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Mate choice and hybrid viability in two species of *Heliconius*

Master's Thesis

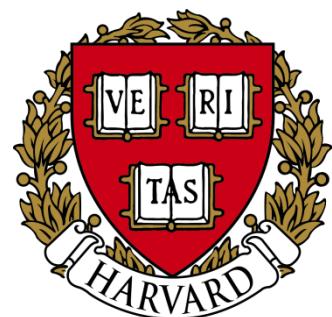
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ABSTRACT

Speciation with gene flow is a hot topic in evolutionary biology considering that gene flow normally tends to homogenize populations. Nevertheless there are some taxa where hybrids between species can be found and are believed to trigger speciation. An example of this is the case of the *Heliconius* butterflies, a recently radiating Neotropical genus which has become a model organism to study speciation. *Heliconius* species have distinctive color patterns that have an important role in mating preferences and mullerian mimicry. *H. pardalinus* and *H. elevatus* are two distinguishable species that are closely related, diverged recently and coexist in sympatry. The two species have distinctive color patterns but show great amounts of admixture in their genome. In this thesis we study the isolation barriers between this two species. To achieve this I studied mate choice and courtship behavior, likelihood of mating within and between species and hybrid sterility and inviability. *Heliconius pardalinus* and *Heliconius elevatus* have strong prezygotic isolation but no postzygotic isolation.

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1. INTRODUCTION

Speciation is the mechanism that creates and maintains species diversity and is one of the fundamental study areas of evolutionary biology. Nevertheless, after more than 150 years of the publication of Darwin's master piece "On the Origin of Species...", the different mechanisms by which speciation can occur are not fully understood and the genetic basis of speciation is still unveiled. The most used definition of a species is the biological definition instated by Mayr in 1963 which defines a species as reproductive isolated populations. This means that very little or no gene flow should exist between two species if hybrids are viable they should be maladapted, sterile or selected against by some other means. Under this definition of species, is easy to understand speciation in an allopatric scenario where the lack of gene flow due to geographic barriers eventually leads to incompatibilities that generate pre-zygotic and post-zygotic barriers. On the other hand, other possible mechanisms such as parapatric speciation (this involves a little but not complete isolation) and sympatric speciation (on which there is no geographical isolation at all) are not so intuitive because gene flow would tend to homogenize the divergent populations (Mayr 1963; Rice and Hostert 1993). For a long time, allopatry was considered the main driver of speciation in nature with a few examples of parapatric speciation and almost no examples of sympatric speciation with the exception of allopolyploidy in plants.

In nature, the biological definition of speciation applies poorly to many cases were species are clearly distinct but still exchange genes with other species e.g. *Heliconius* butterflies (Mallet *et al.* 2007) and *Ficedula* flycatcher (Ellegren *et al.* 2012), especially in systems where a recent adaptive radiation has occurred. Interestingly, Darwin viewed the species as on a continuum of varieties rather than as very different units (Darwin 1869). Only in the last few years with the newly available genomic techniques, opinion on the importance of gene flow in the process of

speciation has started to change. More and more, new cases appear in which hybrids have an important role in speciation by facilitating introgression of adaptive traits or by hybrid speciation (the generation of a new species by hybridization of two existing species). Hybrids do exist and they are more common than we thought: at least 10% of animal and 25% of plant species are known to hybridize (Mallet 2005), which could mean that they have a much more important role than what previously thought.

The best-known examples of hybrid speciation in sympatry and with gene flow are allopolyploid species like many crops and angiosperms e.g. American daisies (Weiss – Schneeweiss *et al.* 2012) that preserved both parental genomes (so have twice the normal chromosome number) and remained isolated because of the ploidy incompatibilities. Another mechanism of speciation with gene flow is homoploid hybrid speciation (HHS) on which the original number of chromosomes is maintained. This is known in flowering plants but is rarer than allopolyploidy due to the conditions it has to fulfill for it to happen; there must be few incompatibilities between the chromosomes of the parent species, and yet a means to maintain reproductive isolation must rapidly evolve. This is usually reached by a combination of ecological speciation and sexual selection (Schluter 2001). Some examples of HHS are: *Helianthus* sunflowers (Ungerer *et al.* 1998) the Italian sparrow (*Passer italiae*) (Hermansen *et al.* 2011), African cichlids (Koblmuller *et al.* 2007), Nicaraguan cichlids (Barluenga *et al.* 2006), *Rhagoletis* fruit flies (Dietmar Schwarz *et al.* 2005), Swallowtail butterflies (Scriber and Ording 2005), *Lycaeides* butterflies (Gompert *et al.* 2006), *Heliconius* butterflies (Salazar *et al.* 2005, 2008, 2010; Mavarez *et al.* 2006) and the Clymene dolphins (*Stenella clymene*) (Amaral *et al.* 2013).

Today the species with a known hybrid origin seem surprisingly common given that HHS is supposed to be highly unlikely. One of the most striking examples of this is found in the

Heliconius butterflies (Nymphalidae: Heliconiinae). This genus has particular characteristics that make it unique among all the butterflies and suitable for speciation studies. They are pollen eaters, have social roosting, long life span (up to six months) and UV (Briscoe *et al.* 2010) and color vision (related to host plant finding and memory). All *Heliconius* are unpalatable to predators and as a consequence they are frequently involved in mullerian mimicry. This characteristic is the reason why among the 43 species in the genus there are hundreds of races with particular color patterns that can be both shared among distantly related species and also completely different among closely related species. The species involved in mullerian mimicry benefit one another by sharing a similar color pattern that has to be learned only once by predators. Color pattern also has a crucial importance in mate choice and sexual selection so that mullerian mimicry can have an important role in ecological speciation and pre-zygotic isolation. In this genus, there are three cases that could be HHS on which adaptive characteristics have been acquired via introgression from *H. melpomene*: *Heliconius heurippa* (Salazar *et al.* 2005, 2008, 2010; Mavarez *et al.* 2006), *Heliconius timareta* and *Heliconius elevatus* (Heliconius Genome Consortium 2012).

Heliconius elevatus is one of the subjects of the current study, and forms part of the recent radiation in the silvaniform clade of *Heliconius*. In this clade *H. elevatus* is the only species that has a rayed color pattern (Fig. 1a) that mimics rayed *H. erato* and *H. melpomene*, as well as a number of other species. All other species show a silvaniform or “tiger” pattern characteristic of ithomiine butterflies, with the exception of *H. besckei* from SE Brazil, which shows a pattern with a red forewing band and yellow hindwing band, and mimics local forms of *H. erato* and *H. melpomene* from this area). *Heliconius elevatus* acquired its rayed pattern from an introgression event from *H. melpomene* (Heliconius Genome Consortium 2012). *Heliconius elevatus* is closely

related to *H. pardalinus*, a typical ithomiine-mimicking silvaniform *Heliconius* (Fig. 1a). The two species diverged recently and coexist in sympatry (see map, Fig. 1b). Something that is interesting to observe in their phylogeny and distribution (Fig. 1b) is that *H. pardalinus* has two subspecies *H. p. butleri* and *H. p. sergestus* which are allopatric; *H. p. sergestus* is restricted to the upper Huallaga valley (a dry area) whereas *H. p. butleri* occupies the Amazonian lowlands broadly in sympatry with *H. elevatus*. At the same time *H. elevatus* and *H. p. sergestus* are parapatric since they can be both found in the transition area between lowland amazon and the higher-dryer area.

Studies of whole-genome sequences of these species show low divergence between the genomes of *H. pardalinus butleri* and *H. elevatus* except for specific regions known as “islands of speciation” within which, in theory, genes coding for important characteristics for speciation, such as color pattern, mating behaviour, and hybrid inviability are expected to be found. These islands of speciation have been identified in *H. pardalinus* and *H. elevatus* (Kryvokhyzha 2014). Nevertheless basic ecological and behavioral information for this species is still missing. This thesis will focus on describing some basic ecological observations of these two species as well as the mate choice preferences and the hybrid viability.

The main objective of this master's thesis is to understand reproductive isolation between *H. pardalinus* and *H. elevatus*, specifically between *H. p. butleri* and *H. elevatus*. To achieve this I studied mate choice by means of a behavioral experiment on courtship behavior. Additionally I estimated the likelihood of mating within and between species. Finally to studied hybrid sterility and inviability, I made pure and hybrid crosses and compared the hatching rates. It is already known that there are no major difficulties in obtaining viable hybrids between these species

(Rosser and Dasmahapatra, unpublished data, 2013). However, we want to document it quantitatively.

