity was concentrated between 17:00 and 22:00, with very little or no activity after midnight. Therefore, for statistical analyses we used time blocks instead of the actual time at which every scan was done. We used 30min intervals between 16:00 and 0:00 and then two blocks until the time at which the moths were collected, one from 0:00 to 6:00 and another from 6:00 to 8:00.

1.3 Statistical analyses

In order to test whether male morphs differed in their activity and if this is affected by their frequencies we first ran a generalized linear mixed effects model (GLMM) with the proportion of flying moths within each time block as the response variable, and treatment and morph as the explanatory variables. We included trial and time blocks as random effects to account for the non-independence of flying across each day and at different times.

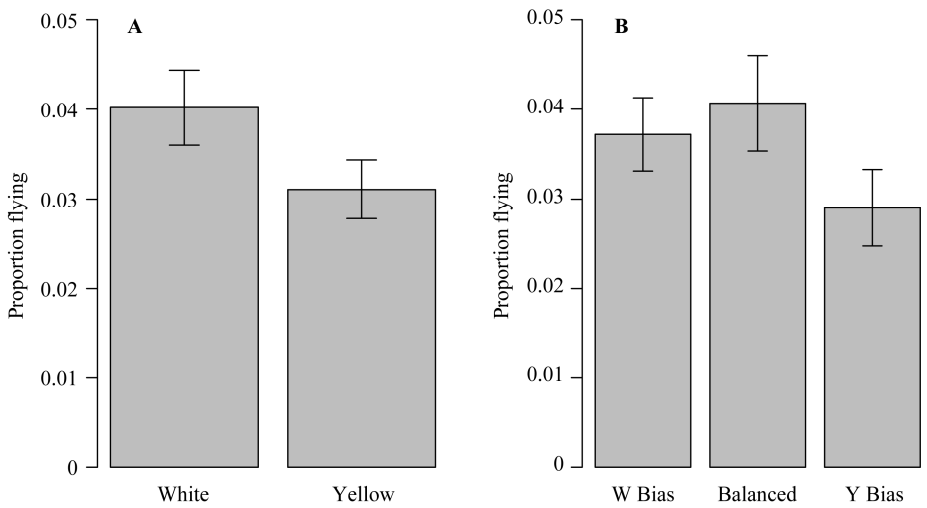

Fig. 2 In panel A graph showing the proportion of flying activity between white and yellow males for all treatments combined. In panel B graph shows differences in activity between all three treatments [White Biased (W Bias), Balanced ratio, and Yellow Biased (Y Bias)]

Bars indicate standard errors.

We explored the differences in activity across time in each trial by running another GLMM for proportion of flying moths, but this time with both morph and the time blocks as the explanatory variables and only trial as the random factor.

2 Results

Our results show that yellow males have less flying activity than white males (Fig. 2; effect= -0.283 ± 0.103, z = -2.748; P = 0.006). Yellow males were less active regardless of the morph ratio (interaction of morph x treatment were all greater than effect=0.282 ± 0.243, z = 1.160; P > 0.246 so the interaction was removed from final analysis). There was, however, significantly less activity for both morphs altogether in the yellow male biased treatment (balanced vs. yellow-biased effect= -0.341 ± 0.126, z = 2.712; P = 0.007) compared to both the balanced ratio and white biased treatment, which were similar to each other (effect= -0.114 ± 0.116, z = -0.980; P = 0.327; Fig. 2).

Noticing that there seemed to be peak activity during certain time blocks (Fig. 3) we then analysed whether there was a difference in the activity schedules and peak activities between morphs. We did so by fitting a GLMM where time was a continuous variable by using the centre time-points of each time-block. To describe the peak, we fit a quadratic component of time. We included the interaction of morph with both the linear and quadratic effects of time to detect differences in the schedule. All parameters of the quadratic curve were significantly different between both morphs (see Table 1). As expected, there was a peak in levels of flying activity for both morphs between 17:00 and 21:00. However, the white morph showed a significantly flatter curve with activity levels spread throughout time and higher activity levels early and late in the curve (Table 1; Fig. 3).

