Analysis of reproductive barrier traits between closely-related neotropical butterflies

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ABSTRACT

Butterflies from the genus Heliconius are a well-studied case of Müllerian mimicry, which means that different species living under similar conditions mimic each other's aposematic signals. However, some species also mimic each other's flight behaviour, to produce an additional cue for the predator's perception. Here, I investigated if the Müllerian mimicry occurs between the *H. melpomene* and *H. elevatus* individuals. *H. pardalinus* were used to check if there wasn't a higher similarity between closely related species (*H. elevatus* in this case). The butterflies were filmed and their wing beat frequency was calculated based on the recordings obtained. The wing beat frequency values for the pair of mimics were more similar than between *H. pardalinus* and *H. elevatus*. In this mimicry pair, a wing motion mimicry was developed to offer one more signal to the predators. Since the birds rely mainly on their vision to catch the prey and have retinas with an up to three times higher flicker-fusion rates than in humans, the similarity in the wing beat frequency between mimics is used by the birds to differentiate them from the edible butterflies.

INTRODUCTION

Heliconius is a Neotropical genus of butterflies known for having a high number of races among all its species, with many cases of convergent evolution between distantly related taxonomic groups and divergent evolution between closely related species. Species from this genus living in the same environment have a convergence in the wing phenotype, and within species there is a great variation in the patterns. Their colourful wings have a function of aposematic warning to the predators since they are all unpalatable to them. The wing colour is under frequency-dependent selection in favour of the most common colour patterns, since the predators learn to avoid them.

There are two types of mimicry: Batesian mimicry (Bates, 1862) and Müllerian mimicry (Müller, 1879). In the Batesian mimicry, a defenceless and harmless species mimics the warning signals of another species that has evolved some kind of defence against a common predator, such as toxins and unpalatability. The harmless species parasitizes the signal, gaining a direct benefit from it (the predators that know the signal will avoid them) without investing in the development of an actual defence. The efficiency of the Batesian mimicry is frequency dependent, because it only works well if the mimics are in low densities. If the imposters occur in high frequencies, it's likely that the predator will meet them more often. Thus, the signal will be dismissed, losing its value, which is the avoidance from the predator. So, the species with the defence may develop other strategies to avoid being mimicked (Srygley & Ellington, 1999). The Müllerian mimicry, however, occurs when a poisonous, unpalatable or venomous mimics another species that also have a defence against a common predator. By mimicking the warning signals, both will have a similar colour pattern, enabling the predator to easily differentiate them from edible prey. In *Heliconius* butterflies, some species have similar occurrence ranges, and across their range in the Neotropics, they converge in the wing colour pattern in several locations (Nadeau et al., 2014). The prey species share the costs of

educating the predator, who, by learning to avoid one of the species, will also learn to avoid the other one.

Mimicry is an example of speciation due to natural selection. It occurs because hybrids with an intermediate phenotype (wing colour pattern) are less adapted and have a lower fitness, losing the mimetic ability. However, the wing colour pattern does not have only the aposematic signalling function, it also serves for assortative mating, being the main cue to which they respond. Hybrid females may also be sterile (Jiggins *et al*., 2001). However, as the great diversity of colour patterns in *Heliconius* suggest, the shifts in the colouration of a certain subspecies wings doesn't always lead to speciation. It only occurs when there is a dramatic enough change in the colour is achieved during the shift to generate pre-mating isolation through mate choice.

The places where different forms of the species meet and hybridize are known as hybrid zones. When a hybrid zone is narrow, it can be maintained by strong selective forces that favour certain forms in certain habitats or even prevent mixing between them (Barton & Hewitt, 1985). In the case of the Neotropical *Heliconius* butterflies, the strong selection in the hybrid zones is performed by the birds through avoidance of the aposematic colour patterns. Many of these hybrid zones are ancient, and were maintained by the selection of certain colour patterns by the predators (Nadeau *et al*., 2014). There are many *Heliconius* hybrid zones across different environments within their range because the subspecies differ in loci that are not related to the wing colour pattern, despite the enhanced divergence in loci controlling the colour patterns. The occurrence of parallel evolution of the mimetic wing colour patterns in this genus of butterflies is explained by the adaptive introgression of loci involved in the mimicry (The *Heliconius* Genome Consortium, 2012).