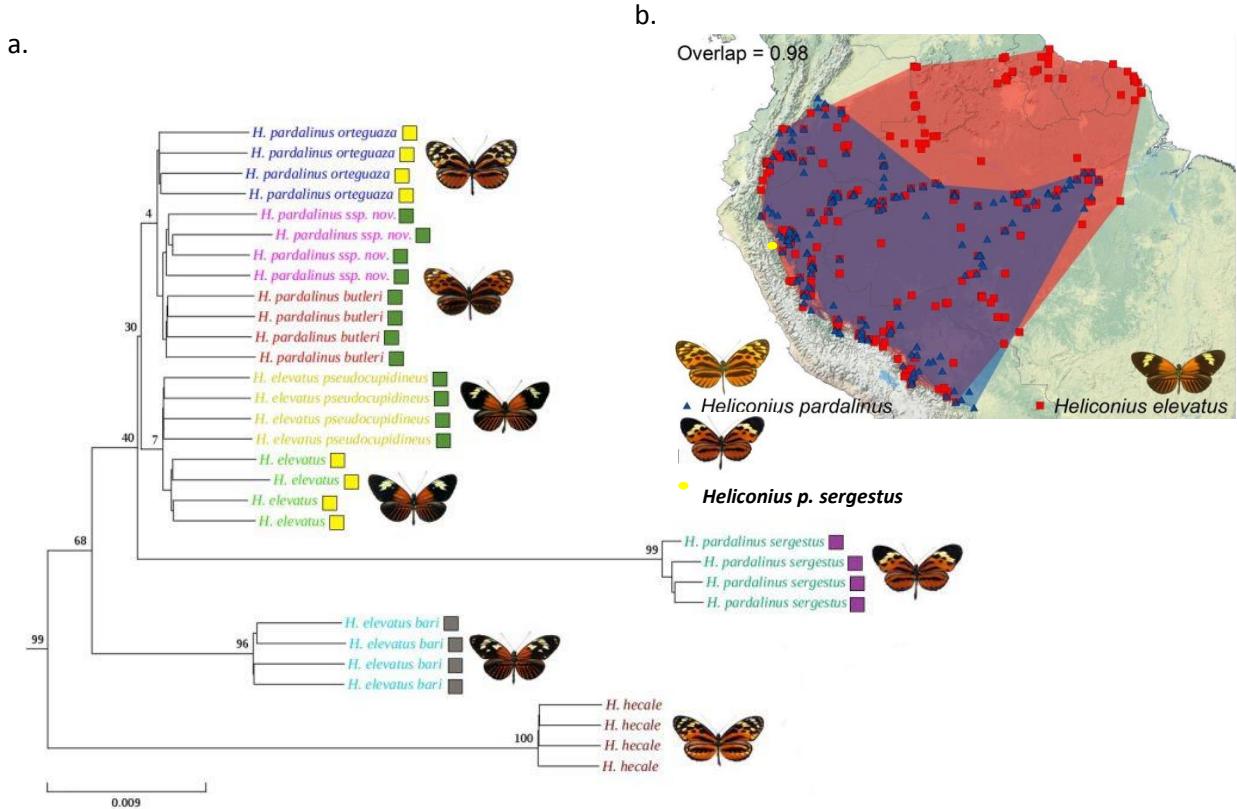


Figure 1. (a) NJ phylogenetic tree of the whole nuclear genome (Kryvokhyzha 2014). (b) Distribution map of *H. elevatus* and *H. pardalinus* (Rosser *et al.* 2012).

2. MATERIALS AND METHODS

Our collaborative group in Tarapoto (San Martín, Perú) consisted of Neil Rosser (Post doc in charge of the project, Dasmahapatra group at York University), Lucie Queste (Master's student in the Dasmahapatra group at York University), Patricia Velado Lobato (MEME master's student / Dasmahapatra group at York University) and myself Carolina Segami (MEME master's student / Mallet group, Harvard University). All the rearing tasks including the feeding, maintenance of the cages, collecting the eggs and care of the caterpillars were shared by

everyone. The behavioral experiments were carried out by Patricia Velado and I. The individual crosses were taken care of by myself and Neil Rosser. I carried out all statistical analyses.

I worked with *Heliconius elevatus* (E), *Heliconius pardalinus butleri* (P), *Heliconius pardalinus sergestus* (S) and the F1 hybrids of *Heliconius pardalinus butleri* x *Heliconius elevatus* (PE); in the tables and graphs I use the following abbreviations respectively: E, P, S and PE. The stock cages of butterflies (of dimensions of 2 x 3 x 2 m³) and all the experiments were conducted outdoors in Tarapoto, San Martín, Perú between September 2014 and January 2015. The butterflies for the species stocks, were collected in different sites nearby Tarapoto. Every stock had host plants (*Passiflora edulis*, *Passiflora nitida*, *Passiflora riparia* or *Passiflora serratodigitata*) and additional plants and flowers to simulate a natural environment, additionally natural pollen sources were provided (*Lantana sp.* and commercial pollen) every day. Artificial nectar was also provided every day and consisted on a solution of water, honey, sugar (approx. 10%). Additional pollen was presented in a small container filled with red and yellow straws to simulate a flower (McMillan *et al.* 1997). Eggs were collected daily and put in plastic pots until they hatched in our rearing room area, and the small caterpillars were placed on tendrils or very tender leaves of shoots of *Passiflora* inside the corresponding rearing cage, where they developed to adulthood. We maintained rearing cages for each stock and for each cross, all under the same local conditions. Shoots were changed as often as necessary depending on density of caterpillars. Every newly emerged butterfly was marked with a unique color code after the wings were fully extended and strong, in order to track the identity of each individual. As well as the stock insectaries, we maintained male and virgin cages that had the same characteristics.

2.1 Mate choice behavior

For the mate choice experiment we used a design that consisted of putting five males of each species (*H. elevatus*, *H. p. butleri*, and *H. p. sergestus*) with one virgin female at a time in a 2 x 2 x 2 m³ cage with characteristics as described above. The males were at least seven days old to ensure sexual maturity (McMillan *et al.* 1997; Naisbit *et al.* 2001). We maintained three experimental cages, and each experiment was observed for a three day maximum period if no mating occurred before the third day, and if the weather was considered good enough to observe courtship behavior i.e. above 20 °C without rain or strong wind (Klein and Araùjo 2010) otherwise we left the experiment for one more day. We used this setup to study two parameters: The probability of mating between and within species (as a proxy for pre-zygotic barriers) and the mate choice behavior by measuring behavioral units already identified by Klein and Araùjo, 2010 for another *Heliconius* species see Table 1., comparing male preferences in the courtship across species and ultimately describing any behavior since the ecology and behavior of this species has not been studied before.

The experiment was monitored every hour for 15 minutes in the most active hours of the day between 10 a.m. and 3 p.m. (Klein and Araùjo, 2010). In each 15 minutes trial we recorded the time, date, weather (Sunny, Cloudy or Rainy), temperature, number of experiment, day of experiment, number of cage, female ID, the different behaviors and copulation.

Table 1. Ethogram of *H. erato Phyllis* male-female interactions modified from (Klein and Araùjo, 2010).