3 Discussion

Colour polymorphisms in aposematic species are challenging to explain in theory because selection by predators is expected to drive the morph with the most efficient signal to fixation (Mallet and Joron, 1999). However, local polymorphism in hindwing colouration is widespread in wood tiger moth males. For the polymorphism to persist there should be no differences in their net fitness, yet survival selection seems to favour yellow hindwing coloured individuals over white ones (Nokelainen et al., 2012; Nokelainen et al., 2013; Nokelainen et al., 2014). Our aim was to test whether fre-

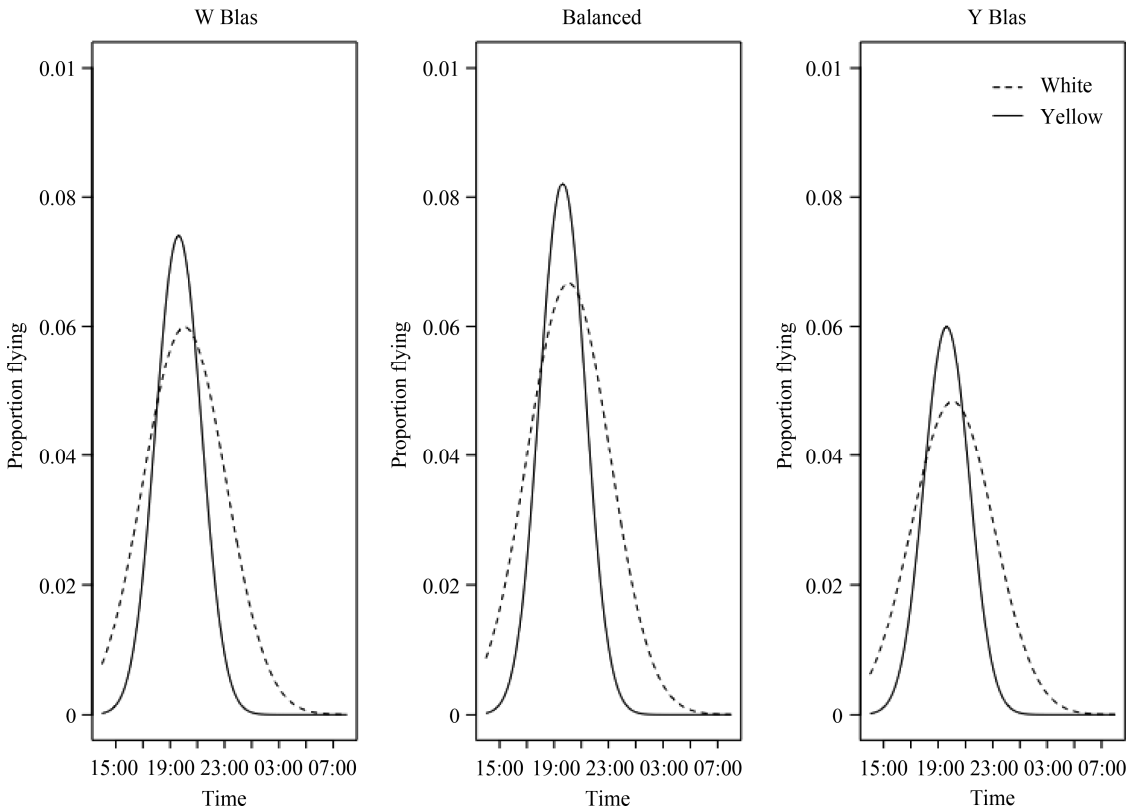

Fig. 3 Proportion of male flying activity in each treatment over time
Hatched lines show the yellow male morph activity whereas the solid lines indicate white males. Error bars indicate standard errors.