The main common predator to the *Heliconius* butterflies are the birds, and they actively hunt the butterflies during their flight. The main sense used by the predators to catch the butterflies is the vision. Birds perceive motion much faster than humans, with their retina having a higher flicker-fusion rate than ours (Frost *et al*., 1994). They can, and must, discriminate a flying animal must faster in order to catch the appropriate prey. An important part of the foraging process is to differentiate the edible prey from the unpalatable ones. Besides the wing colour pattern being used by the butterflies as the sole warning signal, they may develop other means of mimicry, to help to improve the predator's discrimination capabilities. The main way they do so is by mimicking each other's flight behaviour. In some mimicry rings, butterflies from the same clade have a different wing motion that converges to mimic other, distantly related, species' flight (Srygley & Ellington, 1999; Srygley, 1999; Srygley,2007). This mimicry may arise because it's not always easy for a bird to identify its prey only based on the colouration. Also, with more complex signals, it's harder for a Batesian mimic to parasitize the unpalatable *Heliconius* butterflies.

The main objective from this work was to evaluate if two mimic species, *H. elevatus* and *H. melpomene*, mimic each other's flight behaviour. To do so, I compared the flight dynamics of these species to that of *H. pardalinus*, which is closely related to

H. elevatus. Before those experiments, the relation between the flight activity of the butterflies and the environmental temperature and the individual sex.

METHODS

I measured the flight dynamics of three *Heliconius* butterflies species: *H. melpomene*, *H.* elevatus and *H. pardalinus*. All three species occur in the Amazon Basin (Rosser et al., 2009; Brown, 1979). Both *H. pardalinus* and *H. elevatus* are Silvaniforms, a different clade from the *H. melpomene* (Beltran, 2007). In this region, *H. melpomene* and *H. elevatus* are a pair of mimics (The Heliconius Genome Consortium, 2012). By using those three species it was possible to determine if any observed differences was due to the colour pattern mimicry group, the phylogenetic lineage or both.

The flight of the butterflies was recorded in a greenhouse corridor. The corridor was used in addition of a mesh net upon the head level to constrain their flight path as close to a straight way as possible. The camera used to film them was the GoPro Hero4 Black (240 frames/second at 848x480 resolution). Flight sequences analyzed ranged between 1 and 2 seconds of duration. The wing motion was not considered when the butterfly was flying towards the greenhouse glass nor when it was moving the wings while roosting. Before the filming of each butterfly, the corridor's temperature was taken. Each butterfly was filmed ten times under each temperature and, from these, about three videos could be used to make the measurements. However, due to the occurrence of deaths and the limited availability of individuals, not all of them were filmed in this exact number of times.

Two sets of experiments were made. The first one was made just with *H. melpomene* individuals due to availability of butterflies. Before these recordings, I marked each individual by painting dots with coloured pens on their wings, so that each of them would have a unique pattern (there may be an individual variation). Then, the sex of each individual was identified, so that any effects of sex on the wing motion could be evaluated. Also, with these recordings, the temperature was taken before filming to have its effects assessed. The flight dynamics was not expected to differ between males and females, since they were filmed under the same conditions and performing the same behaviour (which was not related to any sexual activity). However, as butterflies are ectotherms and the *Heliconius melpomene* individuals studied come from a tropical region, temperature was expected to have an influence in the wing motion (Heinrich, 1986; Saastamoinen & Hanski, 2008).

The second set of experiments was the recordings of *H. pardalinus* and *H. elevatus*. Nonetheless, there were no *H. pardalinus* individual available, and only two *H. elevatus*, which was not enough for the measurements. So, for this experiment, I manipulated the wing shape of *H. melpomene* individuals using templates from the other two species: *H. pardalinus* and *H. elevatus*. Scanned wings of dead butterflies from each species were used to make the templates. Before manipulating the *H.*

melpomene wings, the individuals were marked and had their sex identified. Then, their flight was recorded and I cut their wings with the templates. Finally, their flight was recorded again, but not immediately after the manipulation. I also took pictures of their wings before and after the manipulation in order to calculate the percentage of the area lost, since it may also affect their wing motion. The temperature of the corridor was taken before each recording. It was expected a difference between the *H. pardalinus* and the *H. elevatus* flight dynamics, as well as a similar motion between *H. elevatus* and *H. melpomene*, since in previous studies (Srygley & Ellington, 1999; Srygley, 1999; Srygley, 2007) the mimic species had a higher similarity than related ones.