Behavior	Description
Male	
Approach	In flight, the male approaches the alighted female with the body directed to her, similarly to the way he does in front of flowers or other objects that attract him
Hovering	The male remains in flight over the alighted female (5–15 cm) without considerable displacement

Alighting on wings	The male in flight, when approaching an alighted female, briefly puts his legs on her anterior wings (<2 s most of the times)
Slow flapping	Alighted, next to the female (<3 cm), the male opens and closes the wings slowly
Androconia exposition	Alighted, next to the female (<3 cm), the male flaps the wings quickly without opening much and separates the forewings from the hindwings, exposing the silver-colored area of the forewings where the androconia are located (Fig. 1b, counter no. 5–15 in the video image)
Abdomen bending	Alighted, next to the female (<3 cm), usually in antiparallel sense or slightly slanted to her, the male bends the abdomen in her direction. Most of the times it is executed simultaneously with androconia exposition
Touching with proboscis	Alighted, the male touches several regions of the female body with the proboscis
Mating	Successfully grabs the female with claspers and initiates spermatophore transfer.
Male and female Flight interaction	Any encounter in flight that does not initiate a pursuit but that is followed by a trajectory change of at least one of the individuals
Flight pursuit	The male pursues the female in a rapid flight with sudden direction changes, touching her often, principally on the posterior and ventral regions

If a mating occurred then both the male and the female were removed immediately after mating had ceased from the experimental cage and replaced by a new male and a new virgin the next day, the mated males were not reused and the females were isolated for egg counting purposes. Females that did not mate were discarded to the stocks or used to generate other crosses. In total 39 experiments were performed, 14 for female *H. elevatus*, 15 for female *H. p. butleri* and 10 for female *H. p. sergestus*.

All the statistical analysis was done in R version 3.1.0 (R Core Team 2014); generalized linear mixed models assuming poisson distribution (appropriate for count data) were generated for each behavior, provided they had enough observations to create a model, and to test for differences of activity of males between species). Count data and activity values were used as response variables in the R package lme4 (Bates *et al.* 2014). Our main interest was to test whether there were interactions between species of the males and females, therefore all models included these

interaction terms. The weather and activity were included in the model because we observed that it may influence the behavior of the butterflies and could explain some variance, I decided to consider them both because it was not clear if they would have different effects. As random effects we used the number of experiment i.e. female identity, the time and date; we think these variables could explain part of the variation and we are not particularly interested in their individual effect. For the model of Hovering we had to deal with overdispersion. One strategy to deal with overdispersion is to add a per-individual random effect that consists of all the observations tested in the model (Pirk *et al.* 2013). I therefore created a variable called “Over” to estimate individual characteristics and added it to the overdispersed model. The models were selected using likelihood ratio test and AIC values and at the same time non-significant variables with no major biological importance were eliminated using backward elimination (Zuur *et al.* 2009). For significance, confidence intervals were created with the profiles of the model generated with likelihood ratios with the commands “profile” and “confint” of the package lme4 (Bates *et al.* 2014). The plots of the behavioral observations were generated with the R package ggplot2 (Wickham 2009).

2.2 Probability of mating (likelihood analysis)

To estimate the probability of mating and male attempts at mating females within and between species in the context of our experiment (one female and three possible outcomes of mating) we used a likelihood approach (McMillan *et al.* 1997) based on a multinomial distribution. For each type of female, the probability of mating P_{ij} was obtained for each combination of i female and j male (P_{1ij} , P_{2ij} and P_{3ij} sum 1) maximizing the \log_e likelihood given by

$$nE\log_e(P_{1ij}) + nP\log_e(P_{2ij}) + nS\log_e(P_{3ij}) \quad (1)$$

Where nE , nP and nS are the number of matings with an *H. elevatus* male, a *H. p. butleri* male and a *H. p. sergestus* male respectively given a virgin female i . The maximum likelihood and probabilities $P_{i \times j}$ for each i female were calculated in R using equation 1. The support limits, equivalent to 95% confidence intervals, were obtained with a two units decrease in \log_e likelihood from the maximum likelihood (Edwards, 1972). Additionally, surfaces of \log_e likelihood were plotted in R for each scenario. This same approach was repeated for observations of abdomen bending which is an actual mating attempt. If the same male was observed to repeat abdomen bending in a same trial it was counted only a single mating attempt. In this case we hope to estimate the probabilities that a male j tries to mate with a female i .

2.3 Crosses and egg hatch rate

I am interested in the study of post zygotic barriers between *H. elevatus* and *H. pardalinus butleri* and for instance I performed pure species crosses and all the possible combinations that I was able to obtain (see table 3). Mated females were isolated in individual cages 1 x 1.5 x 1.8 m³; some of these females came from our mate choice experiment, other matings were obtained by introducing few females in our male cages. The experimental cages were monitored every hour to ensure we obtained matings. Later, the rest of the crosses (mainly *H. elevatus* x *H. p. butleri* and *H. p. butleri* x *H. elevatus*) were obtained by hand pairing the parents. The collected eggs were preserved in plastic containers in our rearing area; these containers were checked every day for hatching larvae to avoid cannibalism. I counted the number of eggs per day for each cross until a maximum of 100 eggs; I also recorded some descriptive information listed in table 5. Finally, I created a binomial distributed generalized linear mixed model with all the data per day (eggs collected per day) that we had to compare the hatching rates across species and type of cross. The model was selected following the same steps mentioned before. In this model I

chose as a random effect the female ID (to account for brood variation) and also a variable called “Over” (which consists on every single observation) as in previous information to deal with overdispersion in the model. A Wilcoxon sum-rank test, a non-parametric test, was performed in order to test for differences between *H. elevatus* and *H. p. butleri* with regard to the numbers of eggs laid per day.

3. RESULTS

While monitoring all the experiments and in particular the behavioral experiment, we were able to observe the whole sequence of behavioral steps for a few mating events. The sequence of the successful courtship was most of the times as follows: Hovering vigorously, alighting on wings, touching with proboscis face to face and finally the male rotates to be by the side of the female and bends the abdomen (see table 1). This sequence could be repeated several times (usually no more than 3) until the male is successful to grab the female with its claspers after an abdomen bending. This happened very fast, usually in less than a minute. If the courtship lasted longer it usually didn't finish in copulation with really few exceptions. Sometimes we observed active competition between males to pair with a female doing all these steps at the same time; in one case this competition was between *H. p. butleri* and *H. p. sergestus* that ended in a hybrid mating between a female *H. p. butleri* and a male *H. p. sergestus*.

3.1 Mate choice behavior

Of the 39 experiments that were performed, we obtained 16 matings: 7 pure crosses of P♀ x P, 5 pure crosses E♀ x E and 4 hybrid crosses P♀ x S. From the behaviors described in Table 1, the only consistent ones that we could observe for males were: Approach, Hovering, Alighting on wings and Abdomen bending. The other described behaviors in table 1 were just not present,

were too fast to observe or too few to consider. In the case of the females we were unable to observe any constant behavior and so it was not recorded. Therefore our observations for mate choice are mainly focusing on male preference. A summary of the observed behavior across all the trials can be observed in figure 2 and figure 3. The data for all behaviors presented a skewed distribution due to the high numbers of zero observations.