Table 1 Binomial GLMM for the effect of time and treatment on flight propensity of both morphs

Random Effects	Variance			
Trial	0.210			
Fixed Effects	Estimate	*SE*	Z-value	*P*-value
(Intercept)	-4.75	0.37	-12.79	< 0.001
Morph (Y)	-3.62	0.74	5.96	< 0.001
Time	0.69	0.12	5.96	< 0.001
$Time^2$	-0.06	0.01	-5.92	< 0.001
Treatment (W-bias)	-0.11	0.12	-0.97	0.333
Treatment (Y-bias)	-0.34	0.13	-2.68	0.007
Morph (Y) × Time	1.42	0.27	5.28	< 0.001
Morph (Y) × $Time^2$	-0.13	0.02	-5.61	< 0.001

quency-dependent differences in flying activity might be a behavioural mechanism compensating for the between-morph differences in predator and female colour preferences. We found no evidence of positive frequency-dependent flight activity in relation to wing colour; i.e. neither morph flew more when more abundant. Instead, the yellow-biased treatment had the lowest flight overall, indicating that frequency may indeed play some role (albeit very small) in morph-related flight activity. However, these differences in flight are most likely related to the morph itself, rather than to the morph ratios. The white wood tiger moth morph displayed higher activity levels, and for longer stretches of time, in all frequency treatments; whereas the yellow morph seemed to focus their flying in correlation to the peak calling activity of female moths. We now discuss the biological relevance of our findings for the wood tiger moth system, and how behavioural mechanisms may aid the general maintenance of polymorphisms in nature.

3.1 Difference in flying activity between morphs

There are several possible explanations why white individuals are more active in flight over time than yellow individuals. First, as white males have a weaker warning signal (Nokelainen et al., 2012; Nokelainen et al., 2014) and less deterrent chemical defences (Rojas et al., unpublished), they may compensate behaviourally for this disadvantage against predators. Predation experiments on static models so far have not taken into account the probability of escape either before or after an attack, and hence might overestimate attacks on white individuals. Instead, the possibility exists that whereas yellow individuals rely on their costly chemicals and efficient warning signal coloration to defend themselves, individuals of the white morph rely on their higher flying activity in order to escape predators while searching for females. This agrees with previous research indicating that butterflies have evolved two alternative anti-predator strategies to avoid birds: unpalatability and escape (Chai, 1996; Pinheiro, 1996). There is evidence suggesting that moths are capable of avoiding predators by flying quickly and performing abrupt changes in direction, especially when despite exhibiting conspicuous colouration they are not well defended chemically (Talianchich et al., 2003). Furthermore, noticeable differences in the flight patterns of palatable and unpalatable butterfly species (Chai and Srygley, 1990) could be reflected in similar ways between morphs of the same species differing in the strength of their chemical defences, as seen in this system.

According to previous studies, birds seem to be important predators of wood tiger moths (Nokelainen et al., 2012; Nokelainen et al., 2014). Thus, it is likely that a higher activity level increases white males' risk of attracting unwanted attention by predators while looking for females. However, given that both morphs are conspicuous (i.e. easily detected) when resting on natural vegetation (Nokelainen et al., 2012), high flying activity may correlate positively with improved escape probabilities of the less well defended white individuals. Furthermore, it is important to note that the activity peak of wood tiger moths does not overlap with either the peak activity of birds (which is earlier) (Lehikoinen et al., 2011) or bats (which is later) (Kosonen, 2008). Therefore, increased flying activity during this "safety-window" may allow white individuals the opportunity to get a female to mate with, while avoiding predation attempts.

Second, yellow individuals may not sustain their flying activity for long periods of time compared to white individuals because of a conflict in resource allocation. It is known that yellow males are better chemically defended than their white counterparts (Rojas et al., unpublished), but this comes at life history-related (Suisto et al., unpublished) and mating (Nokelainen et al., 2012) costs. Therefore, physiological costs associated to the investment in high-quality chemical defences, which is also common in other Lepidopterans (Marden and Chai, 1991), would trade-off with energetic resources required for frequent flying. For example, slow-moving anurans and salamanders, which do not rely on their activity to escape predators, tend to have powerful skin secretions (Duellman and Trueb, 1994). Given this limitation, in our case yellow male moths may focus their flight at only key times, for instance during peak calling activity of females.