The measurement taken from the recordings was the wing beat frequency. In other works done with locomotor mimicry of *Heliconius* butterflies, such as Srygley & Ellington (1999), the asymmetry ratio of the wing motion was measured, as well as the wing beat frequency. The symmetry of the wing motion corresponded to a more sinusoidal motion. Thus, higher asymmetry ratios had a wing motion more distant to a sinusoidal one. However, the asymmetry ratio didn't show very significant results, and the percentage explained of the differences between mimicry groups by the asymmetry ratio was low, compared to the wing beat frequency. The wing beat frequency (Hertz) was calculated by counting the number of wing beats and dividing it by the time (seconds). The beginning of the beat was the starting point of the down stroke, with the wing tips in their highest elevation. To count the wing beats and have an accurate value of the time, I used the GoPro Studio program, which allowed the use of slow motion to view the recordings, consequently enabling the perception of the exact moment of the beginning of the down stroke.

To check the differences of wing beat frequency among temperatures and between the sexes, a Linear Mixed Effect model was used, with the individual identity as the random factor and sex and temperature as the fixed factors. For the wing manipulation experiment, I used a Generalized Linear Model, checking the influence of the manipulations in the wing beat frequency. The statistical analysis was made in R Project for Statistics Computing.

RESULTS

In total, 24 *H. melpomene* individuals were recorded for the first experiments. Ten of them were male, and 14 were female. The males' wing beat frequency (10.51±1.32Hz) was slightly higher than the females' (10.45 ±1.29Hz) (Fig.1). Sex didn't affect the wing beat frequency (χ^2=1.49, p=0.2211).

The recordings were made from 23°C to 28°C. The wing beat frequency increased with the temperature, with the highest value being recorded in 28°C (11.67±1.26Hz) and the lowest in 23°C (8.18±1.34Hz). Temperature affected frequency (χ^2=134.74,

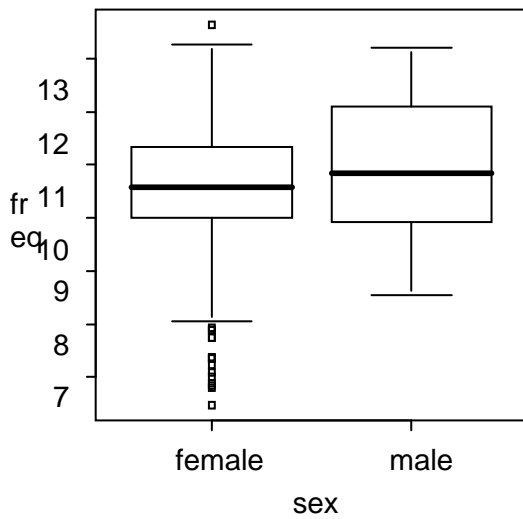p<2.2e-16), increasing it by an average of 0.52±0.04Hz (Fig.2).

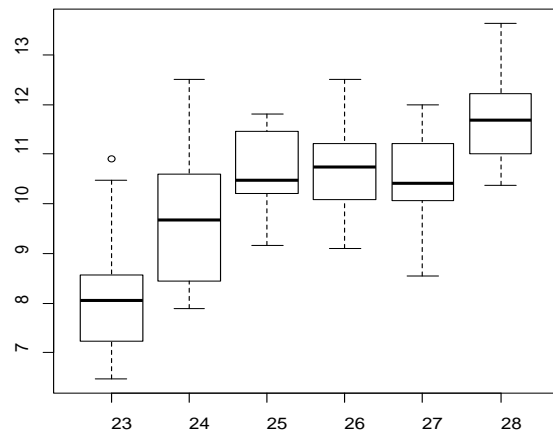**Figure 1**: Differences in wing beat frequency (Hz) between sexes (p=0.2211).

Figure 2: Wing beat frequency for each temperature analysed. The wing beat frequency (Hz) is represented in the y-axis and the temperature (°C) in the x-axis (p<2.2e-16).