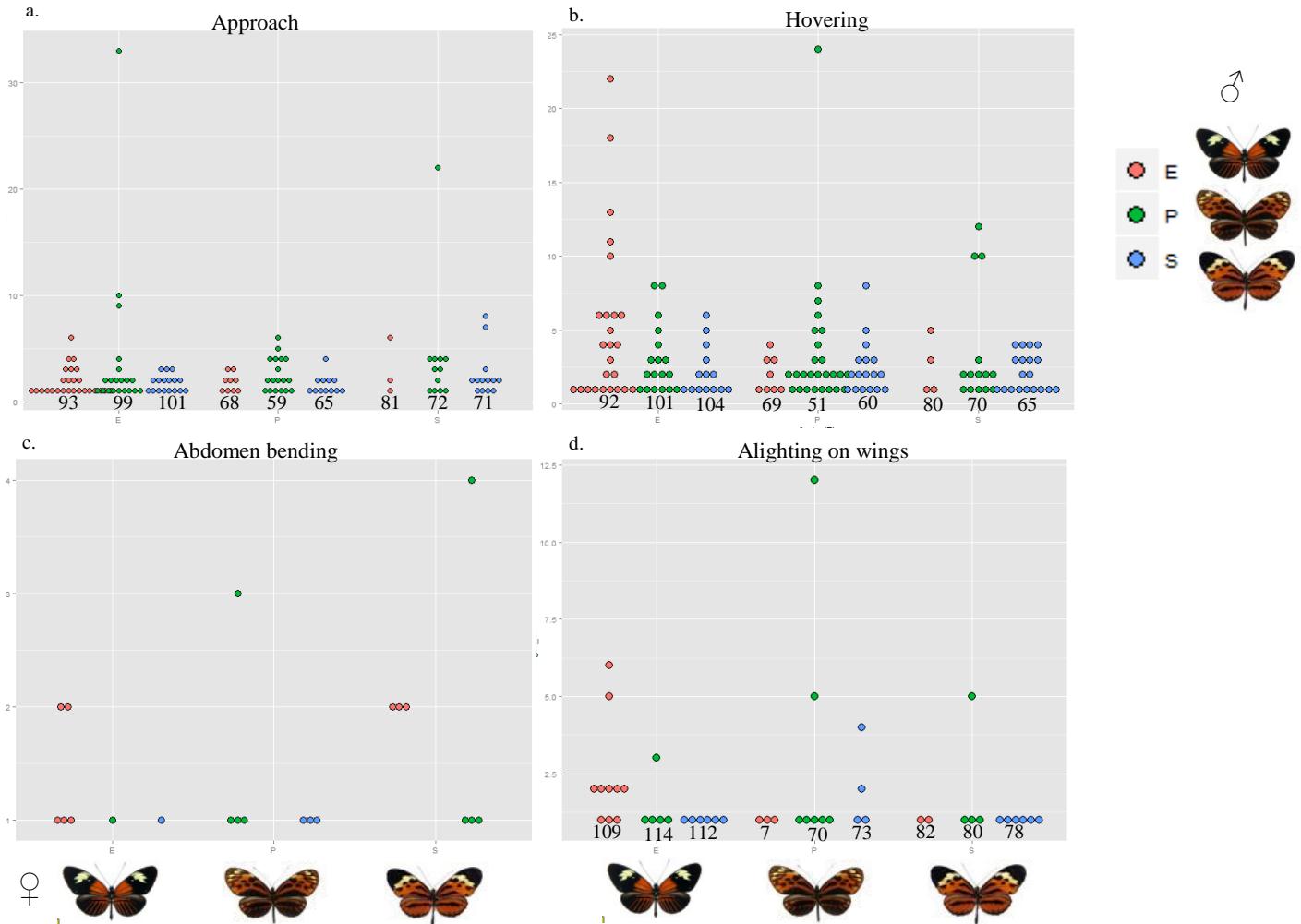


Figure 2. Strip charts of the number of counted behaviors across trials. Each dot represents a 15 minute trial, the y axis indicates the number of times each unit was observed during a single trial. (a) Number of approaches, (b) number of hoverings (c) number of abdomen bendig and (d) number of alighting on wings that were executed by males (legend) towards females (x-axis). The zero values are represented by real numbers.

As can be seen in Figure 2, both approach and hovering were the most common observed behaviors followed by alighting on wings and finally abdomen bending. This makes sense because approach and hovering are part of the first steps in courtship where the males are inspecting the female and therefore are more common. In the other hand abdomen bending and alighting on wings are immediate previous steps to the copulation event so when performing those, the male is already willing to copulate. In appendix 1 there is the table with the coefficients and significance intervals for the generalized linear models that I created to test for interactions and difference between species. In the case of the behavioral unit abdomen bending it was not possible to create the GLMM model due to the small number of observations but instead we analyzed the data using a likelihood approach with the mating behavior below.

The models were used to test the significance of the interactions between males and females that are indicated with an asterisk in the graphs of figure 3. The first thing that is very obvious across the three behaviors is that *H. elevatus* females seem to receive more attention and so might be more attractive to all males. In the other hand we can see that *H. p. sergestus* females are the ones receiving less attention, especially regarding alighting on wings and abdomen bending. Looking at the significant interactions, we can see that in general all the interactions are consistent across the three studied behaviors. Basically all the interactions between *H. p. butleri* : *H. p. butleri* and *H. p. sergestus* : *H. p. sergestus* are significant and the interactions between *H. p. butleri* : *H. p. sergestus* as well as the ones between *H. elevatus* and *H. elevatus* are also significant most of the times. This suggests that all three taxa show a preference for their own species because at the same time *H. p. sergestus* and *H. p. butleri* show a mutual interest. Across the three models we found that overall activity has a significant effect on individual behaviours. This makes sense: the more active the butterflies were, the more likely they were show courtship

behaviour. We also made a model to test which species was the most active throughout the experiments (see table 2.) and *H. p. butleri* was significantly more active than the other two taxa.

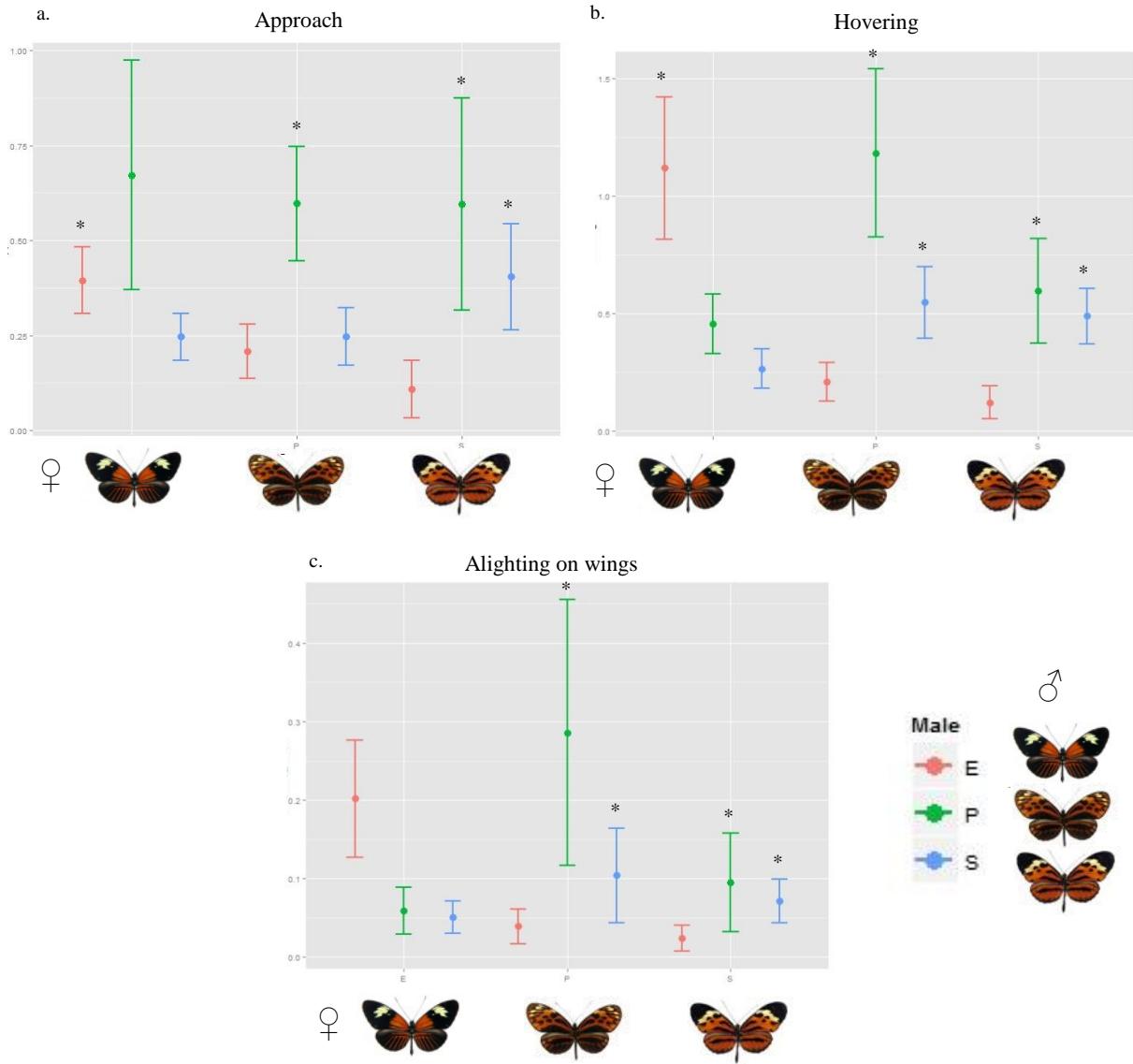


Figure 3. Mean numbers of counted behaviors \pm SE across trials. The y axis indicates the number of times each behavior was observed during a single trial. (a) Number of approaches (b) number of hoverings, (c) number of alighting on wings that were executed by males (legend) towards females (x-axis). The asterisk indicates the significant interactions given by the model.