Third, in organisms that depend on chemical signals (e.g., pheromones) to find resources or mates, such as the wood tiger moth, the efficiency of the flight path is very important (Carde and Willis, 2008). Searching behaviour incurs not only increased predation risk but also increased energetic costs; thus, selection should favour mechanisms that allow for search optimisation (Bell, 1990). The time an individual can devote to searching depends on the value of the resource and the individual's physiological state (Bell, 1990). In the case of females, rapid location is particularly important as signals are ephemeral (Carde et al., 2012) and males compete against each other for access to their potential mates (Greenfield, 1981). Hence, finding a female might be more important than lowering the flight costs associated with searching (Carde et al., 2012). It could be the case that white wood tiger moths have less efficient paths to find pheromone sources, and need to fly for longer in order to mate (Carde and Willis, 2008; Carde et al., 2012). More studies in natural or semi-natural conditions, ideally involving a real measure of predation pressure, are needed before any of these three possible explanations can be fully supported.

3.2 Frequency-dependent flying activity and differences across time

We found no frequency-dependence in the flying activity of each morph, but reduced activity for both morphs in the yellow-biased environment. Overall activity is lower when yellow males are more abundant, but even in that treatment white males are more active. It is possible that the activity levels of each morph were driven by the general activity levels of the population. Therefore, when yellow individuals are more abundant the activity of the white males is reduced too.

The time block with the highest activity for both morphs coincided, not surprisingly, with the times of higher female calling in the wood tiger moth; this timing is common in some Lepidopterans (Charlton and Cardé, 1982) and also Hemipterans (Levi-Zada et al., 2014). The synchrony between male flying activity and pheromone release by females is most likely adaptive, and aimed at guaranteeing high mating success (Levi-Zada et al., 2014). Interestingly, significant differences between their activity curves show that although white males overall have a higher flying activity than yellow males, the spread of this activity between morphs is very different. Specifically, white males fly earlier and finish later; whereas yellow males have a burst of activity that increases steeply but is not sustained for long.

The observed differences in activity spans may be due to morph-specific thermoregulatory abilities. Yellow individuals can afford a sudden and steep increase in activity because they warm up significantly faster than white males (Hegna et al., 2013). Therefore, white males might need to start their activity earlier in order to be at the maximum of their performance when the females are at their peak of pheromone release. Additionally, Crespo et al. (2012) indicates that males of some moth species are capable of taking-off at lower-than-optimal temperatures for flight muscles. This early start can trigger a trade-off with overall suboptimal flight performance (Crespo et al., 2012), but would also allow white males to get access to some females in the population at times when yellow males would not normally fly. Research shows that taking off at higher temperatures allows for flights at a higher speed (Crespo et al., 2014). In the wood tiger moths this could lead yellow males to the pheromone source (i.e. the female) faster, which would translate into overall reduced flying activity but would explain the match between the peak of activity of yellow males and the peak of female calling activity.

In conclusion, classic research on colour polymorphisms in aposematic species has focused on the role of predator learning and signal evolution, due to the strong selection exerted by the high cost of predator attacks. Recent research has shown that many other processes, which can also be frequency-dependent, may lead to similar net fitness benefits across morphs, maintaining polymorphisms. One such process may be related to variation in morph-specific behaviours (Rojas et al., 2014a). Taken all together, our results suggest the existence of a compensatory mechanism enabling the coexistence of both morphs in nature. However, we found no evidence that such mechanism is frequency-dependent in relation to colour. More specifically we suggest that, by flying more overall, white wood tiger moth males could compensate behaviourally for the disadvantages of weaker chemical defences through escape, and of less efficient paths to finding females through prolonged activity. Yellow males, while better protected but less sexually selected, may be able to compensate their lower activity by "flying smart", timing it with the period of higher female calling activity and finding females more quickly. Both strategies, behaviourally compensating the trade-off between warning-signal and sexual selection, could lead to similar overall reproductive success (Gordon et al., 2015) and potentially explain why both morphs are present in such high frequencies in multiple populations. Further research in settings where preda-

tion can be measured, combined with a detailed study of flight trajectories, physiological performance, and the possible interactions occurring between males at high densities, is needed in order to support our 'compensation' hypothesis and, in general, to have a better understanding of the activity patterns of this aposematic species, and their possible role in the maintenance of *P. plantaginis* wing colour polymorphism.

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