In the manipulation experiment, seven *H. melpomene* had their wing cut with the *H. pardalinus* manipulation and eight with the *H. elevatus* manipulation. All of them were recorded before the manipulation. The wing beat frequency was different among the manipulations, with the *H. pardalinus* manipulation having an increase in the wing beat frequency of 0.09±0.27Hz and the *H. elevatus* manipulation decreasing it by 1.02±0.23Hz (p=0.0364). The wing beat frequency with the *H. elevatus* manipulation had a more similar value to that of *H. melpomene,* the control in this case (Fig.3).
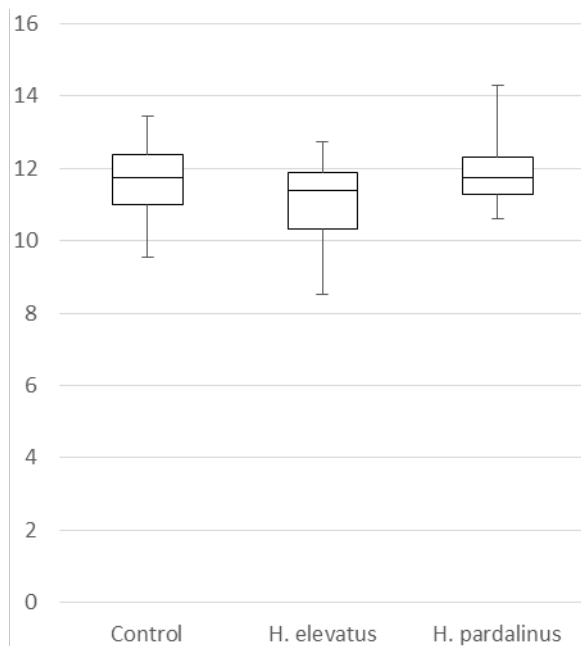

Figure 3: Relation between wing beat frequency (Hz, in the y-axis) and the manipulations (p=0.0364).

DISCUSSION

All the butterfly individuals were filmed flying in a similar context during the first recordings, which was in an almost straight direction, as well as under similar conditions. So, both males and females were filmed displaying the same behaviour in the same environment. Srygley (2007) evaluated the effects of different behaviours in the flying dynamics of *Heliconius* butterflies. The behavioural contexts analysed were: cruise, forage, hostplant search, roost and court. Two of these behaviours were exclusive to one of the sexes, with hostplant search being female-exclusive and court being male-exclusive. Wing beat frequency varied with context, the highest value being for the hostplant search (12.4Hz), followed by the court (12.3Hz). So, even though both behaviours characterised for being displayed by individuals of a single sex were slightly different, it didn't affect the overall average of each sex wing beat frequency. The main function of the wing beat frequency is the generation of lift to keep the butterfly in the air during the flight, and it works equally for male and female insects. Even asymmetry ratio, the other measurement taken in Srygley's (2007) work, didn't show a significant difference among behaviours and between sexes as well, indicating that the flight is not dependent on the sex.

Insects may be regulate their body temperature as endotherms. They can do so by muscular activity (active flight muscles, more specifically), by physiological means or even by certain behaviours, such as basking (Heinrich, 1974). However, even in larger insects, there is a high dependence on the environmental temperature and in sources of heat to regulate their body temperature and, consequently, their metabolic rates, since they dissipate the excess of heat produced physiologically. In a study made with *Coenonympha inormata*, a species of Satyridae butterflies (Heinrich, 1986), the body temperature and ambient temperature were taken and associated with flight activity. When the temperatures were lower, the butterflies flew slowly and occasionally stopping and roosting, with a low flight duration. When the temperatures were higher, the butterflies flew for a longer time without stopping, and when they stopped, it was for a shorter time. In the Glanville fritillary butterfly (*Melitaea cinxia*), the environmental temperature also affected the female reproductive performance (Saastamoinen & Hanski, 2008). The oviposition occurred later in the day when the ambient temperature was lower, leading to a smaller clutch size as well. Also, with lower temperatures, their activity and opportunities for feeding are reduced. Their body temperature during flight increased with the environmental one, with the body mass having no effect. Dreisig (1995) analysed the thermoregulation and activity of male butterflies of two species (*Hipparchia semele* and *Ochlodes venata*). They have a territorial behaviour, defending their mating sites from other males. While perching in the territory, they expose their body to the sun to reach a body temperature as close as possible to the preferred level. At low temperatures, they expose the maximum possible body area to the Sun in order to increase the body temperature. At high temperatures, the butterflies exposed the minimum body area to lessen the heat uptake. Since the butterflies' activity rates depend on the ambient temperature, and their flight is energetically expensive, the results are in accordance with the interaction between the butterflies' activity rates and the temperature. Thermoregulation is an important factor for the butterflies

because they heavily depend on their body temperature control to regulate their behaviour and in lower temperatures, butterflies lose heat more rapidly than at higher temperature conditions (Gilchrist, 1990).