Looking only at the hovering behavior, we see from both figures 2 and 3. that the females *H. elevatus* received the most hovers and males *H. p. butleri* performed most hovers. The three species prefer their own type with significant interactions but both *H. p. sergestus* and *H. p. butleri* have also significantly interact with each other. What we are seeing is a pattern where *H. elevatus* has strong male assortative behavior (as we can see in figure x. it shows no interest in the other two taxa) and in the other hand *H. p. butleri* and *H. p. sergestus* do not seem to discriminate between each other.

In the case of Approach, we find that *H. p. butleri* was the one that performed most approaches and *H. elevatus* the one that performed the least. Once more, the significant interactions show that the all three species interact significantly with their own form, but *H. p. butleri* males also significantly interact with *H. p. sergestus*. Even though *H. p. butleri* does not have a significant interaction with *H. elevatus*, it can be seen by the overlapping of the error bars that they also approached it similarly. *H. p. sergestus* approached *H. elevatus* and *H. p. butleri* similarly but significantly less than itself. In this case both *H. elevatus* and *H. p. sergestus* males do not show any interest in the other two species.

In the last observed behavior of which I have a model (Alighting on wings), it can be observed that again the tendency to interact significantly with their own type is maintained (even though the *H. elevatus* interaction with *H. elevatus* is not significant the error bars show a difference). In this case is clear that there is no assortative behavior between *H. p. butleri* and *H. p. sergestus* while they both show very little interest on *H. elevatus*.

Even if I was not able to use the abdomen bending data to create a model it is interesting to mention the results recorded taking into account that abdomen bending its considered a firmly

attempt to mate by the male, also a likelihood analysis with this data is presented below. In figure 2c it can be observed that *H. elevatus* maintains a strong preference for itself and that never tried to mate with *H. p. butleri*. What is remarkably interesting is the fact that *H. elevatus* did try to mate with S even though in the models it never shows a preference for it and in the behavioral stripchart (Fig. 2) we can see that it always shows less courtship behavior towards *H. p. sergestus* than towards *H. p. butleri*. In the other hand *H. p. butleri* tries to copulate with *H. p. butleri* and *H. p. sergestus* almost indiscriminately and *H. p. sergestus* tries to copulate mainly with *H. p. butleri* and never with itself. We also never got a pure *H. p. sergestus* mating in the experiment.

Table 2. Coefficients and confidence intervals of GLMM of the response variable of Activity. The significant values are shown in bold characters.

Fix effects	β (95% CI)
Intercept	1.09 (0.95,1.23)
Male P	0.11 (0.02,0.2)
Male S	0.04 (-0.06,0.13)
Rainy	-0.28 (-0.56,-0.001)
Sunny	0.02 (-0.10,0.14)
Random effects	σ^2 (95% CI)
Time	0.1665 (0.10,0.23)
Date	0.2587 (0.18, 0.36)

3.2 Crosses and egg hatch rate

We managed to obtain 20 broods and 1390 eggs over the span of three and a half months (table 3). Some crosses were more difficult to obtain and so we got very few broods or none at all. That was the case of back crosses with *H. p. butleri*.

The F1 hybrids (Fig. 4b) had a phenotype similar to the *H. elevatus* rayed pattern. Especially the hind wings resemble a lot the rays of *H. elevatus*. In the other hand the fore wing still look a lot like *H. elevatus* (Upper individual in Fig. 4a) with the yellow patch and the black tips but if

looked closely the spots typical of the tip of *H. p. butleri* can be seen. There was a little variance between the F1 phenotypes, some individuals were darker and resembled more to *H.elevatus* (Fig. 4b) and others were lighter and the spots in the tips of the hind wings were more evident (Individual facing down in Fig. 4a).

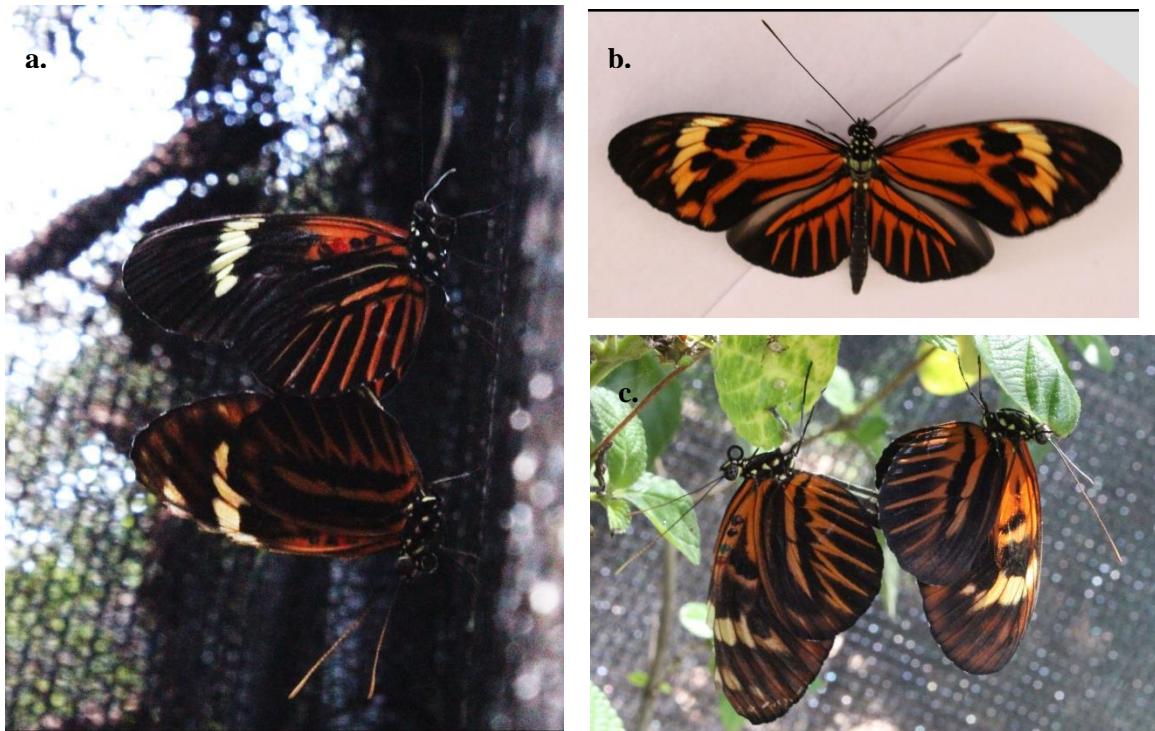


Figure 4. Hybrid phenotypes and matings. (a) backcross mating between a male *H. elevatus* and a female F1 hybrid. (b) Hybrid phenotype. (c) F1 x F1 mating.

Table 3. Hatch rate of pure, hybrid and back cross broods.

Cross type	Female	Male	Numb er of Broods	Numbe r of eggs	Mean hatch rate
Pure	E	E	4	339	0.8178136
	P	P	5	563	0.7062077
Hybrid F1	P	E	4	260	0.7361873
	E	P	1	37	0.7297297
Hybrid F2	PE	PE	4	108	0.5402947
	PE	E	1	22	0.8636364
Backcr oss	E	PE	1	61	0.9016393

In table 4 we have the coefficients and confidence intervals for the binomial GLMM of hatch rate, the model shows no significant difference between females or males (E, P and PE) but it shows that the interaction of PE females and PE males lowers the hatch rate significantly. This is also evident in the box plot (fig. 4).

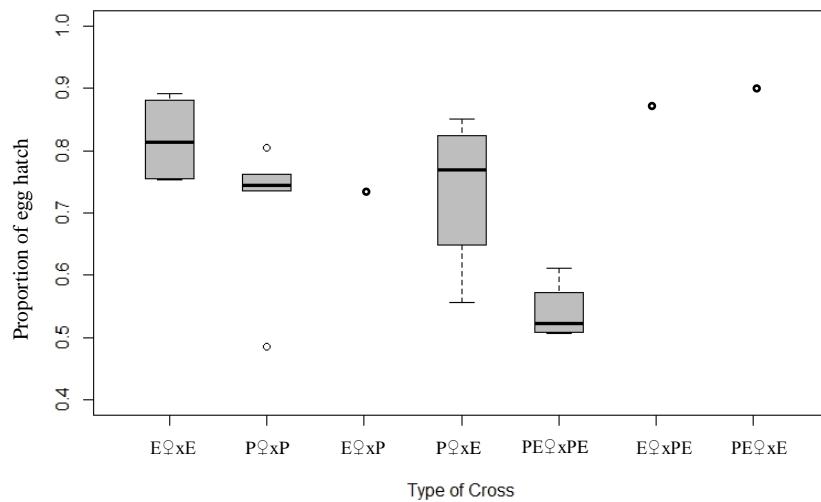


Figure 4. Box plot of the brood mean hatch rates per cross.

Table 4. Coefficients and confidence intervals for GLMM of Hatch rate. The significant values are shown in bold characters.

Fix effects	β (95% CI)
Intercept	1.85 (1.33,2.38)
Female P	-0.54 (-1.31,0.23)
Female PE	0.37 (-1.43,2.36)
Male P	-0.15 (-1.52,1.28)
Male PE	1.08 (-0.33,2.62)
Female P : Male P	0.04 (-1.56,1.58)
Female PE : Male PE	-3 (-5.49,-0.70)
Random effects	σ^2 (95% CI)
Female ID	0.32 (0,0.59)
Over	1.08 (0.84,1.36)

Additional observations that were recorded during the behavioral trials and the egg counted are listed below in table 5 along with a box plot of the mean eggs per day layed by females (fig. 5). Regarding the mating time, it is important to mention that even though it is clear that most of the matings occurred around noon and the earliest mating that was observed in the experiments was 9:10, in our stock cages we observed matings happening as early as 8:00 am. A Wilcoxon rank sum test showed that there was a significant difference between the mean eggs per day of *H. elevatus* and *H. p. butleri* ($W=9$, $p=0.0359$). In the other hand the F1 hybrids (PE) seem to have an intermediate phenotype. It is important to mention that we noticed that all the matings that we had occurred on days on which the maximum temperature was at least 33.2 °C.

Table 5. Additional recorded data regarding the matings
The data in this table was taken from both the experimental behavior crosses and the additional crosses for the egg counting.

	Species	Min	Mean	Max
Days until laying	E	2	3.4	5
	P	1	3.778	6
	PE	1	1.75	3
Mean eggs per day	E	1.4	1.79	2.96
	P	1.35	3.46	5
	PE	1.28	2.78	4.9
Mating time	E	9:10	11:49	15:15
	P	10:00	12:25	14:30
Mating duration	E	2:05	2:30	3:12
	P	1:08	1:56	2:25

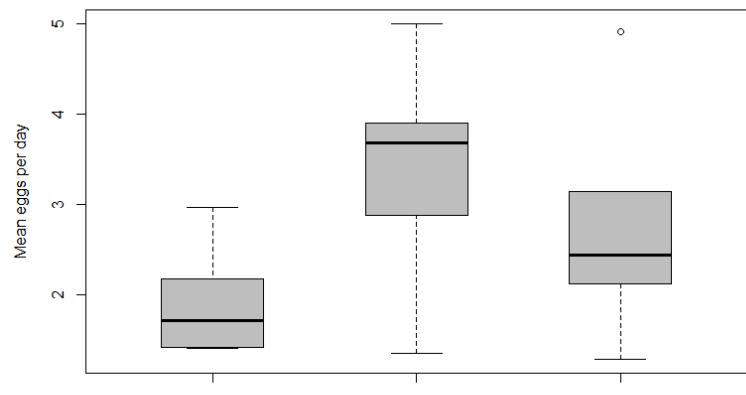


Figure 5. Boxplot of the brood means of the laid eggs per day.

3.3 Probability of mating (likelihood analysis)

In table 6 we have the maximum likelihood values of the probabilities of the different combinations of mating from our observations mentioned at the beginning of the mate choice behavior results. No *H. p. sergestus* females mated in any experiment, so we have no findings for relative probabilities of mating for this type of female. It is very unlikely that an *H. elevatus* male would mate with a female *H. p. butleri* and in the other hand is equally likely that a *H. p. butleri* or a *H. p. sergestus* male would mate with a female *H. p. butleri*. In the case of *H. elevatus* females is highly unlikely that a nonspecific mating happens. This patterns can be better visualized in the likelihood surfaces in figure 6.

Table 6. Maximum likelihood values and support limits for mating probabilities. The table shows the estimated probabilities ($P1_{ij}$, $P2_{ij}$, $P3_{ij}$) of a female i mating with a male j .

Parameter	Value	Support limits	Maximum Likelihood
$P1_{PxE}$	0.0	(0.0-0.17)	
$P2_{PxP}$	0.63	(0.34-0.86)	-7.32
$P3_{PxS}$	0.36	(0.13-0.65)	
$P1_{ExE}$	0.98	(0.98-0.96)	
$P2_{ExP}$	0.0	(0.0-0.03)	-0.10
$P3_{ExS}$	0.0	(0.0-0.03)	

In table 7, like in the previous case, we have the values of the probabilities of the different combinations of mating attempts calculated with a likelihood analysis from our observations of matings and abdomen bending. As we can see in figure 8. the probabilities and confidence intervals of *H. p. butleri* and *H. elevatus* females remain the same which could suggest that the mating probability in this case is strongly influenced by male preference. In the case of *H. p. sergestus* it is clear that it has no preference for itself under the experimental conditions.

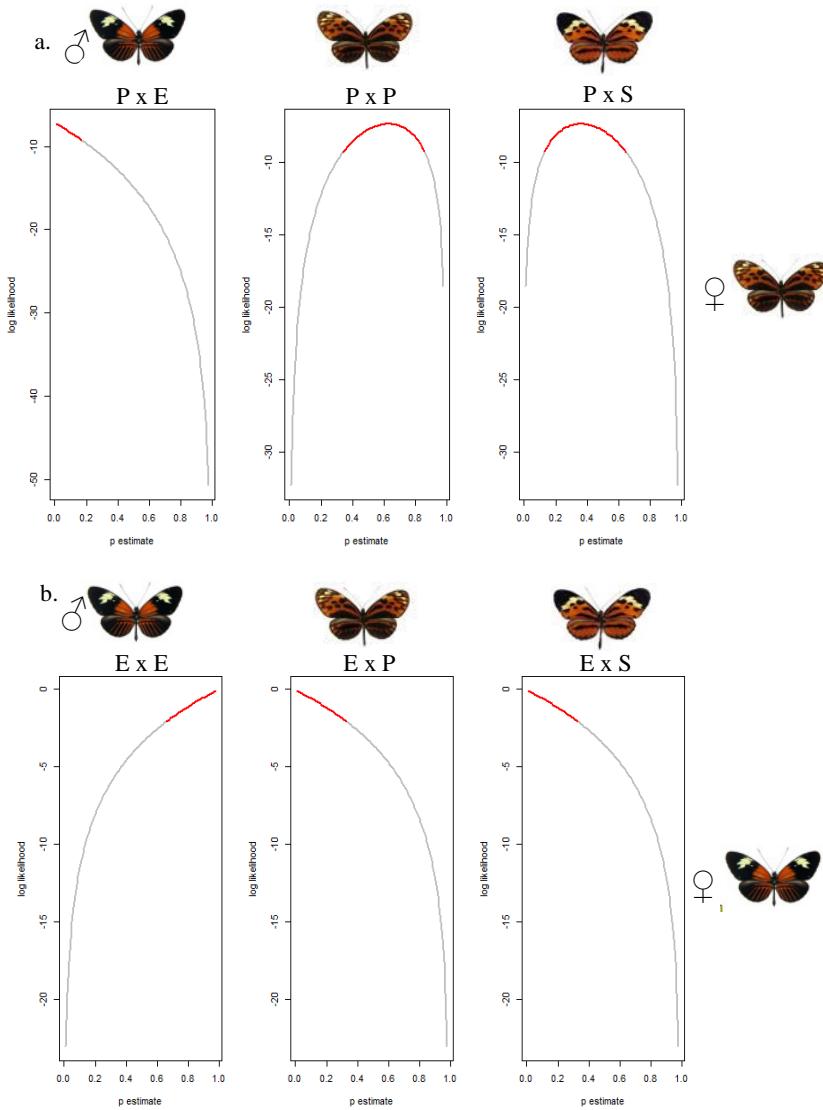


Figure 6. Likelihood surfaces for the probability of mating with (a) a female *H. p. butleri* and (b) a female *H. elevatus*. When the support intervals overlap, as in (a) between *H. p. butleri* and *H. p. sergestus*, there is no significant difference between the estimated probabilities.