The wing manipulation result was in accordance with the hypothesis of wing motion mimicry. The *H. elevatus* manipulation had a closer wing beat frequency value to its mimic species (in this case the *H. melpomene* individuals before manipulation) than to the closer related species, which is the *H. pardalinus* (Fig.3). The divergence between closely related species may occur due to reproductive isolation by differences in wing colour pattern. However, a shift in the species habitat is more likely to produce a pre- and post-mating, and the assortative mating would just reinforce the difference between the species (Jiggins *et al.*, 2001). Body temperature differences may cause the variation in the wing beat frequency between the different groups of mimics in these butterflies (Srygley & Chai, 1990). However, it was already stated that there is a difference between two different mimicry groups without any differences in body temperature among the individuals (Srygley, 1999). The difference in wing beat frequency between species is unlike to influence on the reproductive isolation, since its main adaptive function is probably the wing motion mimicry.

The main reason for the convergence of the wing beat frequency between mimics is to serve as an extra cue for the distinction of Müllerian mimicry groups. By having a similar wing motion, both species are better identified by their predators, the birds, during the flight. Also, it is harder for any Batesian mimics to successfully parasitize the unpalatable *Heliconius* butterflies.

Birds such as the rufous-tailed jacamars (*Galbula ruficauda*) select their prey based on their movement, as well as their wing colour pattern (Chai, 1986). The associations between butterflies' colour pattern, flight behaviour and body shape rapidly enables the predator to assess the palatability of its prey (Chai & Srygley, 1990). Probably the distinction between the palatable and the unpalatable is much quicker and more effective when cues other than just the colour pattern are involved. The wing motion is probably the first recognizable visual characteristic of a butterfly, since the bird must catch it during the flight, and before it can spot the details in the colour pattern. Similarly, people can recognize friends and acquaintances just by the way they walk, even when the face cannot be seen (Jokisch *et al.*, 2006).Palatable butterflies fly faster and more erratic than the unpalatable ones, since they need to avoid the predators for not having a secondary defence (Srygley & Chai, 1990). Under the same predatory pressure, the aposematic signals of distasteful butterflies are predicted to converge (Joron & Mallet, 1998).

Although the speed of the butterflies' wing beat may be too fast for the perception of human eyes, the birds probably can notice the difference in the wing beat frequency between the difference mimetic groups. Humans have a motion discrimination that requires the integration of speed for 100 to 200 ms in the downstroke of the wing to obtain a correct speed signal. The acceleration, as well as other higher-order signals, may require more time to distinguish (Frost *et al.*, 1994). According to Srygley and Ellington (1999), the difference between the downstroke duration from

butterflies from two different groups (the *cydno-sapho* and the *melpomene-erato*) was in average 12 ms. This difference is not detectable by humans. There isn't data available for birds to make a comparison. However, using the flicker-fusion rates from the birds' retina in comparison to the humans', in some birds it is from 1.5 to 3 times faster than in humans (74-145Hz in pigeons, Frost *et al.*, 1994; and 50-100Hz in humans, McKee & Watamaniuk, 1994). Birds discriminate wing strokes that, over a 50 ms time-span, differ in velocity and acceleration. So, they perceive motion two to four times faster than humans (Srygley & Ellington, 1999). This would enable them to recognize the differences in the wing beat frequency of different *Heliconius* butterflies.

The locomotor mimicry is also likely to be developed between species that share an environment where the predator visibility is limited or the colour pattern has a reduced function in the prey detection, such as during the night (when predators use the wing motion perception through echolocation, for instance). In these situations, the perception of the butterflies' wing motion is essential to differentiate the edible from the unpalatable prey.

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