Table 7. Maximum likelihood probabilities and support limits for male mating attempts. The table shows the probabilities ($P1_{ij}$, $P2_{ij}$, $P3_{ij}$) of a male j attempting to mate with a female i .

Parameter	Value	Support limits	Maximum Likelihood
$P1_{PxE}$	0.0	(0.0-0.11)	
$P2_{PxP}$	0.6	(0.38-0.8)	-12.21
$P3_{PxS}$	0.39	(0.19-0.61)	
$P1_{ExE}$	0.84	(0.57-0.96)	
$P2_{ExP}$	0.08	(0.01-0.32)	-6.79
$P3_{ExS}$	0.08	(0.01-0.32)	
$P1_{SxE}$	0.42	(0.13-0.65)	
$P2_{SxP}$	0.57	(0.22-0.75)	-4.85
$P3_{SxS}$	0.0	(0.0-0.22)	

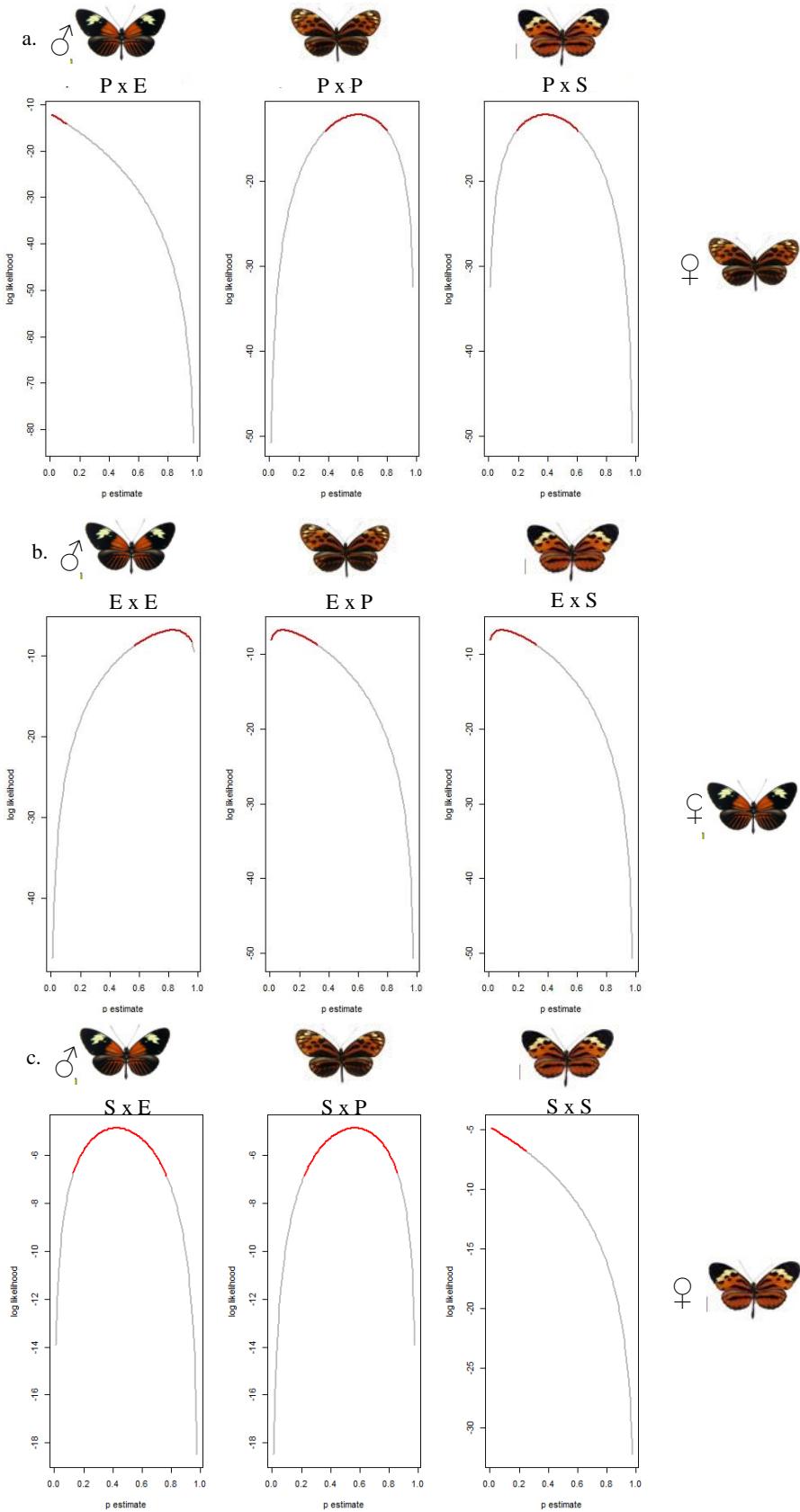


Figure 8. Likelihood surfaces for the probability of a male mating attempt with (a) female *H. p. butleri* and (b) female *H. elevatus*, and (c) female *H. p. sergestus*. When support limits overlap, as in (a) between *H. p. butleri* and *H. p. sergestus*, there is no significant difference between the estimated probabilities.

4. DISCUSSION

This is the first study about the courtship behavior, fertility and hybrid sterility on these two species of *Heliconius*. *H. elevatus* and *H. pardalinus* show very strong assortative mating and no apparent hybrid inviability. This is consistent with the hypothesis of a hybrid origin for *H. elevatus*.

4.1 Mate choice behavior

As it can be seen in the results, I was not able to identify all the behaviors described for *H. erato Phyllis* in (Klein and Araùjo, 2010). This could be due to the different experimental design and the different available tools to observe the behavior. One of the main differences with their methodology is the number of butterflies we had interacting, while they had one female and one male we had 15 males and one female which can alter the normal behavior. It is also worth mentioning that Klein and Araùjo describe all the behaviors using video footage of continuous observation for at least an hour long. For this reason it is not sure if the missing behavior is due to actual differences between our species and *H. erato Phyllis* either because they just behave differently or because of the differences in the experiment conditions. Additionally in the case of the females, some of the behaviors were age related and they used both young and old females while we used young females.

Heliconius pardalinus butleri males turned out to be the most active in the experiments and this coincides with the fact that *H. p. butleri* was the one that performed more hovers and approaches. We are not sure if this was because they adapted better to the cages than the other two, but the fact that there were no significant differences between *H. elevatus* and *H. p. sergestus* makes it unlikely. In the case of the females, I didn't record the activity, but as a personal observation it

was evident that *H. p. sergestus* were less active than females *H. elevatus* and *H. p. butleri*, in fact they rarely move even to get food. This it is very important because in our results female *H. p. sergestus* were the less attractive and we had no mating events despite the fact that males showed a similar behavior than the other two species and that we got hybrid matings between male *H. p. sergestus* and females *H. p. butleri*. Their lack of activity could have been the reason why males didn't show much interest and no matings were achieved, this case of female inactivity leading to no mating success was seen before in hybrid *Anartia* females (Davies *et al.* 1997).

Even if there were no significant interactions between *H. elevatus* and the other species, *H. elevatus* females received more approaches and more hovers than any other female. This could be because male elevatus displayed sexual courtship almost exclusively towards female elevatus whereas pardalinus and sergestus were divided but also because the color red proper of the elevatus rayed pattern attracts all *Heliconius* in general () see appendix.

The main conclusion of the behavioral results is that *H. elevatus* seems to have strong assortative mating behavior (across all courtship behaviors) while *H. p. butleri* and *H. p. sergestus* do not seem to have assortative mating preference between them but they do with *H. elevatus*. This is telling us that the prezygotic barriers of isolation are very strong within *H. elevatus* and *H. pardalinus* but basically nonexistent between the two subspecies of pardalinus. This could be due to the importance of color pattern as it has been shown previously in *Heliconius* (Jiggins *et al.* 2001, 2004) although pheromones can't be ruled out in this case.

4.2 Crosses and egg hatch rate

The results of our hatching rate model show a significant low hatching rate (proxy of fertility) in the F1 x F1 hybrid cross compared to the pure crosses but normal hatching rates in the back crosses. This could suggest that the F2 broods would have lower fitness due to some hybrid infertility related maybe to Haldane's rule (the heterogametic sex shows lower fertility). Nevertheless, if this would be the case the back cross with a female F1 should have also a lower hatching rate. It is important to mention that even though the back crosses have very high hatching rate, the sample size is very low (one cross in each direction and only 22 eggs in the F1 female x *H. elevatus* male) and one backcrossed hybrid female stopped laying eggs after laying 2 eggs. In spite of these facts, the most probable explanation of this result is that there is an inbreeding effect on the hatching rate () since the parents are siblings. This would mean that there is no evidence for any kind of sterility or lower fitness in the hybrids which means that there are no post zygotic barriers at all between *H. elevatus* and *H. pardalinus*. Based on the evidence, it seems that Haldane's rule doesn't hold in this case and this would match with what predicted by (Kryvokhyzha 2014) based on admixture levels of the Z chromosome.

An interesting result was the significant difference in eggs laid per day between *H. p. butleri* and *H. elevatus* and even more interesting is that the F1 hybrids seem to have an intermediate phenotype for this trait. This could perhaps suggest codominance in the genes regulating this trait.

4.3 Probability of mating (likelihood analysis)

The results of the likelihood analysis of mating, are consistent with the results of the courtship behavior. Therefore, *H. elevatus* has strong assortative mating while *H. p. sergestus* and *H. p.*

pardalinus have no assortative mating between them. When comparing with the probabilities calculated with the abdomen bending data, there are little changes on the likelihood surfaces for *H. elevatus* and *H. p. butleri* females. For this data there are likelihood surface graphs for *H. p. sergestus* females but the results are unexpected and do not match the behavioral result. No male *H. p. sergestus* tried to mate with a female *H. p. sergestus* whereas *H. p. butleri* and *H. elevatus* tried more than once. For Abdomen bending we have very few observations

As mentioned in the results, some crosses were extremely hard to obtain and so that gives us further prove that some combinations have very low likelihood of occur. For instance from our data it seems very unlikely that a mating between *H. p. butleri* and *H. elevatus* could occur in nature and in fact we had to hand pair them to get the hybrid cross. In the case of *H. p. butleri* and *H. p. sergestus*, it was easier to obtain the crosses in both directions without using hand pairing. In the case of the hybrids, they have a phenotype more similar to *elevatus* and two back crosses were easily obtained by introducing hybrid females in the *elevatus* male cage. This would suggest that even if it is very unlikely that a hybrid mating between *H. p. butleri* and *H. elevatus* occurs in nature, if it happens, the progeny would be able to backcross easily with *H. elevatus*. This would generate high admixture among the two species which would explain the topography of the phylogeny (Figure). In the case of *H. p. butleri*, backcrosses seem more difficult to obtain and thus I had not a single one.

4.4 Hybrid origin of *Heliconius elevatus*?

H. elevatus appears to be a very nice example of HHS as it fulfills the necessary conditions. My results confirm that there are strong prezygotic barriers and no postzygotic barriers between *H. elevatus* and *H. pardalinus* which suggests that prezygotic barriers caused the reproductive

isolation between them. What appears to be the main cause of the prezygotic isolation is the color pattern difference found to be also the trait that triggered speciation in *Heliconius Heurippa* (Jiggins *et al.* 2001; Merril *et al.* 2011). The trait of color pattern can be considered a “magic trait” that is involved in ecological speciation and at the same time in mate choice (sexual selection). Thus it can cause a rapid speciation process. Additionally *H. elevatus* has had an introgression event precisely of color pattern from *H. melpomene* (Heliconius consortium 2012) that gave it its actual rayed phenotype. Finally *H. elevatus* is sympatric with both *H. pardalinus* and *H. melpomene*. The only thing missing, would be to map the Qtl for color pattern and see if they overlap with the islands of speciation identified by (Kryvokhyzha 2014).

5. CONCLUSION

H. elevatus and *H. pardalinus* have very strong prezygotic barriers and no post zugotic barriers. They have similar behaviors and differ in levels of activity and some particular traits such as laid eggs per day. They share a very broad sympatric distribution and it is likely that *H. elevatus* has a hybrid origin with *H. melpomene* and *H. pardalinus* as “parent” species. Further studies on the color pattern preferences of the hybrids would help on the understanding of the mechanism of color pattern inheritance which in this case is a key to understand the hybrid speciation process.

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Appendix I.

Table 1. Coefficients and confidence intervals of generalized linear mixed effect models of the behavioral units. The significant values are shown in bold characters.

	Hovering	Approach	Alighting on wings
Fix effects	β (95% CI)	β (95% CI)	β (95% CI)
Intercept	-4.44 (-5.89, -3.12)	-2.9 (-3.84,-2.01)	-4.23 (-5.9,-2.85)
Female P	-1.36 (-2.78, -0.03)	-0.55 (-1.47, 0.34)	-1.57 (-3.32,-0.03)
Female S	-2.25 (-4.08, -0.72)	-2.07 (-3.23, -0.96)	-2.32 (-4.42, -0.59)
Male P	-0.82 (-1.94, 0.28)	0.4 (0.04, 0.78)	-1.39 (-2.32,-0.59)
Male S	-1.21 (-2.41, -0.05)	-0.46 (-0.94,0.00)	-1.35 (-2.36, -0.52)
Raining	-0.01 (-1.96, 1.71)	0.06 (-1.31, 1.35)	-2.72 (-4.84, -1.07)
Sunny	0.45 (-0.22, 1.13)	0.5 (-0.15, 1.17)	0.17 (-0.69, 1.15)
Activity	0.22 (0.01, 0.44)	0.22 (0.1, 0.35)	0.31 (0.1,0.53)
FemaleP : MaleP	2.76 (0.95,4.62)	0.69 (0.02, 1.39)	3.24 (1.87,4.9)
FemaleS : MaleP	2.30 (0.31, 4.47)	1.3 (0.53, 2.15)	2.73 (1.1,4.78)
FemaleP : MaleS	2.33 (0.45,4.24)	0.65 (-0.16, 1.48)	2.44 (0.92,4.2)
FemaleS : MaleS	3.07 (1.07,5.27)	1.96 (1.11, 2.89)	2.53 (0.8,4.64)
Random effects	σ^2 (95% CI)	σ^2 (95% CI)	σ^2 (95% CI)
Date	0.00 (0.00,0.69)	0.82 (0.41,1.32)	1.48 (0.93,2.38)
Time	0.41 (0.00, 1.06)	1.49 (1.13,1.97)	0.95 (0.44,1.66)
Over	3.35 (2.05, 4.45)	NA	NA
Experiment	NA	0.59 (0.29,1.01)	0.83 (0.34,1.56)

Appendix II.



Figure 1. Intense red colour displayed by flying elevatus.

Erklärung zur Masterarbeit

Hiermit versichere ich, dass die vorliegende Arbeit von mir selbstständig verfasst wurde und dass keine anderen als die angegebenen Quellen und Hilfsmittel benutzt wurden. Diese Erklärung erstreckt sich auch auf in der Arbeit enthaltene Graphiken, Zeichnungen, Kartenskizzen und bildliche Darstellungen.

Master's thesis statement of originality

I hereby confirm that I have written the accompanying thesis by myself, without contributions from any sources other than those cited in the text and acknowledgements. This applies also to all graphics, drawings, maps and images included in the thesis.

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Name und Vorname:

Julia Carolina Segami Marzal

Titel der Arbeit:

Mate choice and hybrid viability
of two species of *Heliconius*

Arbeitsgruppe, in der die Arbeit angefertigt wurde:

Mallet lab, OEB, Harvard University

Betreuer/in der Arbeit:

James Mallet

Unterschrift Betreuer/in:



Weitere/r Prüfende/r der Arbeit:

September 2014

Datum des Beginns der Arbeit:

February 2015

Datum der Abgabe der Arbeit:

Unterschrift des Vorsitzenden des Prüfungsausschusses EES
oder eines Bevollmächtigten

Bei Unterbrechungen:

Die Masterarbeit wurde angefertigt

Vom..... bis

Vom..... bis

Unterschrift Betreuer/in: