The contribution of female mate choice and pupal mating to the reproductive isolation between the butterflies *Heliconius erato* and *Heliconius himera*

By:

ELIZABETH A. EVANS

In partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

IN

BIOLOGY

DEPARTMENT OF BIOLOGY

FACULTY OF NATURAL SCIENCES

UNIVERSITY OF PUERTO RICO RIO

PIEDRAS CAMPUS

DEFENDED MARCH 17, 2022

SAN JUAN, PR

This thesis has been accepted by the faculty of the

DEPARTMENT OF BIOLOGY
FACULTY OF NATURAL SCIENCES
UNIVERSITY OF PUERTO RICO
RIO PIEDRAS CAMPUS

In partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE
IN
BIOLOGY

Thesis Committee:

_____
Riccardo Papa, Ph.D., Advisor

_____
Tugrul Giray, Ph.D.

_____
Miguel Acevedo, Ph.D.

_____
W. Owen McMillan, Ph.D.

March 17, 2022

Table of Contents

List of Figures

List of Tables

Abstract

Alternative mating tactics, mutual mate choice, and multiple mating cues can contribute to reproductive isolation but are rarely all addressed in case studies of speciation. Here, we dissected the behavioral components of assortative mating using the parapatric butterflies *Heliconius erato cyrbia* and *Heliconius himera*. We tested male and female preference, the role of geography, the use of color or pheromones in female choice, and male choice in pupal mating, and an alternative mating tactic. We found evidence of female preference for conspecific males in both species, while only *H. erato* males showed evidence of courtship preference. Female *H. himera* rejected males with blocked androconial pheromones over controls but did not show bias against males with altered coloration. In the context of pupal mating, an alternative mating strategy, we observed only *H. erato* males were less likely to make interspecies mating mistakes. This work underscores the importance of collecting behavioral data to the study of speciation and highlights how multiple traits can contribute to reproductive isolation even between closely related species.

Keywords

Assortative mating, speciation, multimodal mating cues, mutual mate choice, alternative mating tactics

Introduction

Behavior and Speciation

Diversity in morphology and coloration often what we use to distinguish between animal species, but also a great deal of what allows similar species to evolve independently and coexist in nature are differences in behavior. Closely related species can differ in almost every aspect of their behavior, including daily activity patterns (Bertossa et al., 2013; Davison, McMillan, Griffin, Jiggins, & James L. B. Mallet, 1999; Groot, 2014), food and habitat preferences (Daniel

I. Bolnick et al., 2009; Estrada & Jiggins, 2002; MacCallum et al., 1998; Merrill et al., 2013; Turissini et al., 2017), breeding seasons (Boumans et al., 2017; Hillis, 1981; Marshall & Cooley, 2000), migration patterns (Turbek et al., 2018), and mating preferences(Ficarrotta et al., 2021; Rossi et al., 2020; Uy et al., 2018; Yukilevich & Peterson, 2019). Under the biological species concept, to maintain species boundaries, species are maintained by geographically separation or genetic divergence to the extent that hybrids are either inviable or infertile(Jerry Coyne & H. Allen Orr, 2004). However, literature reports that many animal species remain distinct genetically and morphologically despite producing fertile hybrids and inhabiting the same place, and in these cases, behavior may be particularly important in reproductive isolation. Preferences for different habitats and shifts in breeding seasons can contribute to speciation by preventing males and females from two different species from interacting in a mating context even though they inhabit the same general area(Jerry Coyne & H. Allen Orr, 2004; Van Belleghem et al., 2016). Hybrids between some species exhibit maladaptive behavior of critical functions such as migration(Pärssinen et al., 2020), food location, or habitat preference(Pärssinen et al., 2020; Turissini et al., 2017)– all potential forms of post-zygotic isolation . Finally, one of the most common behavioral barriers is divergent mating preferences, which cause individuals of newly developing species to mate assortatively(Jerry Coyne & H. Allen Orr, 2004; Kirkpatrick & Ravigné, 2002).

As many species are kept apart by a combination of barriers rather than just assortative mating, assortative mating is also the sum of multiple behavioral traits and divergent mating cues. At a minimum, a mating interaction between two species involves two species and two sexes. In many cases, one species can exhibit stronger mate preference than the other (Beysard et al., 2015a; Hochkirch & Lemke, 2011; Mérot et al., 2017a; Merrill et al., 2011a; Polačik &

Reichard, 2011; Yukilevich, 2012a). In theory, behavioral isolation can be caused by both male

and female choice or either alone. Among *Drosophila* species, female and male preferences have

been demonstrated to evolve separately, with female choice predominating in sympatric species

but male and female choice evolving at similar rates in allopatry (Yukilevich & Peterson, 2019).

Within-species mating preferences can sometimes involve combinations of types of mating

cues(Girard et al., 2015) such as visual, auditory, chemical, or behavioral. However, there are

few studies reporting on these multiple aspects between species. In some cases, each sex may

choose based on different cues. For example, in many butterflies, females chose mates using

male androchonial pheromones(Costanzo & Monteiro, 2007; Darragh, Vanjari, Mann, Gonzalez-

Rojas, et al., 2017b; González-Rojas et al., 2020) or male-specific coloration or UV

signals(Ficarrotta et al., 2021) that are not present in females. Furthermore, the behavioral

repertoire of many animals also includes alternative male mating tactics, which typically allow

males to sidestep female choice. Wildly different rates of hybridization between species have

been observed in calling vs. satellite behavior in spring peepers (Stewart et al., 2017), cuckolding

vs. parental tactic in sunfish (Garner & Neff, 2013), and pupal vs. adult mating in *Heliconius*

(Munoz et al., 2010). Despite these individual cases, the multiple components of mating behavior

are most often not dissected in the study of pre-mating isolation. In part, this is because

behaviors can be difficult to quantify and are time-consuming to measure in many organisms

Study System Background

     One example of a group that has been extensively studied in the context of speciation, but

studies of mating behavior have heavily focused on one aspect of assortative mating are the

Heliconius butterflies. The *Heliconius* genus is composed of over four dozen species and

hundreds of morphologically divergent forms at various levels of reproductive isolation(Kozak et

al., 2015). In particular, the comparison of reproductive barriers at multiple species pairs in the Melpomene/Cydno/Silvaniform (MCS) clade of *Heliconius* have shown the relative importance of premating isolation relative to postzygotic isolation early in the speciation process (Mérot et al., 2017a; Merrill et al., 2011a). Male mate preference for species-specific color pattern in this group serves as a "textbook" example of magic trait speciation, which occurs when a trait diverges between species for ecological reasons (in this case warning color pattern) but also causes reproductive isolation (color pattern is also used a mating cue). However, several more recent studies are beginning to paint a more complicated picture of how reproductive isolation evolved in *Heliconius*. Firstly, while male mating preference based on coloration is clearly makes an important contribution to speciation in this clade (Jiggins et al., 2001a; M. R. Kronforst et al., 2006; Merrill et al., 2011a), female choice based on pheromones has been demonstrated both within-species (Darragh, Vanjari, Mann, Gonzalez-Rojas, et al., 2017b; González-Rojas et al., 2020; Southcott & Kronforst, 2018b) and between species (Mérot et al., 2015a) in the MCS clade (Darragh, Vanjari, Mann, Gonzalez-Rojas, et al., 2017b; González-Rojas et al., 2020; Southcott & Kronforst, 2018b). Secondly, while male mate preferences based on color may evolve as a by-product of natural selection for color pattern, several studies found evidence for the role of reinforcement (natural selection against hybrids) in the evolution of mate preference(Jiggins et al., 2001a; M. Kronforst et al., 2007; Rosser et al., 2019a).

In contrast to the clade including *H. melpomene* and *H. cydno* and close relatives, relatively little research on mating behavior has been done on sister species in the H. *erato*, *H. sapho*, *H. sara* (ESS) clade (frequently referred to as the 'pupal mating' clade). The pupal mating clade last shared a common ancestor with members of the MCS clade (part of the 'adult mating' clade) about 12 million years ago and contains nearly half the described *Heliconius*

species(Kozak et al., 2015). Species from these two lineages often show near-perfect wing pattern mimicry when their ranges overlap, however, they have diverged in mating behavior, offering an excellent natural replicate to study the process of speciation. Males of species in this group engage to various levels in an alternate mating tactic where males find females as larvae and pupae and mate the female on the pupae, sometimes fighting with other males(Deinert et al., 1994; Mendoza-Cuenca & Macías-Ordóñez, 2010; Thurman et al., 2018). In the most extreme form, males commit to mating before observing the female color pattern and females have no possibility of directly choosing mates(Deinert et al., 1994). The single study of pupal mating between species showed that male preference almost completely disappeared when males were given access to pupal females(Munoz et al., 2010).

Here, we examine the mating behavior of two incipient species in the ESS clade-*Heliconius erato* and *Heliconius himera. H. himera* replaces the more widespread *H. erato* across southern Ecuador and northern Peru in the semi-arid habitats of the Andean valleys. In southern Ecuador, *H. himera* and *H. erato cyrbia* overlap in a narrow hybrid zone with a low frequency of hybrids, which approximately corresponds to the center of a transition between vegetation types (JIGGINS et al., 1996; Mallet et al., 1998b). *Heliconius erato* and *H. himera* show some evidence for adaptive changes to different environmental conditions but similar hostplant choice between species (Davison, McMillan, Griffin, Jiggins, & James L. B. Mallet, 1999; JIGGINS et al., 1996; Montgomery & Merrill, 2017). Recent, population genomic data and modelling suggest that these species likely arose initially in allopatry and show evidence of recent genetic introgression that is asymmetrical(Van Belleghem et al., 2020). This pattern could mean that there is strong natural selection against hybrids, raising the possibility that reinforcement could affect mating behavior in this group in addition to the divergence in warning

color. Previous research on the species pair demonstrated strong assortative mating preferences, but no hybrid inviability or sterility (McMillan et al., 1997b; Merrill et al., 2014b). However, other than documenting the pattern of hybridization in the wild and the presence of assortative mating, the relative of male choice, female choice, pheromones, and pupal mating behavior in assortative mating has not been studied in these species.

Study questions and hypotheses

The main aim of this thesis project was to identify novel behavioral components of assortative mating between *Heliconius erato cyrbia* and *Heliconius himera* beyond those already described for male preferences based on color pattern. An important consideration in this aim was that males in the clade including *Heliconius erato* and *Heliconius himera* can use alternative mating tactics (e.g., both adult mating and pupal mating). Below I describe aspects of adult and pupal mating studied here.

I. Adult Mating

1. **What is the contribution of male and female choice to assortative mating in the case of *Heliconius erato cyrbia* and *Heliconius himera*?**

We considered three alternative hypotheses to explain assortative mating between *H. erato* and *H. himera*: 1) Male choice alone drives assortative mating patterns, 2) Female choice alone drives assortative mating, and 3) Both female and male choice drive assortative mating. If male choice alone causes assortative mating, we expect to see that males court females of their own species more frequently than heterospecific females and importantly that male mating effort predicts mating outcome. Likewise, if female choice alone drives assortative mating, we expect females to reject courtship from heterospecific males more often than males of their own species, and that the

frequency of rejection behaviors would predict mating outcome. If both male and female choice are important, we would expect both males and females to exhibit behavior biased towards their own species, and models including male and female behaviors would better predict mating outcome than either sex's behavior alone.

2. *Did reinforcement contribute to the observed levels of male and female preferences?*

We evaluated the hypothesis that reinforcement has also contributed to reproductive isolation. Reinforcement is difficult to directly assess but is expected to create a pattern of stronger assortative mating in populations closer to a hybrid zone than between distant populations where interactions between species are not possible. Therefore, we would predict that if reinforcement has acted on male preference, *H. himera* males from near the hybrid zone with *H. erato* would show stronger bias towards courting conspecifics over heterospecific females than *H. himera* males from a distant population. Likewise, we would predict that if reinforcement has acted on female preference, *H. himera* females from near the hybrid zone with *H. erato* would show stronger rejection bias towards courting heterospecific over conspecific males than *H. himera* females from a distant population. Secondly, if reinforcement has contributed to male or female preference, we would expect to observe a corresponding decrease in mating frequency when a heterospecific pair includes a *H. himera* from a population close to the *H. erato cyrbia* hybrid zone compared to *H. himera* from an allopatric population.

3. *Do females base mating decisions on color pattern or chemical cues?*

We evaluated two separate, non-mutually exclusive hypotheses about female mate choice cues: 1) Females choose mates based on androchonial pheromones and 2) Females choose mates based on whether the males have a similar color pattern. If the hypothesis that females choose males based

on androconial pheromones, we predict that females will more often reject courtship of males with blocked androconial regions than males with unblocked wings. Consequently, we would predict that females should more often end up mating with control males than blocked males. Similarly, if females choose males based on coloration, we predict that females will more often reject courtship of males with abnormal color patterns than males with natural color patterns. We would further predict that females should more often end up mating with control males than males with altered color patterns. If the predictions of both hypotheses are correct, then we could conclude that females use both pheromones and color patterns as mating cues.

II. Pupal Mating

4. *Do males discriminate between larvae and pupae of their own species?*

The factors controlling individual variation in pupal mating behavior in *Heliconius* are poorly understood, although many hypotheses have been proposed including male size, the number of female larvae, individual genetic factors, and larval feeding(Deinert et al., 1994; Estrada & Gilbert, 2010; Mendoza-Cuenca & Macías-Ordóñez, 2010; Thurman et al., 2018). In the context of pupal mating behavior between species, it is unclear if males can distinguish larval and pupal species. Here we used multiple hypotheses testing to compare which of these hypotheses best explains male behavior. We predicted that if males could discriminate between female species in the larval and pupal stage, males would be more likely to visit female larvae and pupae of their own species. If males preferred immature females of their own species, we predicted that they would hover and sit for a longer duration on female pupae and larvae of their own species. If either prediction is true, the models for these responses including the interaction between male species and female species would better predict pupal mating behavior than any other models.

Is male-male competition more intense between males of the same species?

Once a male has found a female pupa, he often must compete with other males for access(Deinert et al., 1994). In general, male size is expected to determine the outcome of competition. However, it is unclear how the dynamics of male-male competition between species compare to within species interactions and no attention has been paid to this issue. We speculated that males might more aggressively defend pupae from males of their own species, or that one species might be generally more aggressive than the other. Because there is no literature on this subject in *Heliconius*, we used multiple hypothesis testing to determine if the amount of male-male competition was changed by whether the males were of the same species or not. We expected to observe that if males interacted with other males differently depending on whether they were the same or different species, then models including an interaction term between interacting male species would fit better the observed frequency of male-male interactions. Furthermore, we also predicted that if male-male competition was dependent on male species, we would observe a pattern in the outcome of male-male interactions such as whether a male is displaced by an approaching male.

Methods

Butterflies and husbandry

H. himera replaces the more widespread *H. erato* across southern Ecuador and northern Peru in the semi-arid habitats of the Andean valleys (JIGGINS et al., 1996). In southern Ecuador, *H. himera* and *H. erato cyrbia* overlap in a narrow hybrid zone, which approximately corresponds to the center of a transition between semi-arid and wet forest vegetation. For our mating experiments, we established two stocks of *H. himera*. First, butterflies were collected outside, but

close to, the *H. erato cyrbia* hybrid zone near Vilcabamba, Lojas province Ecuador. The second stock was established with individuals collected in Peru, far from the hybrid zone. The *H. erato cyrbia* stock was established from individuals collected in Balsas, Ecuador, near the hybrid zone. We were not able to establish an allopatric stock of *H. erato*. Adult butterflies were kept in cages inside a screened greenhouse with artificial sugar water feeders, potted *Psychotria* ('hot lips plant'), *Psiguria sp.*, and occasionally *Lantana camara* for pollen. Butterflies laid eggs and caterpillars were fed *Passiflora biflora,* which is a suitable host to both species although not native in either species' range.

All mating experiments were performed at the insectaries of the Smithsonian Tropical Research Institute in Gamboa, Panama between October 2014 and August 2016. All experiments were conducted in 4.5m x 3.8m x 2.5m cages within screened greenhouses where butterflies had access to artificial sugar water feeders, potted *Psychotria*, and *Psiguria* for pollen as in the stock cages. For adult mating experiments, we used virgin females in the first 3 hours after first flying from the pupa. All males used in experiments were a minimum of 4 days old to ensure sexual maturity and were without significant wing damage. Males used for the adult mating experiments were with only other virgin males of their own species. Males used in the pupal mating experiment were not controlled for exposure to females or mated males.

Mutual mate choice

To understand the relative importance of both male and female behavior in assortative mating, we made behavioral observations of both sexes during no-choice mating trials. First, we introduced a newly emerged virgin female of either *H. himera* or *H. erato cyrbia* to a cage holding six males of either *H. himera* or *H. erato cyrbia*. We used six males instead of a single male with the intent of increasing the likelihood of observing interspecific courtship. For 120 minutes after

introducing the female to the cage (or until mating), we recorded male approach, courtship, and abdomen bending. When we observed courtship, with or without abdomen bending, we recorded the seven female responses during courtship (**Table 1**). All male and female behaviors were scored as present or absent during a minute, following the semi-quantitative approach of previous *Heliconius* studies. If mating did not occur during the initial observation period of 120 minutes, the cage was monitored every daylight hour until either coupling or 48 hours had elapsed, which ever occurred first. At the end of each trial that ended in mating, the mated male and one random male from the remaining five males were replaced for subsequent trials. If the trial did not conclude in mating, two random males out of the six were replaced for the subsequent trial.

We estimated mating rates of no choice trials using a logistic regression model of mating outcome with an interaction term between male species and female species. To assess whether female rejection or male courtship behavior better predicted the mating outcome of the no-choice trials, we used a multiple hypothesis testing approach to compare several candidate logistic regression models of mating outcome. We compared three models using AICc weight with the following predictors, respectively, (1) female rejection, (2) male effort, and (3) both female rejection and male effort. The strength of female rejection was calculated by the number of courtship minutes a female opened her wings in response spent over the total number of courtship minutes by the male (0-never opened wings; 1-always opened wings). Males' effort was estimated as the number of minutes spent courting over the total number of trial minutes (0-never courted; 1-courted every minute of the trial). AICc weights were tabulated using the package AICcmodavg(Mazerolle, Marc J., 2020).

To assess whether females and males behaved preferentially towards potential conspecific mates, we modeled the proportion of minutes during which males engaged in behavior towards

females using a quasibinomial generalized linear model. Similarly, female behavior was modeled as the proportion of minutes during which males courted the female that she responded with a given behavior. A quasibinomial (rather than binomial model) was used to model behavior in both cases because our response variables were the result of multiple Bernoulli trials (in this case, each minute for male behavior or each courtship minute for females). This type of data is often overdispersed, which can result in an underestimation of the p-value and standard error. Therefore, a more conservative approach is to implement a quasibinomial model, which adds a dispersion parameter to the equation that has the effect of increasing the standard error, but not the point estimate of the coefficients. We tested for the significance of predictors in quasibinomial models using an F-test, as a true AIC or log-likelihood cannot be calculated for a quasibinomial model. For all generalized linear models, the confidence intervals for the estimated effect of male or female species in terms of the responses were calculated using the effect package in R(Fox & Hong, 2009).

Geographic patterns of mating behavior

Reinforcement, or the increase in reproductive isolation due to natural selection against hybrids, is sometimes observed in cases of secondary contact, as well as being relevant to models of speciation with gene flow. While reinforcement is difficult to demonstrate directly, an expected pattern of reinforcement is the observation of pre-mating isolation that is stronger in populations where hybridization is occurring than those further away from the contact zone. To test for a geographical pattern of mating preferences, we compared no-choice trials using *H. himera* from Ecuador (near the hybrid zone with *H. erato cyrbia*) and *H. himera* from Peru (far from the hybrid zone *H. erato cyrbia*). We were not able to establish a stock of allopatric *H. erato cyrbia* and were only able to evaluate geographic patterns of mating behavior in *H. himera*.

To test for differences in mating preferences between populations of *H. himera*, we analyzed the subset of the no-choice trial data that involved trials between species (*H. himera* female with *H. erato* males or *H. erato* female with *H. himera* males). We predicted that if reinforcement were acting on male mate preferences, *H. himera* males from Ecuador would be less likely to approach and court *H. erato* females relative to *H. himera* males from Peru. Using a subset of the data that only included trials between *H. himera* males and *H. erato* females, we modeled the proportion of minutes associated with male courting behavior using a quasibinomial model with the male population (Ecuador or Peru) as a predictor. We compared the model with the population as a predictor and the model without using an F-test. Similarly, we predicted that if reinforcement were acting on female preference in *H. himera*, female *H. himera* from Ecuador would reject the courtship of *H. erato* males more frequently relative to *H. himera* females from Peru. Using data from trials of *H. himera* females with *H. erato* males, we compared quasibinomial models of the proportion of courtship minutes that a female spent in a 'wings open' position with and without the female population (Ecuador or Peru) as a predictor using an F-test. Finally, we also examined whether *H. erato* females and males behaved differently towards potential *H. himera* mates from Ecuador or Peru using the same approach, although this would not necessarily be predicted if reinforcement has occurred.

Role of visual and pheromonal cues in female choice

Recent work on *Heliconius* butterflies demonstrates that both color patterns and chemical cues can be involved in mating decisions. However, the relative importance of color and chemical signals for females assessing potential mates remains unclear. To assess the role of male color pattern and androconial pheromones in female choice in *H. himera*, females were given the choice of a control male or a male who had been experimentally manipulated to eliminate male wing

color or pheromone release. Experimental set-up and behavioral scoring for choice trials were similar to the no-choice trials (see 'Mutual Mate Choice'), with the exception that the female response and male behavior was recorded separately for the courtship of treated and control males.

For the color-blocking experiments, we used black Sharpie® makers to color in all the red and yellow wing regions of males, producing a butterfly whose wings were entirely black on both sides. To control any effects related to handling or the scent of the maker, three control males were colored with black Sharpie® in the black wing regions (along the edges of color elements) so that the butterfly appeared unaltered. We took care not to avoid altering the androconial scales where pheromones are produced. To observe if the coloring treatment affected UV signals also known to be present in *Heliconius* yellow, we took photos of wings with the control and treatment with a lens that allowed only UV wavelengths to pass (**Figure 2**).

For the pheromone-blocking experiments, we masked the androconia in the upper ventral hindwing (where the fore and hindwing overlap) of the treatment males using Revelon® Liquid Quick Dry nail varnish. After application, we manually immobilized the butterflies for 3 minutes, holding the fore- and hindwings separate to prevent bonding of the wings. We did not find it necessary to anesthetize the butterflies for this procedure. To control for the scent of the nail varnish, we applied varnish to the dorsal side of the androconial region of control males. Nail varnish was applied to the butterfly a minimum of 24 hours before and maximum of three days before the experiment.

To determine whether the proportion of females mating with control or treated males was unequal, we used an exact binomial test. To test whether females had responded differently to the courtship of control males and altered males (pheromones or color pattern), we modeled 'female

response' (the proportion of courtship minutes with or without female response) using a generalized linear mixed model with binomial error distribution and logit link function using the lme4 package in R. In the full model, we included 'male treatment' as a fixed effect and 'individual female' as a random effect. To test for significance, we compared this model to a random effect only null model using a likelihood ratio test. Because male behavior can also affect the probability mating, we also tested for difference in courtship behavior between treatment and control males following the same procedure.

Pupal mating

Males of pupal mating species such as *H. erato* and *H. himera* engage in both adult and pupal-mating, although there is variation between individuals, populations, and species in which mating tactic is favored (Mendoza-Cuenca and Macías-Ordóñez, 2010b; Thurman et al., 2018). The factors which contribute to individual and between- species differences in pupal mating behavior, however, are not well understood. For this reason, we designed an experiment that included all potential inter- and intra-species interactions within the hybrid zone: (a) heterospecific male-male interactions, (b) conspecific male-male interactions, (c) heterospecific male-female interactions, and (d) conspecific male-female interactions.

In the experimental cage, we placed eight potted *Passiflora biflora* plants and randomly assigned late fourth or fifth instar female larvae to each of these plants. Larval sex was determined using external sex-specific abdominal pits, which have not been formally described in *Heliconius* larvae but were consistent in pattern and location with those observed in other lepidopteran families (Underwood, 1994). Next, three mature males of *H. erato cyrbia* and three males of *H. himera* were individually labeled in the forewing band and introduced into the experimental cage. Males were given one of three markings, which were chosen because they were easily

distinguished in video footage: I, III, or ---. Two separate experiments were carried out, one with *H. himera* from Ecuador and one with *H. himera* from Peru. We recorded age and triplicate forewing measurements for each male. Replacement larvae were added as females emerged or to replace larvae that died. While ratios of immatures of each species remained equal and there was never more than one larva per plant, the absolute number varied between two and four larvae due to the availability of female late instar larva. After each female emerged, the mated male and a randomly selected male of the other species were replaced with a new male of their own species.

To capture male behaviors towards larva and pupa, we recorded daily 30-minute video observations in randomized order, between 7:00hrs and 14:00hrs, of each female larva or pupa using a GoPro Silver Hero3+ (1080p, 30fps). On the morning of a female's emergence, cameras were set-up at sunrise and recordings were started when the first male took flight (we never observed males roosting on pupas) and continued until either mating occurred or the female flew from her pupal shell. Male-female and male-male interactions were later quantified using the video event recorder Solomon Coder (Péter, 2017). We scored (a) approach, (b) hovering, and (c) sitting behavior by males as well as male-male interactions which occurred around the larva or pupa. Individual males were identified in the video by the markings in their forewing band.

To anlayze the factors affecting male behavior, we compared 11 candidate models for two response variables: (1) whether males approached the larva and pupa (treated as a binary response: 'approach' or 'no approach') and, given an approach, (2) how much time they spent near a larva or pupa (treated as a continuous proportion, the fraction of the observation that was spent around larva or pupa, bounded by 0 and 1). Candidate models were based on previous descriptions of pupal mating, as well as to address the current objective of determining if pupal mating behavior differs between closely related species. We analyzed larva and pupa observations separately in

order to allow for the case in which predictors of male behaviors differ dramatically between life stages. In both model types, the ID of the male was used as an intercept-only random effect to account for the repeated observations of the same male. Male approaches to female larvae or pupae were modelled using a generalized linear mixed model with binomial error distribution with a clog log link function and the logarithmic function of the length of the observation as an offset to account for variation in observation time(Bolker, 2019). We initially modeled the proportion of time spent engaging in behavior towards a larva or pupa using beta mixed models, in the 'glmmTMB' package for R(Brooks et al., 2017). In the case of the beta mixed models, it was determined that the variation explained by the individual random effect approached 0 in all candidate models, so we then conducted beta regressions with only fixed effects using the beta using the 'betareg' package for R(Grün et al., 2012). Models were compared using the AICc values in the 'AICcmodavg' package for R(Mazerolle, Marc J., 2020).

In some pupal-mating *Heliconius* species, males frequently fight with other males for access to pupa. It is unknown whether the dynamics of male competition change when competing males are members of different species. We used our video observations to score male-male interactions in which one male (male 1) that was in the process of hovering or sitting on a female when a second male approaches (male 2). As the camera view was focused on the larva or pupa, interactions that occurred outside the focal area around the larva or pupa could not be scored. Male-male interactions were further scored based on the outcome of the interaction as either: (1) 'male 2' displaces 'male 1' and follows him away from the larvae or pupa, (2) 'male 2' does not displace 'male 1' and instead himself flies away, (3) 'male 2' joins 'male 1' in hovering over the larva or pupa, and (4) the 'male 2' displaces 'male 1' and takes his place sitting or hovering over the larva or pupa. Differences between the outcome of male-male interactions were evaluated

using Fisher's test for independence. Finally, we modeled the number of interactions a male initiated, regardless of outcome, with other males using a generalized linear mixed model with a Poisson distribution and log link function. Here, we used an offset for the amount of time the male who was approached (male 1) spent hovering over the pupa (since per our definition an interaction could only occur during this time) and the two individual males as 'random effects' to account for repeated observations of the same male. We used a multiple hypothesis testing approach compared models that included the interaction term between the approaching (male 2) and hovering (male 1) male's species with a constant-only null.

Results

Mutual mate choice

In the no-choice trials, mating occurred more frequently between conspecific males and females than between heterospecific males and females (**Table 2**). Unexpectedly, we observed a much lower rate of mating for within-species trials of *H. himera* compared to within-species trials of *H. erato.* Regarding between-species trials, the direction of the pairing (e.g. male *H. himera* with female *H. erato* versus male *H. erato* with female *H. himera*) did not change how likely they were to mate. Of the three models tested to explain mating outcome based on the behavior (e.g. female behavior only, male behavior only, and female and male behavior), the best model for mating outcome included both male and female behavior (ΔAICc of 8.37 between this model and the next best, AICc weight = 98.5%)(**Table 3**).

Females of both H. *himera* and *H. erato* responded differently to the courtship of conspecific versus heterospecific males (**Figure 4**). *H. erato* females performed 'wing opening' (F-test, $F_{49,48}=6.2248$, p=0.01609) and 'orientation change' (F-test, $F_{49,48}=9.9159$, p=0.002817) behaviors more often in response to the courtship of *H. himera* males than males of *H. erato*. Similarly, female *H. himera* performed 'wing opening' more often towards *H. erato* males than *H. himera* males (F-test, $F_{60,59}=4.6466$, p=0.0352) but did not differ in their orientation behavior (F-test, $F_{60,59}=0.0169$, p=0.897). None of the other female response behaviors differed in response to the courting male's species for *H. erato* (**Error! Reference source not found.**, **Table 4**) or *H. himera* (**Figure 6**, **Table 5**).

Male preference was asymmetric with only *H. erato* showing preference towards conspecific females (**Figure 7**). *H. erato* males courted *H. erato* females ($F_{53,52}=7.1348$, p=0.01007) more often than *H. himera* females, although they approached *H. himera* females at

a similar rate ($F_{53,52}=0.3024$, p=0.5847) ,(**Figure 8**,**Table 6**). Despite their apparent preference, male *H. erato* nevertheless courted *H. himera* females at least once in 25 of 33 trials with courtship progressing to abdomen bending in 15 of these trials (**Table 2**). *H. himera* males' approach ($F_{71,70}=0.0881$) and courtship ($F_{71,70}=0.2559$, p=0.6145) behavior was not biased by female species (**Figure 9**,**Table 7**). In fact, *H. himera* males performed 'abdomen bending' at least once towards *H. erato* females in 24 of 29 trials, whereas they did so in only 28 of 43 trials with *H. himera* females (**Table 2**).

Geographic patterns of mating behavior

Female *H. himera* and mated with male *H. erato* at a similar rate regardless of whether the female was from the Ecuadorian population (1 out of 17 trials resulted in mating) or Peruvian population (2 out of 16 trials resulted in mating). Similarly, *H. himera* males from Ecuador and Peru also mated with *H. erato* females at similar rates (2 out of 15 and 1 out of 15 trials resulted in mating, respectively). The low frequency of mating observed in same-species *H. himera* trials was roughly consistent between trials involving *H. himera* from Peru (8 out of 21 trials resulted in mating) and *H. himera* from Ecuador (6 out of 15 trials ending in mating).

Male *H. himera* from Ecuador and Peru did not differ towards *H. erato* females in their rate of approach ($F_{28,27}=0.0944$, p=0.7611) and courtship ($F_{28,27}=0.177$, p=0.6773) (**Figure 10**, **Table 8**). *H. himera* females from Ecuador and Peru also did not respond differently towards the courtship of *H. erato* males with wing opening ($F_{24,23}=0.2025$, p=0.657, **Figure 11**, **Table 9**). However, *H. himera* females from the Peru population showed decreased frequency of flight ($F_{24,23}=20.178$, p=0.0068) and abdomen lowering response to courtship ($F_{24,23}=7.952$, p=0.0097). We also noted an unexpected increase in wing opening response of *H. erato* females to the courtship of *H. himera* males from Peru compared with *H. himera* from Ecuador ($F_{28,27}=6.2518$,

p=0.01878) (**Figure 11**, **Table 10**). *H. erato* males did not behave differently towards *H. himera* females of Ecuador and Peru (**Figure 13**,**Table 11**)

Role of wing coloration and pheromones in female choice

Of the 20 color-blocking trials that ended in mating (a total of 38 trials were attempted), ten *H. himera* females mated the control and ten mated the all-black male. We found no difference in the female response towards control males or all-black males in terms of 'wing opening' response (2ΔlnL =1.5019, df =1, p=0.2204; **Figure 14**) and 'orientation change' (2ΔlnL =0.0028, df=1, p=0.9579; **Figure 14**) or any other response behavior (**Table 12**, **Figure 15**). The treatment males approached females more frequently than control males (2ΔlnL =8.7682, df=1, p=0.003065) but were not significantly more likely to court them (2ΔlnL =3.0928, df=1, p=0.07864, **Figure 16**, **Table 13**).

In contrast to the color-blocking experiment, of the 16 pheromone-blocking trials that resulted in mating (out of 34 total trials), 13 females mated the pheromone-intact male and only 3 mated the pheromone blocked males (exact binomial test, p=0.02127). Females responded with rejection behavior to courtship by pheromone-blocked males differently than pheromone-intact males by 'wing opening' (2ΔlnL =6.0908, df=1, p=0.01359; **Figure 14**, **Table 14**), 'orientation change' (2ΔlnL =4.3489, df=1, p=0.03703,**Figure 14**), 'flying' (2ΔlnL =4.4189, df=1, p=0.03554; **Figure 17**) and 'crawling' (2ΔlnL =5.5594, df=1, p=0.01838; **Figure 17**). 'Wing closing' occurred more frequently during courtship by control males (2ΔlnL =6.0908, df=1, p=0.01359; **Figure 17:** Female response to courtship of males with normal and blocked pheromones.). However, we found that courtship rate differed between male types (2ΔlnL=24.427, df=1, p=7.719e-07, **Figure 18**, **Table 15**), with control males courting more often that pheromone-blocked males.

Pupal mating

General observations of pupal mating

Of the 59 female larvae placed in the pupal mating experiments, 42 pupated and only 20 emerged as well-formed adults. Of the 20 healthy females that emerged, 9 were mated before flying away from the pupa, 8 were mated after flying away from the pupa, and 3 remained unmated after two days in the enclosure with the males (

Figure 21: Percent of time spent hovering and sitting on pupae by species

Top left: *H.erato* males in experiment 1 in which *H.himera* males and female larva were from Peru population. Top right: *H.himera* in experiment 1. Bottom left: *H.erato* males in experiment 2 in which *H.himera* males and female larva were from Ecuador population. Bottom right:

H.himera in experiment 2. Each point represents the percent of time that an individual male spent hovering or sitting (total time of behavior divided by total time of all observations), the line segments merely connect points corresponding to the same individual. Only males with at least 5 days of observation with larvae of both species shown. The Y-axes are not identical across plots.

Table 16). Of the 20 *H. erato* and 20 *H. himera* males that had at least one week of observations, all males were observed 'approaching' a larva or pupa at least once, with the exception of one *H. erato* male. Furthermore, of those 39 males that did approach larva or pupa, all but two males of each species (18 out of 20 *H. himera* males and 17 out of 19 *H. erato* males) were at some point observed also hovering over a larva or pupa after approaching. However, only half of *H. himera* (10 of 20 males) and a fifth of *H. erato* (4 of 20 males) were observed engaging in the more intense behavior of 'sitting' on a pupa. Two *H. himera* males were clear outliers, spending two or three times as much time engaging in pupal hovering behavior as other males (**Figure 20**).

We did not observe the more extreme form of pupal mating typical of *H. charithonia* and *H. hewitsonii* in which males spend the night on a pupa and penetrate the pupal casing well before emergence. We did observe two pupal-mating events in which an *H. himera* male was 'sitting' on the pupa for several hours prior to emergence and then successfully clasped on to the female during emergence. In a third case, an *H. himera* male was 'sitting' on the pupa for several hours but was disturbed minutes before the female's emergence by external events but returned to mate immediately after emergence. In the other six cases of pupal mating, males approached and mated with females that had already emerged but were still hanging from the pupa, unable to fly or reject the male.

Heliconius caterpillars go through a *wandering phase* in which they stop feeding and search for a pupation site. During our experiment, we observed an unanticipated difference in pupation site choice between the two species (**Error! Reference source not found.**). Specifically, we observed that *H. himera* larvae tended to pupate near the base of the plant (immediately above the soil and below the rim of the potted plant) whereas *H. erato* primarily pupated on the leaves or stems of the main body of the plant. Pupation site preference evaluated as 'base of plant', 'on plant', and 'off-plant' was significantly different between species (fisher exact test of independence, p-value=4.97x10-6, N=42).

We observed a high rate of interspecific mating during the pupal mating experiments, with 6 of 17 observed mating events taking place between males and females of different species. This number included: four incidents of pupal mating between *H. himera* males and *H. erato* females, one adult mating between a *H. himera* male and a *H. erato* female, and a single pupal mating between a *H. erato* male and a *H. himera* female. There was no clear difference in male behavior between the two experiments (with *H.himera* from Peru and *H.himera* from Ecuador) so we chose to combine the experiments for analysis but incorporated the male population in a candidate model (**Figure 20**,**Figure 21**). To better understand if males showed a preference for conspecific larva and/or pupa or other factors, we compared candidate models for 'hovering' and 'approach' behavior.

Male behavior towards female larvae
First, we compared models representing different hypotheses about what factors predict whether male finds and approaches a larva. Considering 'approach' behavior towards larva only, the best candidate model we tested included both larval feeding status and male size (ΔAICc = 3.58 between this model and the next best, AICc weight = 71%; **Table 17**). Specifically, the model

predicts that males visit larvae who are still feeding more often than they visit larvae who are in the final day before pupation (the wandering stage) (**Table 18**, **Figure 22**). The model also predicts that larger males are more likely to approach larvae than smaller males. The males used in the experiment varied in forewing measurements ranged between 3cm and 3.8cm (**Figure 23**). The model including larval feeding status only also received some support (AICc weight=12%).

Next, we compared beta regression models representing different hypotheses about what factors predicts how much time a male will spend around a larva once he found it. Of the candidate models, the best model for the relative amount of time males spent around larvae included both 'male species' and 'larva species' as predictors (ΔAICc = 1.57 AICc weight = 58%; **Table 19**). This model predicts that male *H. himera* will spend more time visiting larvae (independent of the larval species) than *H. erato* males (**Table 20**, **Figure 24**). The model predicts that both *H. erato* and *H. himera* males spent more time around *H. erato* larvae than *H. himera* larvae. The model which included an interaction term between 'male' and 'larval species also received some support (AICc weight = 26%).

Male behavior towards female pupae
The best candidate model for predicting whether males approached a pupa included an interaction term between male and pupal species, although the interaction term was not significant (ΔAIC =1.85 between this model and the next best, AIC weight = 50%; **Table 21**, **Table 22**). This model predicted that both *H. himera* and *H. erato* approach *H. erato* female pupa more frequently, but that the bias is slightly more pronounced for *H. himera* males (**Figure 25**). However, two other models also received support, including the model which included only pupal species (AIC weight = 20%) and the model which included both pupal and male species but no interaction term (AIC weight = 17%).

Finally, we compared beta regression models for the proportion of the total observation a male spent visiting a pupa if the male has already found and approached the pupae. None of the candidate models scored better than the constant-only null model (AICc weight=24%, ΔAICc = 1.06 between this model and the next best model), **Table 23**). We used a stepwise model selection algorithm (stepGAIC) as implemented in the 'gamlss' R package to see if any unexpected combination of predictors would produce a model better than the null model, however, this procedure also returned the null model.

Male-Male interactions

We observed a total of 166 male-male interaction events in which a male approached another male who was either 'hovering' or 'sitting' on a larva or pupa (**Table 24**). In 77 of these interactions, the second male (the approaching male) scared off the first male ('resident' male at the pupa or larva) and followed him away from the pupa or larva. In 63 of events, the second male did not cause the first male to fly away, instead, the second male departed, and the first male remained at the pupa or larva. In 18 events, the second male joined the first male and they both hovered/sat on the larva/pupa at the simultaneously. In the remaining 8 interactions, the second male scared off the first male and then lingered around the larva or pupa. The outcome of male-male interactions could be in part predicted by the species of the first and second male (Exact test of independence, p = 0.01417). When considering *H. erato-H. erato* interaction events (N = 54), *H. erato* males were twice as likely to flee (N = 36), then to stay in place (N = 18), when a second *H. erato* male approached. When considering only *H. himera-H. himera* interactions (N = 43), *H. himera* males were slightly more likely to stay in place (N = 25), than flee (N = 18), when a second *H. himera* male approached.

We also modelled the frequency that males approached other males independent of the outcome of the interaction. The best candidate model we tested to predict the frequency of male-male interactions occurred included the stage of the immature and the interaction between the second (approaching) and first (resident) male species (AICc weight=100%, ΔAICc =25.18 between this model and the next best) (**Table 25**). The model predicts that *H. erato* males are more likely to approach another *H. erato* male compared to a *H. himera* male, whereas *H. himera* were not predicted to target other males according to species (**Figure 26**). Surprisingly, the model also predicts that males were more likely to approach other males around larvae rather than pupae (Figure 19b).

Discussion

Female and male mate choice contribute to assortative mating

Female mate choice is widespread in butterflies but has only recently come into focus in *Heliconius*(Darragh, Vanjari, Mann, Gonzalez-R, et al., 2017; González-Rojas et al., 2019; Southcott & Kronforst, 2018a). Due to the nature of pupal mating, it has been thought that females of pupal mating species do not contribute to reproductive isolation because they are mated during or too soon after emergence to reject males. However, more recent work suggests adult (non-pupal) mating regularly occurs in wild populations of *H. erato* and to a lesser extent *H. charithonia* (Mendoza-Cuenca & Macías-Ordóñez, 2010; Thurman et al., 2018). Here, we showed that female *H. erato* and *H. himera* that are only a few hours old can and frequently do physically refuse mating attempts, primarily by opening their wings. We further confirmed that wing opening can be considered a rejection behavior because our models showed female wing opening in response to courtship is negatively correlated to successful mating. Furthermore, we demonstrated that females engaged in typical rejection behavior more often in response to courtships by heterospecific males than conspecific males.

Estimates of the cross-species mating probability between *H. erato* and *H. himera* in the wild have been estimated at about 5% (Mallet et al., 1998a; Merrill et al., 2014a). This differs from the results of this thesis and a previous studies that found that between-species mating mistakes occur about 10% of the time in adult mating no-choice trials(McMillan et al., 1997a).

Unlike female choice, male mating preferences for females with their own warning color patterns have a well-established role in the speciation process of *Heliconius* (Mérot et al., 2015b, 2017b; Merrill et al., 2011b, 2014a; Munoz et al., 2010). Here, we confirmed previous results that *H. erato cyrbia* males prefer conspecific females but also found an absence of male preference in *H. himer*a(Merrill et al., 2014a). Asymmetry in mating has been noted in many

sister species pairs in *Heliconius* as well as in diverse animal groups such as fruit flies, salamanders, and voles(Beysard et al., 2015b; Mérot et al., 2017b; Merrill et al., 2011b; Yukilevich, 2012b). The cause of this type of asymmetry is unknown, but several authors have argued that it is evidence of asymmetric reinforcement(Beysard et al., 2015b; Yukilevich, 2012b). Regardless of the cause, our results differ notably from the previous investigation of *H. erato cyrbia* and *H. himera* which concluded that male mate preference was found in both species based on male approach to mounted wings of dead females(Merrill et al., 2014a). It is unclear whether approaches to models predict how males behave with live females. Females have also been shown to approach paper model wings as much as males based on coloration(Finkbeiner et al., 2017), although female to male approach is not a part of normal mating sequence in *Heliconius*(Klein & de Araújo, 2010). In contrast, our experiments demonstrated that males of both species approached live conspecific and heterospecific females at similar rates, but *H. erato* males were more likely to advance from approach to courtship with females of their own species.

Is then male or female preference more important to reproductive isolation? Some have argued that male choice has a proportionally larger effect because it chronologically occurs first (100% of matings will not occur if males do not attempt to mate). In our model comparison analysis, the combination of male courtship frequency and female rejection frequency best predicted whether mating occurs across all species combinations. However, there are two pieces of evidence that female choice has a possibly dominant contribution to reproductive isolation specifically. Firstly, despite evidence of male preference in *H. erato* and no male preference in *H. himera*, the rate of mating in trials between males and a female of a different species did not depend on which species was male or female. Secondly, we observed that while *H. erato* males

appear to prefer females of their own species more than *H. himera* females, in nearly half of experiments they were sufficiently interested in that it is reasonable to think that mating would have occurred in the absence of any female resistance.

No clear evidence of reinforcement in *H. himera*

Heliconius are one of the "textbook" examples of pleiotropy (also known as "magic traits") in speciation, but an alternative hypothesis is that mate preference evolved or was strengthened through reinforcement, or natural selection against hybrids. In support of this hypothesis, there are several examples of geographic patterns of mate preference consistent with reinforcement between sister species of *Heliconius*(Jiggins et al., 2001b; M. R. Kronforst et al., 2007; Rosser et al., 2019b). Furthermore, there is independent evidence of selection against hybrids, as rare hybrid color patterns are attacked by birds more often than the more common patterns of the pure species(Merrill et al., 2012). However, we did not observe a geographical pattern of male or female preference in *H. himera* that would be consistent with reinforcement. *H. erato* females rejected *H. himera* males from Peru more than those from Ecuador, however this outcome is not predicted under a pattern of reinforcement. While this is difficult to explain by reinforcement, the geographical context of speciation between is muddied by two additional hybrid zones with other races of *H. erato* in Peru in which the level of reproductive isolation is unknown(Van Belleghem et al., 2020).

Furthermore, our study did not fully address the issue of reinforcement between these two species, because we did not test for geographic patterns in *H. erato* mate preferences or in the context of pupal mating. Firstly, based on the observation of strong male preference in *H. erato* (but not *H. himera*), future work should address the possibility that reinforcement has acted on male behavior in *H. erato* rather than *H. himera.* In support of this, recent genomic studies show

evidence of recent introgression of *H. himera* alleles into *H. erato* but not in the reverse, which might indicate natural selection may be much stronger against *H. erato* alleles in the habitat of *H. himera* (Van Belleghem et al., 2020). Secondly, while we conducted separate pupal mating trials with *H. himera* from Peru and Ecuador, we did not have a sufficient experimental design to conclude whether or not there is a geographic pattern of pupal mating. However, if hybridization does indeed happen more frequently via pupal mating than adult mating, then it would stand to reason that reinforcement would have more opportunity to act on traits that contribute to pupal-mating mistakes rather than traits of importance to adult mating.

Females use pheromonal cues and not visual cues to select mates

Completely removing male color pattern in our experiments did not affect female behavior in *H. himera*, suggesting that species-specific color patterns are not essential for females to accept a mate. This squares with recent work in the *H. melpomene* clade which found that chemical cues are important in both intraspecies female choice as well as between species in the case of co-mimics *H. melpomene malleti* and *H. timareta florencia*(Darragh, Vanjari, Mann, Gonzalez-R, et al., 2017; González-Rojas et al., 2019). The authors of the *H. melpomene malleti* and *H. timareta florencia* study further ruled out the use of color pattern cues with a color-blocking experiment similar to the one we did. However, regarding pupal mating *Heliconius* species, in contrast to our results, the two existing studies found some evidence for color-pattern based female choice. In one experiment, painting *H. charithonia* males black decreased male mating success in adult mating but not in pupal mating, which the authors took as implying latent female choice. Additionally, *H. erato demophoon* females approached and "courted" models more often if they had yellow or UV reflectance more closely matching *Heliconius* that the yellow of more distantly related *Eueides* butterflies.(Finkbeiner et al., 2017)

There were a few limitations to our color-blocking study. Firstly, our experimental design did not explicitly test the role of UV coloration in female choice, although both male and female wing patterns in *Heliconius* do reflect UV. In our experiments, we observed in UV photography that black sharpie absorbed UV. It is nevertheless possible that UV signals may be less distinguishable in a shaded greenhouse but could become relevant in true field conditions where sunlight could increase the contrast between UV and non-UV reflecting patterns. In support of this, we did observe an unexpectedly low mating rate in *H. himera* which could suggest experimental conditions were not ideal for mating. Secondly, because our design was limited to one species, our experimental design also did not test the alternative explanation that female specifically reject elements of another species color patterns, rather than matching her own (i.e., in the scenario that a *H. himera* female will mate with males with yellow or black forewing patterns, but not red like *H. erato cyrbia*). We also did not test the cues involved in female choice in *H. erato cyrbia*. Therefore, further work is necessary to completely rule out female choice by color pattern.

In contrast to the color-blocking results, *H. himera* females strongly rejected males with blocked androconial pheromones. Our results, combined with previous reports from a number of other *Heliconius* species, this suggest that pheromones are an essential cue used in female mating decisions and are involved in speciation(Darragh, Vanjari, Mann, Gonzalez-Rojas, et al., 2017a; González-Rojas et al., 2019). However, it is important to note that like many butterflies, pheromone blends in *Heliconius* may be highly complex and species can differ both in whether individuals compounds are present or their relative concentrations (rather than a complete absence of shared compounds as in our experiment)(Darragh, Vanjari, Mann, Gonzalez-Rojas, et al., 2017a; González-Rojas et al., 2019; Mann et al., 2017). It is interesting to note that *H. himera*

females were not more likely to change orientation in response to courtship of heterospecific males, but this behavior was observed when rejecting pheromone-blocked males. It is possible that this rejection behavior could be specifically linked to the lack of appropriate pheromone cues. Our experiments demonstrate that female response behavior can be measured, and is linked to their mate choice, which could allow researchers to more easily dissect the role of individual pheromonal differences in female choice.

Pheromone based female choice contrasts to color pattern -based male preference in that it is less clear what, if any, ecological factors cause pheromones to diverge between newly forming species. It has been proposed that pheromone composition and detection can rapidly diverge between sister species primarily via reinforcement. However, we found no evidence of reinforcement on female mating behavior in *H. himera*. Eavesdropping predators or parasitoids can also use mating pheromones; therefore, predator or parasitoid community composition or abundance could influence pheromone composition in different environments. Abiotic factors, in particular humidity, can impair the detection and production of some pheromones. In theory, this could lead to natural selection of pheromone composition or reception systems. The habitats of *H. erato* and *H. himera* show differences in temperature and humidity; therefore, more care should be taken to evaluate and control the effect of the environmental conditions in further analysis of pheromonal production and behavioral preferences. The other scenario possibility is that pheromones diverge through a mutation-order process, rather than through ecological divergence.

Pupal mating may be a source of hybridization
Male *Heliconius* discover larvae by volatile chemicals released from damaged hostplants, but our results suggest for the first time that males may also recognize species-specific cues

intrinsic to the larvae. *H. erato* males showed a behavioral pattern consistent with a preference for larvae of their own species and at least a greater likelihood of finding pupae of *H. erato* pupae. Only one of the six cross-species mating was by a *H. erato* male. Additionally, *H. erato* males were more likely to initiate competitive interactions with *H. erato* males. However, *H. himera* males also seemed to show a subtle preference for *H. erato* larvae in our experiment. This result was in line with adult mating observations, where *H. himera* males courted at least once more often in trials with *H. erato* females than with *H. himera* females. In the future, how males distinguish between immature species should be studied as differences in coloration, UV signals, and chemical cues of larvae have not been described in *H. erato* or *H. himera*.

In addition to larval feeding, most pupal mating studies have proposed a link between a male's size and success using mating behavioral strategy (Deinert et al., 1994; HernÁndez & Benson, 1998; Mendoza-Cuenca & Macías-Ordóñez, 2010). Nevertheless, under greenhouse conditions, the vast majority of both small and large males in our experiments engaged in some amount of pupal mating behavior. However, a handful of *H. himera* males did appear to be extreme outliers in the amount of time invested in pupal mating behavior and in the fact that they sat on the pupa on the morning of emergence. Notably, the only two pupal matings by *H. erato* males in the wild recorded in literature were by the same male(Thurman et al., 2018). So, while there does not appear to be a strict, absolute relationship between male size and presence of pupal mating behavior in controlled conditions, it does suggest there is extreme variation in pupal mating behavior between males. Therefore, further work could consider whether this variation is due to genetic, environmental, or social factors other than male size.

If representative of wild behavior, the high proportion of pupal matings between *H. himera* males and *H. erato* females observed in this study would seem to be inconsistent with the

maintenance of species barriers**.** Estimates of the cross-species mating probability between *H. erato* and *H. himera* in the wild have been estimated at about 5% (Mallet et al., 1998a; Merrill et al., 2014a). This differs from the results of this thesis and a previous studies that found that between-species mating mistakes occur about 10% of the time in adult mating no-choice trials(McMillan et al., 1997a). Mating behavior in the wild is not commonly observed, so it is not clear whether the high rate of pupal mating observed in the caged experiment reflects behavior in the hybrid zone. Previous reports of *H. erato demophoon* in Panama suggested that adult mating was more common than pupa mating. However, pupal mating behavior is thought to depend on population density in *H. charithonia*[6]. In the only similar study in Heliconius, strong assortative mating also disappeared between *H. erato chestertonii* and *H, erato venus* when males were given access to female pupae rather than adult females.(Munoz et al., 2010)

During this thesis research, there were a few unexpected observations of species-specific behavior which may affect the proportion of pupal mating to adult mating**.** Firstly, we observed that some females in both species emerged early in the morning before the first flight of males. In the climate of the hybrid zone, *H. erato cyrbia* males first become active in the morning later than *H. himera* and also take longer to develop from egg to adult(Davison, McMillan, Griffin, Jiggins, & Mallet, 1999). While this difference has been attributed to adaptation to cooler temperatures by *H. himera*, if the timing of female emergence or of first flight of males is different in this habitat between species, this may effectively prevent pupal mating from occurring, at least by *H. erato* males in the hybrid zone. Secondly, we observed a difference in pupation site between *H. himera* and *H. erato.* It is unknown whether or how this behavior would translate to a natural environment, where hostplants do not grow in pots. But is possible

that by pupating near the ground, *H. himera* pupae are less likely to be found and pupally mated as was observed in our experiments.

Conclusion

Looking more closely at the case of *Heliconius erato* and *Heliconius himera* was fruitful because we 1) identified female choice as an important component of assortative mating 2) demonstrated this preference is most likely based at least in part on male androconial pheromone composition rather than coloration and 3) demonstrated that female preference can be studied quantitatively by observable responses to male courtship. This information will allow future researchers to elucidate the specific pheromones and genes which might be involved in pheromone-based mate choice. Although not conclusive, pupal mating behavior should be considered in future studies because there was some evidence that *H.erato* males did distinguish between female immatures. The potential significance of the pupation location and emergence timing should also be explicitly studied. Beyond that, we are in a better position to comment on the mode of speciation in this particular case. The results of this thesis suggest that there are likely several traits involved in reproductive isolation even very early in the speciation process. The apparent importance of pheromones to reproductive isolation might suggest that other processes besides the natural selection for warning coloration, such as either reinforcement, sensory drive, or other processes were contributing to speciation.

Acknowledgments

References

Bertossa, R. C., van Dijk, J., Diao, W., Saunders, D., Beukeboom, L. W., & Beersma, D. G. M.
(2013). Circadian Rhythms Differ between Sexes and Closely Related Species of Nasonia
Wasps. *PLOS ONE*, *8*(3), e60167. https://doi.org/10.1371/journal.pone.0060167

Beysard, M., Krebs-Wheaton, R., & Heckel, G. (2015a). Tracing reinforcement through
asymmetrical partner preference in the European common vole Microtus arvalis. *BMC
Evolutionary Biology*, *15*(1), 170. https://doi.org/10.1186/s12862-015-0455-5

Beysard, M., Krebs-Wheaton, R., & Heckel, G. (2015b). Tracing reinforcement through
asymmetrical partner preference in the European common vole Microtus arvalis. *BMC
Evolutionary Biology*, *15*(1), 170. https://doi.org/10.1186/s12862-015-0455-5

Bolker, B. (2019). Logistic regression, accounting for differences in exposure. *RPubs by
RStudio*. https://rpubs.com/bbolker/logregexp

Boumans, L., Hogner, S., Brittain, J., & Johnsen, A. (2017). Ecological speciation by temporal
isolation in a population of the stonefly Leuctra hippopus (Plecoptera, Leuctridae).
Ecology and Evolution, *7*(5), 1635–1649. PubMed. https://doi.org/10.1002/ece3.2638

Brooks, M., Kristensen, K., van Benthem, K., Magnusson, A., Berg, C. W., Nielsen, A., Skaug,
H., Mächler, M., & Bolker, B. (2017). GlmmTMB Balances Speed and Flexibility
Among Packages for Zero-inflated Generalized Linear Mixed Modeling. *R Journal*, *9*,
378–400. https://doi.org/10.32614/RJ-2017-066

Costanzo, K., & Monteiro, A. (2007). The use of chemical and visual cues in female choice in
the butterfly Bicyclus anynana. *Proceedings of the Royal Society B: Biological Sciences*,
274(1611), 845–851. https://doi.org/10.1098/rspb.2006.3729

Daniel I. Bolnick, Lisa K. Snowberg, Claire Patenia, William E. Stutz, Travis Ingram, & On Lee
Lau. (2009). Phenotype-Dependent Native Habitat Preference Facilitates Divergence

between Parapatric Lake and Stream Stickleback. *Evolution*, *63*(8), 2004–2016.

https://doi.org/10.1111/j.1558-5646.2009.00699.x

Darragh, K., Vanjari, S., Mann, F., Gonzalez-R, M. F., Morrison, C. R., Salazar, C., Pardo-Diaz,

C., Merrill, R. M., McMillan, W. O., Schulz, S., & Jiggins, C. D. (2017). Male sex

pheromone components in Heliconius butterflies released by the androconia affect female

choice. *BioRxiv*. https://doi.org/10.1101/033506

Darragh, K., Vanjari, S., Mann, F., Gonzalez-Rojas, M. F., Morrison, C. R., Salazar, C., Pardo-

Diaz, C., Merrill, R. M., McMillan, W. O., Schulz, S., & Jiggins, C. D. (2017a). Male sex

pheromone components in Heliconius butterflies released by the androconia affect

female choice. *PeerJ*, *5*, e3953. https://doi.org/10.7717/peerj.3953

Darragh, K., Vanjari, S., Mann, F., Gonzalez-Rojas, M. F., Morrison, C. R., Salazar, C., Pardo-

Diaz, C., Merrill, R. M., McMillan, W. O., Schulz, S., & Jiggins, C. D. (2017b). Male sex

pheromone components in Heliconius butterflies released by the androconia affect female

choice. *PeerJ*, *5*, e3953–e3953. PubMed. https://doi.org/10.7717/peerj.3953

Davison, A., McMillan, W. O., Griffin, A. S., Jiggins, C. D., & James L. B. Mallet. (1999).

Behavioral and Physiological Differences between Two Parapatric Heliconius Species.

Biotropica, *31*(4), 661–668. JSTOR.

Davison, A., McMillan, W. O., Griffin, A. S., Jiggins, C. D., & Mallet, J. L. B. (1999).

Behavioral and physiological differences between two parapatric Heliconius species.

Biotropica, *31*. https://doi.org/10.1111/j.1744-7429.1999.tb00415.x

Deinert, E. I., Longino, J. T., & Gilbert, L. E. (1994). Mate competition in butterflies. *Nature*,

370(6484), 23–24. https://doi.org/10.1038/370023a0

Estrada, C., & Gilbert, L. E. (2010). Host plants and immatures as mate-searching cues in

    Heliconius butterflies. *Animal Behaviour*, *80*(2), 231–239.

    https://doi.org/10.1016/j.anbehav.2010.04.023

Estrada, C., & Jiggins, C. (2002). Patterns of pollen feeding and habitat preference among

    Heliconius species. *Ecological Entomology*, *27*(4), 448–456. AGRICOLA.

    https://doi.org/10.1046/j.1365-2311.2002.00434.x

Ficarrotta, V., Hanly, J. J., Loh, L. S., Francescutti, C. M., Ren, A., Tunström, K., Wheat, C. W.,

    Porter, A. H., Counterman, B. A., & Martin, A. (2021). A genetic switch for male UV-

    iridescence in an incipient species pair of sulphur butterflies. *BioRxiv*,

    2021.05.21.445125. https://doi.org/10.1101/2021.05.21.445125

Finkbeiner, S. D., Fishman, D. A., Osorio, D., & Briscoe, A. D. (2017). Ultraviolet and yellow

    reflectance but not fluorescence is important for visual discrimination of conspecifics by

    Heliconius erato. *The Journal of Experimental Biology*, *220*(7), 1267.

    https://doi.org/10.1242/jeb.153593

Fox, J., & Hong, J. (2009). Effect Displays in R for Multinomial and Proportional-Odds Logit

    Models: Extensions to the effects Package. *Journal of Statistical Software*, *32*(1), 1–24.

    https://doi.org/10.18637/jss.v032.i01

Garner, S. R., & Neff, B. D. (2013). Alternative male reproductive tactics drive asymmetrical

    hybridization between sunfishes (Lepomis spp.). *Biology Letters*, *9*(6), 20130658–

    20130658. PubMed. https://doi.org/10.1098/rsbl.2013.0658

Girard, M. B., Elias, D. O., & Kasumovic, M. M. (2015). Female preference for multi-modal

    courtship: Multiple signals are important for male mating success in peacock spiders.

Proceedings of the Royal Society B: Biological Sciences, *282*(1820), 20152222. https://doi.org/10.1098/rspb.2015.2222

González-Rojas, M. F., Darragh, K., Robles, J., Linares, M., Schulz, S., McMillan, W. O., Jiggins, C. D., Pardo-Diaz, C., & Salazar, C. (2019). Chemical signals act as the main reproductive barrier between sister and mimetic Heliconius butterflies. *BioRxiv*, 856393. https://doi.org/10.1101/856393

González-Rojas, M. F., Darragh, K., Robles, J., Linares, M., Schulz, S., McMillan, W. O., Jiggins, C. D., Pardo-Diaz, C., & Salazar, C. (2020). Chemical signals act as the main reproductive barrier between sister and mimetic Heliconius butterflies. *Proceedings of the Royal Society B: Biological Sciences*, *287*(1926), 20200587. https://doi.org/10.1098/rspb.2020.0587

Groot, A. (2014). Circadian rhythms of sexual activities in moths: A review. *Frontiers in Ecology and Evolution*, *2*. https://doi.org/10.3389/fevo.2014.00043

Grün, B., Kosmidis, I., & Zeileis, A. (2012). Extended Beta Regression in R: Shaken, Stirred, Mixed, and Partitioned. *Journal of Statistical Software*, *48*(11), 1–25. https://doi.org/10.18637/jss.v048.i11

HernÁndez, M. I. M., & Benson, W. W. (1998). Small-male advantage in the territorial tropical butterfly Heliconius sara (Nymphalidae): A paradoxical strategy? *Animal Behaviour*, *56*(3), 533–540. https://doi.org/10.1006/anbe.1998.0840

Hillis, D. M. (1981). Premating Isolating Mechanisms among Three Species of the Rana pipiens Complex in Texas and Southern Oklahoma. *Copeia*, *1981*(2), 312–319. JSTOR. https://doi.org/10.2307/1444220

Hochkirch, A., & Lemke, I. (2011). Asymmetric mate choice, hybridization, and hybrid fitness in two sympatric grasshopper species. *Behavioral Ecology and Sociobiology*, *65*, 1637–1645.

Jerry Coyne & H. Allen Orr. (2004). *Speciation* (1st ed.). Sinauer Associates.

JIGGINS, C. D., MACMILLAN, W. O., NEUKIRCHEN, W., & MALLET, J. (1996). What can hybrid zones tell us about speciation? The case ofHeliconius eratoandH. himera(Lepidoptera: Nymphalidae). *Biological Journal of the Linnean Society*, *59*(3), 221–242. https://doi.org/10.1006/bijl.1996.0063

Jiggins, C. D., Naisbit, R. E., Coe, R. L., & Mallet, J. (2001a). Reproductive isolation caused by colour pattern mimicry. *Nature*, *411*(6835), 302–305. https://doi.org/10.1038/35077075

Jiggins, C. D., Naisbit, R. E., Coe, R. L., & Mallet, J. (2001b). Reproductive isolation caused by colour pattern mimicry. *Nature*, *411*, 302.

Kirkpatrick, M., & Ravigné, V. (2002). Speciation by Natural and Sexual Selection: Models and Experiments. *The American Naturalist*, *159*(S3), S22–S35. https://doi.org/10.1086/338370

Klein, A. L., & de Araújo, A. M. (2010). Courtship behavior of Heliconius erato phyllis (Lepidoptera, Nymphalidae) towards virgin and mated females: Conflict between attraction and repulsion signals? *Journal of Ethology*, *28*(3), 409–420. https://doi.org/10.1007/s10164-010-0209-1

Kozak, K. M., Wahlberg, N., Neild, A. F. E., Dasmahapatra, K. K., Mallet, J., & Jiggins, C. D. (2015). Multilocus species trees show the recent adaptive radiation of the mimetic heliconius butterflies. *Systematic Biology*, *64*(3), 505–524. https://doi.org/10.1093/sysbio/syv007

Kronforst, M. R., Young, L. G., & Gilbert, L. E. (2007). Reinforcement of mate preference among hybridizing Heliconius butterflies. *Journal of Evolutionary Biology*, *20*(1), 278–285. https://doi.org/10.1111/j.1420-9101.2006.01198.x

Kronforst, M. R., Young, L. G., Kapan, D. D., McNeely, C., O'Neill, R. J., & Gilbert, L. E. (2006). Linkage of butterfly mate preference and wing color preference cue at the genomic location of wingless. *Proceedings of the National Academy of Sciences*, *103*(17), 6575. https://doi.org/10.1073/pnas.0509685103

Kronforst, M., Young, L., & Gilbert, L. (2007). Reinforcement of mate preference among hybridizing Heliconius butterflies. *Journal of Evolutionary Biology*, *20*(1), 278–285. PubMed. https://doi.org/10.1111/j.1420-9101.2006.01198.x

MacCallum, C. J., Nürnberger, B., Barton, N. H., & Szymura, J. M. (1998). Habitat Preference in the Bombina Hybrid Zone in Croatia. *Evolution*, *52*(1), 227–239. JSTOR. https://doi.org/10.2307/2410938

Mallet, J., McMillan, W. O., & Jiggins, C. D. (1998a). Estimating the Mating Behavior of a Pair of Hybridizing Heliconius Species in the Wild. *Evolution*, *52*(2), 503–510. JSTOR. https://doi.org/10.2307/2411085

Mallet, J., McMillan, W. O., & Jiggins, C. D. (1998b). ESTIMATING THE MATING BEHAVIOR OF A PAIR OF HYBRIDIZING HELICONIUS SPECIES IN THE WILD. *Evolution*, *52*(2), 503–510. https://doi.org/10.1111/j.1558-5646.1998.tb01649.x

Mann, F., Vanjari, S., Rosser, N., Mann, S., Dasmahapatra, K. K., Corbin, C., Linares, M., Pardo-Diaz, C., Salazar, C., Jiggins, C., & Schulz, S. (2017). The Scent Chemistry of Heliconius Wing Androconia. *Journal of Chemical Ecology*, *43*(9), 843–857. https://doi.org/10.1007/s10886-017-0867-3

Marshall, D. C., & Cooley, J. R. (2000). REPRODUCTIVE CHARACTER DISPLACEMENT AND SPECIATION IN PERIODICAL CICADAS, WITH DESCRIPTION OF A NEW SPECIES, 13-YEAR MAGICICADA NEOTREDECIM. *Evolution*, *54*(4), 1313–1325. https://doi.org/10.1111/j.0014-3820.2000.tb00564.x

Mazerolle, Marc J. (2020). *AICcmodavg: Model selection and multimodel inference based on (Q)AIC(c)* (R package version 2.3-1) [Computer software]. https://cran.r-project.org/package=AICcmodavg

McMillan, W. O., Jiggins, C. D., & Mallet, J. (1997a). What initiates speciation in passion-vine butterflies? *Proc Natl Acad Sci U S A*, *94*. https://doi.org/10.1073/pnas.94.16.8628

McMillan, W. O., Jiggins, C. D., & Mallet, J. (1997b). What initiates speciation in passion-vine butterflies? *Proceedings of the National Academy of Sciences*, *94*(16), 8628. https://doi.org/10.1073/pnas.94.16.8628

Mendoza-Cuenca, L., & Macías-Ordóñez, R. (2010). Female asynchrony may drive disruptive sexual selection on male mating phenotypes in a Heliconius butterfly. *Behavioral Ecology*, *21*(1), 144–152. https://doi.org/10.1093/beheco/arp163

Mérot, C., Frérot, B., Leppik, E., & Joron, M. (2015a). Beyond magic traits: Multimodal mating cues in Heliconius butterflies. *Evolution*, *69*(11), 2891–2904. https://doi.org/10.1111/evo.12789

Mérot, C., Frérot, B., Leppik, E., & Joron, M. (2015b). Beyond magic traits: Multimodal mating cues in Heliconius butterflies. *Evolution*, *69*(11), 2891–2904. https://doi.org/10.1111/evo.12789

Mérot, C., Salazar, C., Merrill, R. M., Jiggins, C. D., & Joron, M. (2017a). What shapes the continuum of reproductive isolation? Lessons from Heliconius butterflies. *Proceedings of*

the Royal Society B: Biological Sciences, *284*(1856), 20170335.

https://doi.org/10.1098/rspb.2017.0335

Mérot, C., Salazar, C., Merrill, R. M., Jiggins, C. D., & Joron, M. (2017b). What shapes the

continuum of reproductive isolation? Lessons from Heliconius butterflies. *Proceedings of*

the Royal Society B: Biological Sciences, *284*(1856), 20170335.

https://doi.org/10.1098/rspb.2017.0335

Merrill, R. M., Chia, A., & Nadeau, N. J. (2014a). Divergent warning patterns contribute to

assortative mating between incipient Heliconius species. *Ecol Evol*, *4*.

https://doi.org/10.1002/ece3.996

Merrill, R. M., Chia, A., & Nadeau, N. J. (2014b). Divergent warning patterns contribute to

assortative mating between incipient Heliconius species. *Ecology and Evolution*, *4*(7),

911–917. https://doi.org/10.1002/ece3.996

Merrill, R. M., Gompert, Z., Dembeck, L. M., Kronforst, M. R., McMillan, W. O., & Jiggins, C.

D. (2011a). MATE PREFERENCE ACROSS THE SPECIATION CONTINUUM IN A

CLADE OF MIMETIC BUTTERFLIES. *Evolution*, *65*(5), 1489–1500.

https://doi.org/10.1111/j.1558-5646.2010.01216.x

Merrill, R. M., Gompert, Z., Dembeck, L. M., Kronforst, M. R., McMillan, W. O., & Jiggins, C.

D. (2011b). Mate preference across the speciation continuum in a clade of mimetic

butterflies. *Evolution*, *65*(5), 1489–1500. https://doi.org/10.1111/j.1558-

5646.2010.01216.x

Merrill, R. M., Naisbit, R. E., Mallet, J., & Jiggins, C. D. (2013). Ecological and genetic factors

influencing the transition between host-use strategies in sympatric Heliconius butterflies.

Journal of Evolutionary Biology, *26*(9), 1959–1967. https://doi.org/10.1111/jeb.12194

Merrill, R. M., Wallbank, R. W. R., Bull, V., Salazar, P. C. A., Mallet, J., Stevens, M., & Jiggins, C. D. (2012). Disruptive ecological selection on a mating cue. *Proceedings. Biological Sciences*, *279*(1749), 4907–4913. PubMed. https://doi.org/10.1098/rspb.2012.1968

Montgomery, S. H., & Merrill, R. M. (2017). Divergence in brain composition during the early stages of ecological specialization in Heliconius butterflies. *Journal of Evolutionary Biology*, *30*(3), 571–582. https://doi.org/10.1111/jeb.13027

Munoz, A. G., Salazar, C., Castano, J., Jiggins, C. D., & Linares, M. (2010). Multiple sources of reproductive isolation in a bimodal butterfly hybrid zone. *Journal of Evolutionary Biology*, *23*(6), 1312–1320. https://doi.org/10.1111/j.1420-9101.2010.02001.x

Pärssinen, V., Hulthén, K., Brönmark, C., Skov, C., Brodersen, J., Baktoft, H., Chapman, B. B., Hansson, L.-A., & Nilsson, P. A. (2020). Maladaptive migration behaviour in hybrids links to predator-mediated ecological selection. *Journal of Animal Ecology*, *89*(11), 2596–2604. https://doi.org/10.1111/1365-2656.13308

Péter, A. (2017). *Solomon Coder* (beta 17.03.22) [Computer software]. https://solomoncoder.com

Polačik, M., & Reichard, M. (2011). Asymmetric Reproductive Isolation between Two Sympatric Annual Killifish with Extremely Short Lifespans. *PLOS ONE*, *6*(8), e22684. https://doi.org/10.1371/journal.pone.0022684

Rosser, N., Queste, L. M., Cama, B., Edelman, N. B., Mann, F., Mori Pezo, R., Morris, J., Segami, C., Velado, P., Schulz, S., Mallet, J. L. B., & Dasmahapatra, K. K. (2019a). Geographic contrasts between pre- and postzygotic barriers are consistent with reinforcement in Heliconius butterflies. *Evolution*, *73*(9), 1821–1838. https://doi.org/10.1111/evo.13804

Rosser, N., Queste, L. M., Cama, B., Edelman, N. B., Mann, F., Mori Pezo, R., Morris, J., Segami, C., Velado, P., Schulz, S., Mallet, J. L. B., & Dasmahapatra, K. K. (2019b). Geographic contrasts between pre- and postzygotic barriers are consistent with reinforcement in Heliconius butterflies. *Evolution*, *73*(9), 1821–1838. https://doi.org/10.1111/evo.13804

Rossi, M., Hausmann, A. E., Thurman, T. J., Montgomery, S. H., Papa, R., Jiggins, C. D., McMillan, W. O., & Merrill, R. M. (2020). Visual mate preference evolution during butterfly speciation is linked to neural processing genes. *Nature Communications*, *11*(1), 4763. https://doi.org/10.1038/s41467-020-18609-z

Southcott, L., & Kronforst, M. R. (2018a). Female mate choice is a reproductive isolating barrier in Heliconius butterflies. *Ethology*, *124*(12), 862–869.

Southcott, L., & Kronforst, M. R. (2018b). Female mate choice is a reproductive isolating barrier in Heliconius butterflies. *Ethology : Formerly Zeitschrift Fur Tierpsychologie*, *124*(12), 862–869. PubMed. https://doi.org/10.1111/eth.12818

Stewart, K. A., Hudson, C. M., & Lougheed, S. C. (2017). Can alternative mating tactics facilitate introgression across a hybrid zone by circumventing female choice? *Journal of Evolutionary Biology*, *30*(2), 412–421. https://doi.org/10.1111/jeb.13017

Thurman, T. J., Brodie, E., Evans, E., & McMillan, W. O. (2018). Facultative pupal mating in Heliconius erato: Implications for mate choice, female preference, and speciation. *Ecology and Evolution*, *8*(3), 1882–1889. https://doi.org/10.1002/ece3.3624

Turbek, S. P., Scordato, E. S. C., & Safran, R. J. (2018). The Role of Seasonal Migration in Population Divergence and Reproductive Isolation. *Trends in Ecology & Evolution*, *33*(3), 164–175. https://doi.org/10.1016/j.tree.2017.11.008

Turissini, D. A., Comeault, A. A., Liu, G., Lee, Y. C. G., & Matute, D. R. (2017). The ability of

    Drosophila hybrids to locate food declines with parental divergence. *Evolution*, *71*(4),

    960–973. https://doi.org/10.1111/evo.13180

Underwood, D. L. A. (1994). Methods for sexing Lepidoptera larvae using external morphology.

    Journal of the Lepidopterists' Society., *48*(3), 258–263.

Uy, J. A. C., Irwin, D. E., & Webster, M. S. (2018). Behavioral Isolation and Incipient

    Speciation in Birds. *Annual Review of Ecology, Evolution, and Systematics*, *49*(1), 1–24.

    https://doi.org/10.1146/annurev-ecolsys-110617-062646

Van Belleghem, S. M., Cole, J. M., Montejo-Kovacevich, G., Bacquet, C. N., McMillan, W. O.,

    Papa, R., & Counterman, B. A. (2020). Selection and gene flow define polygenic barriers

    between incipient butterfly species. *BioRxiv*, 2020.04.09.034470.

    https://doi.org/10.1101/2020.04.09.034470

Van Belleghem, S. M., De Wolf, K., & Hendrickx, F. (2016). Behavioral adaptations imply a

    direct link between ecological specialization and reproductive isolation in a sympatrically

    diverging ground beetle. *Evolution; International Journal of Organic Evolution*, *70*(8),

    1904–1912. https://doi.org/10.1111/evo.12998

Yukilevich, R. (2012a). ASYMMETRICAL PATTERNS OF SPECIATION UNIQUELY

    SUPPORT REINFORCEMENT IN DROSOPHILA. *Evolution*, *66*(5), 1430–1446.

    https://doi.org/10.1111/j.1558-5646.2011.01534.x

Yukilevich, R. (2012b). ASYMMETRICAL PATTERNS OF SPECIATION UNIQUELY

    SUPPORT REINFORCEMENT IN DROSOPHILA. *Evolution*, *66*(5), 1430–1446.

    https://doi.org/10.1111/j.1558-5646.2011.01534.x

Yukilevich, R., & Peterson, E. K. (2019). The evolution of male and female mating preferences

in Drosophila speciation*. *Evolution*, *73*(9), 1759–1773.

https://doi.org/10.1111/evo.13752

Figures and Tables

Figure 1: Ranges of *H. himera* and <u>*H. erato.*</u>

Table 1: Description of male and female behaviors scored during behavioral experiments.

Female courtship response	Description
Perching	*Sitting in place on any surface*
Flight	*Flight*
Walking	*Walking along any surface*
Abdomen raising/lowering	*Abdomen lifted between wings; Abdomen exposed below wings*
Wing opening/closing	*Wings completely or partially opened; wings together*
Changed orientation	*Changes the direction of the body without translocation*

Male behavior- adult	Description
Approach	*flies within 20cm of and oriented towards female with brief pause or change of direction upon reaching female*
Courtship	*Includes any of the following (≥5 seconds): hovering in place over female, chasing female, landing on female, touching female with proboscis or antennae, walking around female,*
Abdomen bending	*male curves abdomen toward female while perched next to female*
Mating	*copulation in which male transfers a spermatophore to female*

Male behavior- larva/pupa	Description
Approach	*Flies within 20 cm of and oriented towards immature*
Hovering	*Flies within 20 cm of and remains in 20cm radius roughly oriented towards immature (>1 sec)*
Sitting	*Male perches on immature (≥ 1 sec)*
Pupal mating- pharate	*male who is sitting in pupa prior to emergence mates female before, during, or immediately after emergence*
Pupal mating- teneral	*male approaches and mates a newly emerged female who has yet to fly from pupa (includes cases where female has crawled off pupa onto nearby branches)*
Adult mating	*mating that occurs after a newly emerged female's first flight*
Male-male interaction	*male approaches another male who is already hovering, or sitting on an immature female*
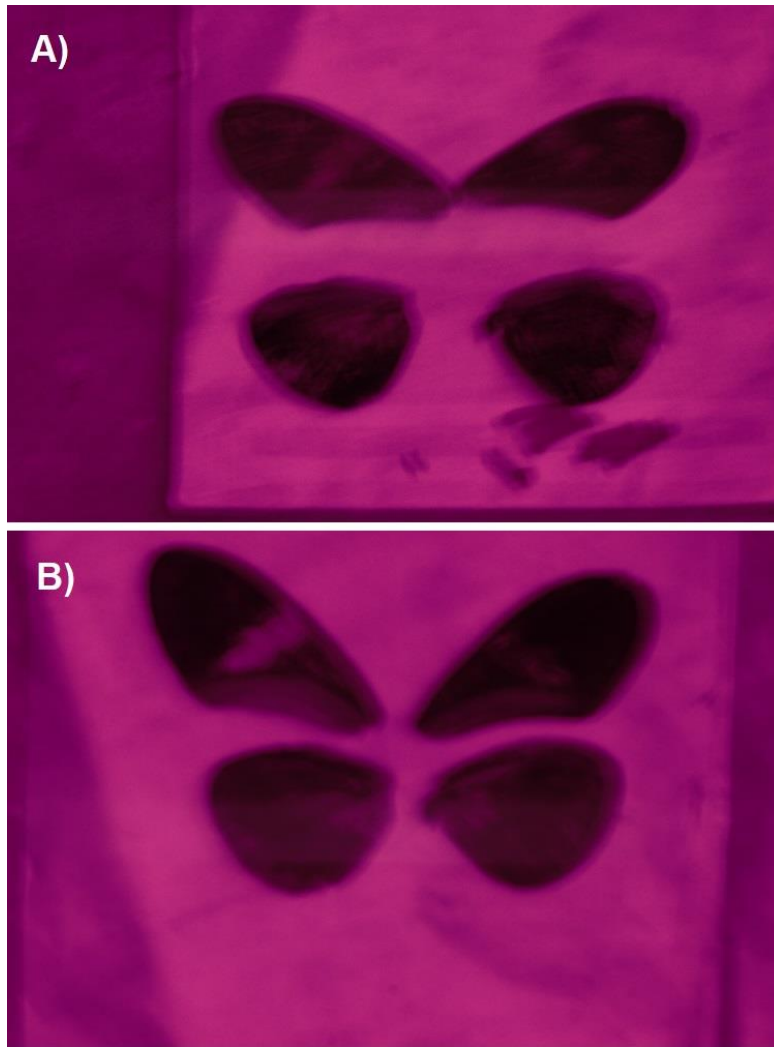
Figure 2:UV photography of *H.himera* wings from color blocking experiments

Photographs taken using a UV lens of the A) dorsal and B) ventral surface of *H.himera* wings**.** On the left of both images, yellow and red patterns were colored with black permanent marker and right side and control with black regions marked.

Table 2: Mating outcomes of no-choice trials.

Estimate of mating rate in no-choice trials and 95% confidence intervals were calculated from a logistic regression of mating outcome predicted by an interaction between male species and female species. The proportion of trials in which males courted or abdomen bending was observed at least once during the 120-minute observation are listed in the right most columns.

Species Pair	Total trials	Trials mated	Estimated proportion mating 95 % CI	Trials with courtship	Trials with abdomen bending
H. erato ♂ x H. erato ♀	21	19	0.90 (0.69,0.98)	21/21	17/21
H. erato ♂ x H. himera ♀	33	3	0.09 (0.03,0.25)	25/33	15/33
H. himera ♂ x H. erato ♀	29	4	0.14 (0.05,0.31)	29/29	24/29
H. himera ♂ x H. himera ♀	43	16	0.37 (0.24,0.52)	36/43	28/43
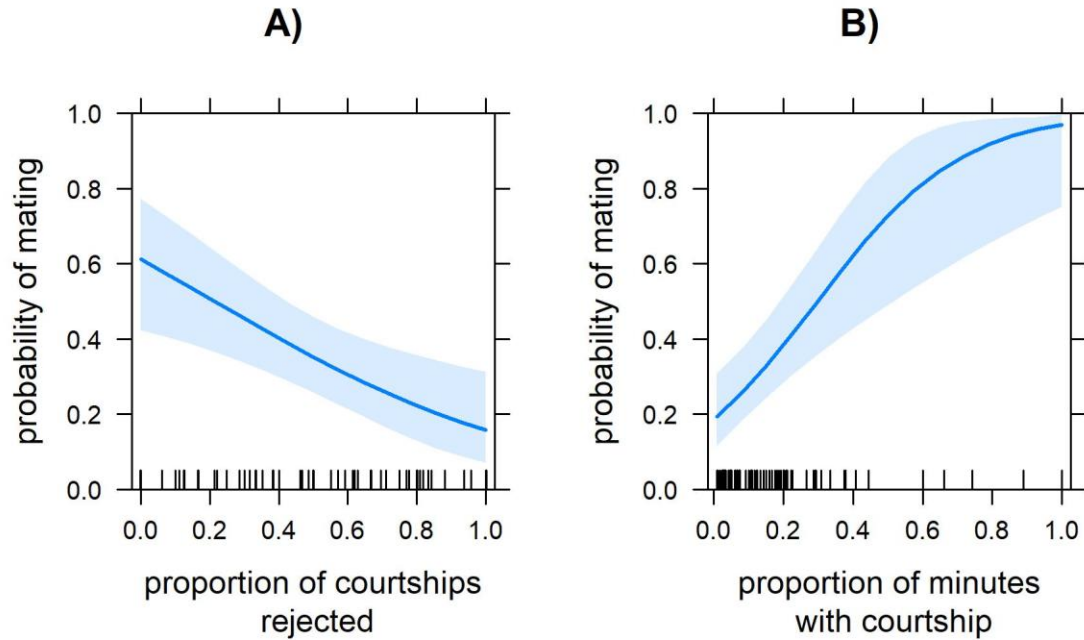
Figure 3: The effect of female rejection and male courtship on probability of mating.

Female rejection was calculated as the proportion of minutes with male courtship that a female responded by opening her wings. Male effort was calculated as the proportion of total minutes that males courted the female. Black tick marks on the bottom of graph represent the values which occurred in the data.

Table 3: Comparison of candidate models for mating outcome.

	null	model 1	model 2	model 3
intercept	-0.57 (0.20)**	0.18 (0.34)	-1.25 (0.28)***	-0.37 (0.39)
female rejection		-1.54 (0.59)**		-2.13 (0.71)**
courtship			4.11 (1.27)**	4.91 (1.40)***
AIC	147.10	141.82	132.83	124.47
Δ AIC	22.64	17.35	8.37	0
AIC Weight	0.00	0.02	1.50	98.48
BIC	149.81	147.24	138.25	132.59
Log Likelihood	-72.55	-68.91	-64.42	-59.23
Deviance	145.10	137.82	128.83	118.47
Num. obs.	111	111	111	111

***p < 0.001, **p < 0.01, *p < 0.05
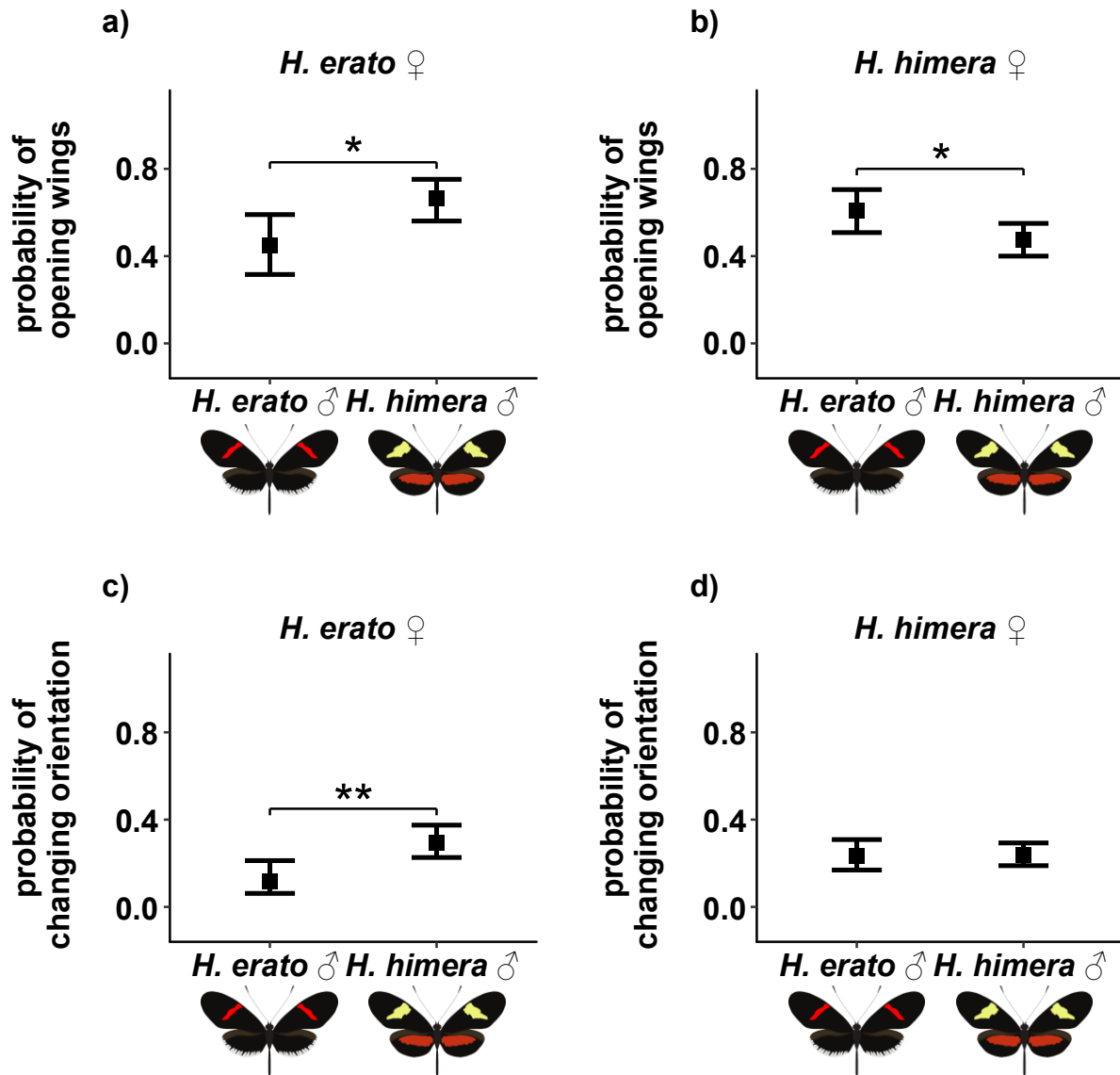
Figure 4: Effect of male species on the probability of female response to courtship.

Figure 3a an 3b show the probability of a female opening her wings in response to male courtship by *H. erato* and *H. himera* females respectively. Figure 3c and 3d show probability of changing orientation in response to a male courtship by *H. erato* and *H. himera* females respectively. Error bars and black squares are the 95% confidence intervals and mean estimate calculated from the quasibinomial models. * p<0.05, **p<0.01, ***p<0.001

Table 4: Quasibinomial models of female responses to courtship by *H. erato* females.

	Opened Wings	Closed Wings	Changed Orientation	Abdomen lowered	Abdomen raised	Perched	Flying	Crawling
(Intercept)	-0.20 (0.28)	0.51 (0.25)*	-2.01 (0.35)***	-1.92 (0.41)***	2.37 (0.46)***	3.82 (0.56)***	-2.07 (0.35)***	-1.87 (0.31)***
male_species H. himera	0.88 (0.36)*	-0.52 (0.30)	1.14 (0.39)**	-0.37 (0.54)	0.31 (0.59)	0.25 (0.72)	-0.10 (0.43)	-0.24 (0.39)
Deviance	213.95	152.82	123.45	153.39	110.45	34.84	90.37	94.18
Num. obs.	50	50	50	50	50	50	50	50

***$p < 0.001$, **$p < 0.01$, *$p < 0.05$
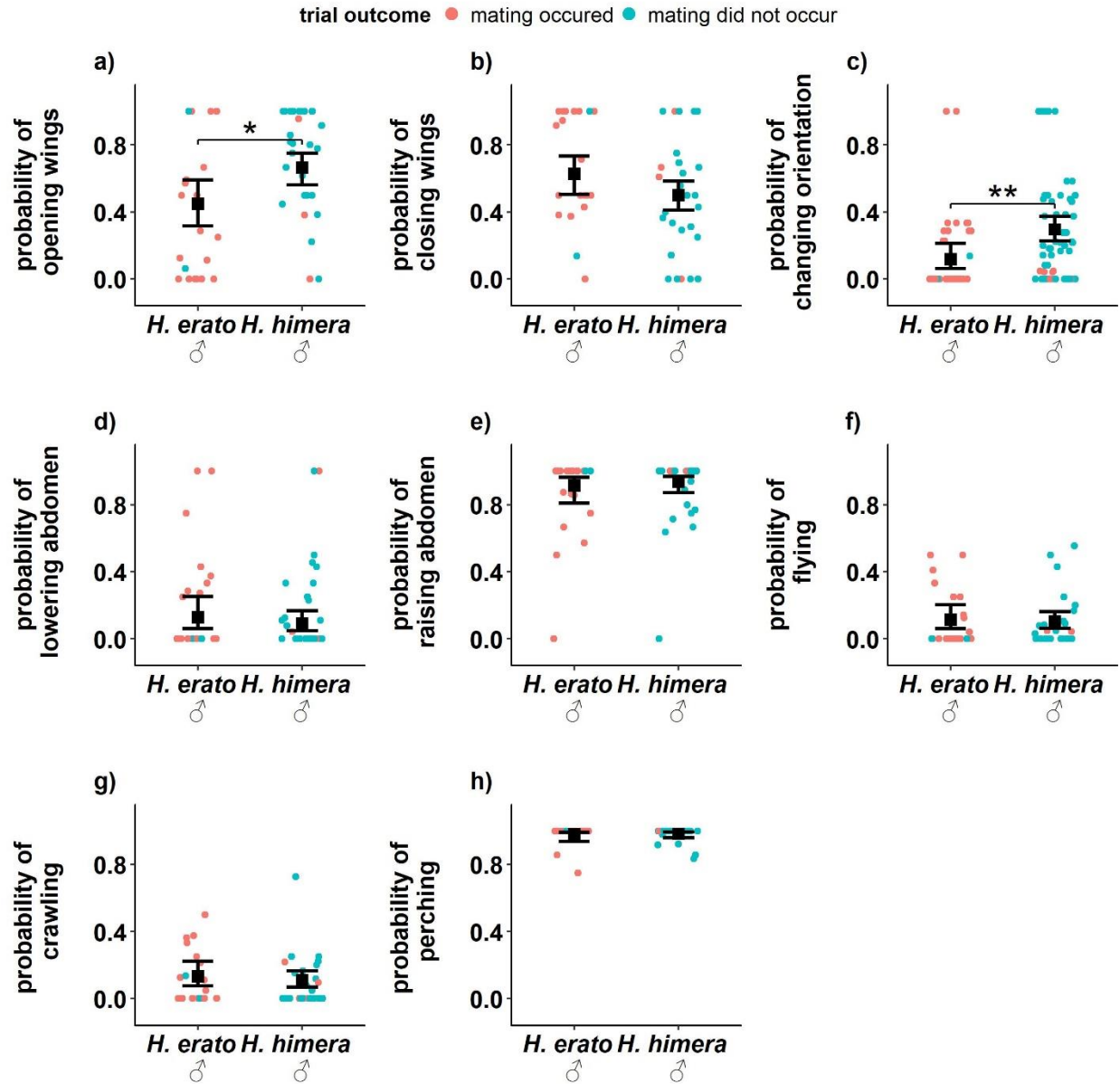
Figure 5: Probability of male species on *H. erato* female responses to courtship.

Effect of male species predicted by quasibinomial models. Black squares represent the point estimate and the black bars are 95% CI. Individual dots represent individual female responses calculated as the number of courtship minutes a female responded with a particular behavior divided by total number of courtship minutes.

Table 5: Quasibinomial models of female responses to courtship by *H. himera* females.

	Opened Wings	Closed Wings	Changed Orientation	Abdomen lowered	Abdomen raised	Perched	Flying	Crawling
(Intercept)	0.45 (0.21)*	-0.05 (0.20)	-1.20 (0.20)***	-2.96 (0.43)***	3.68 (0.69)***	4.20 (1.00)***	-1.75 (0.20)***	-1.96 (0.26)***
male_species H. himera	-0.55 (0.26)*	0.47 (0.25)	0.03 (0.24)	-0.02 (0.54)	-0.76 (0.78)	-0.38 (1.17)	-0.23 (0.26)	-0.50 (0.36)
Deviance	142.52	139.61	102.23	68.11	75.04	61.78	69.41	83.17
Num. obs.	61	61	61	61	61	61	61	61

***$p < 0.001$, **$p < 0.01$, *$p < 0.05$
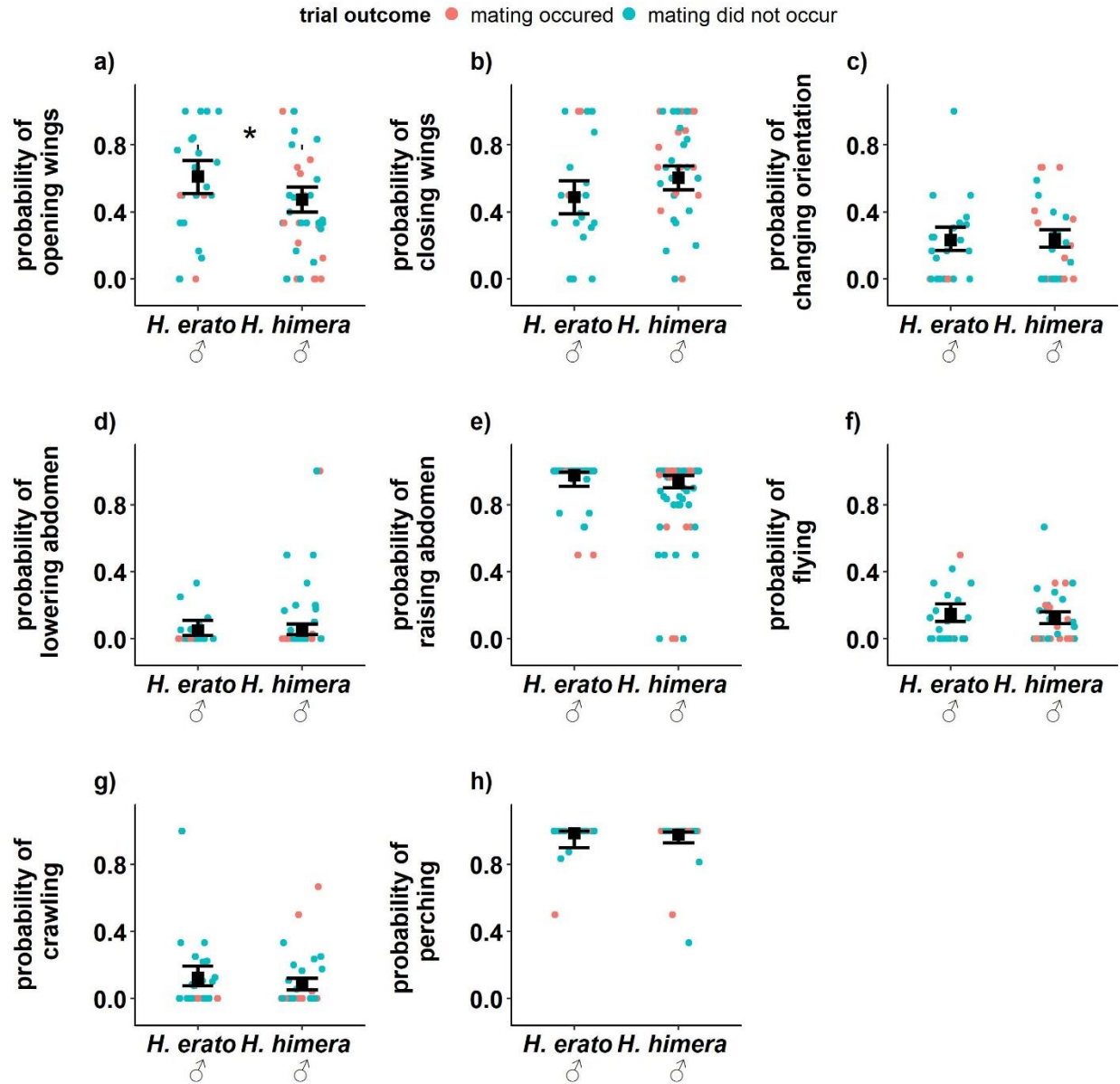
Figure 6: Probability of male species on *H. himera* female responses to courtship.

A-H:Effects predicted by quasibinomial models. Black squares represent the point estimate and the black bars are 95% CI. Individual dots represent individual female responses calculated as the number of courtship minutes a female responded with a particular behavior divided by total number of courtship minutes.

Figure 7: Male courtship towards conspecific and heterospecific females.

Effects of female species on the courtship probability of a) male H. himera and b) male H. erato. predicted by quasibinomial models. Black squares represent the point estimate and the black bars are 95% CI. Individual dots represent experimental data calculated as the number of courtship minutes males courted divided by total number of minutes.

Table 6: Quasibinomial models male *H. erato* behavior towards conspecific or heterospecific females.

	Approaches	Courtship	Abdomen Bending
(Intercept)	-0.20 (0.25)	-1.78 (0.26)[***]	-2.72 (0.41)[***]
female_speciesH. himera	0.16 (0.29)	-0.95 (0.35)[**]	-0.79 (0.55)
Deviance	1120.61	455.41	434.57
Dispersion parameter	12.38	10.44	19.85
Num. obs.	54	54	54

[***]$p < 0.001$, [**]$p < 0.01$, [*]$p < 0.05$

Table 7: Quasibinomial models male *H. himera* behavior towards conspecific or heterospecific females.

	Approaches	Courtship	Abdomen Bending
(Intercept)	-0.38 (0.14)**	-2.06 (0.26)***	-2.68 (0.32)***
female_speciesH. himera	0.05 (0.18)	-0.18 (0.36)	-0.46 (0.48)
Deviance	1072.42	1162.00	845.86
Dispersion parameter	14.02	20.53	19.12
Num. obs.	72	72	72

***$p < 0.001$, **$p < 0.01$, *$p < 0.05$
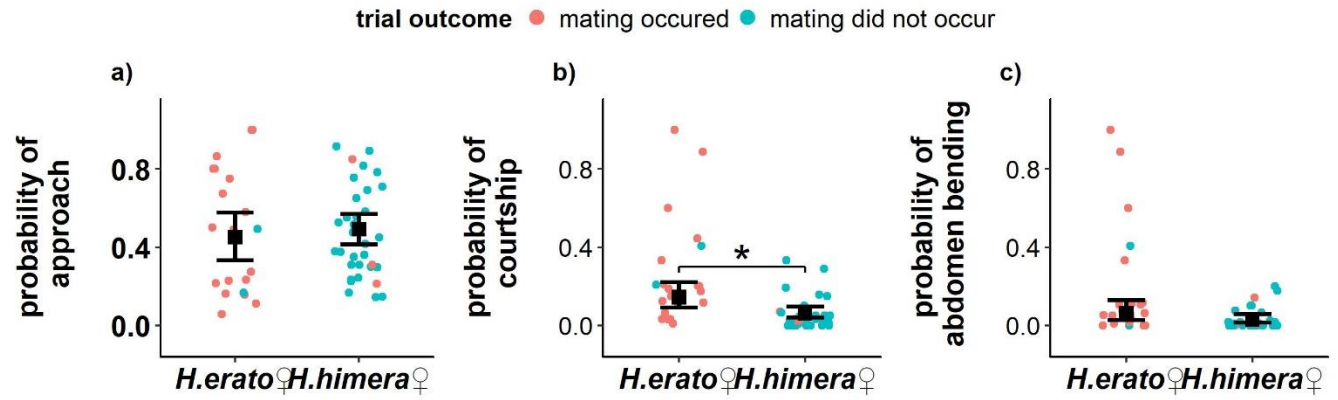
Figure 8: *H. erato* male behavior separated by approach, courtship, and abdomen bending towards conspecific and heterospecific females.

A-C. Effects of female species on the courtship probability of male *H. erato* predicted by quasibinomial models. Black squares represent the point estimate and the black bars are 95% CI. Individual dots represent experimental data calculated as the number of courtship minutes males courted divided by total number of minutes.
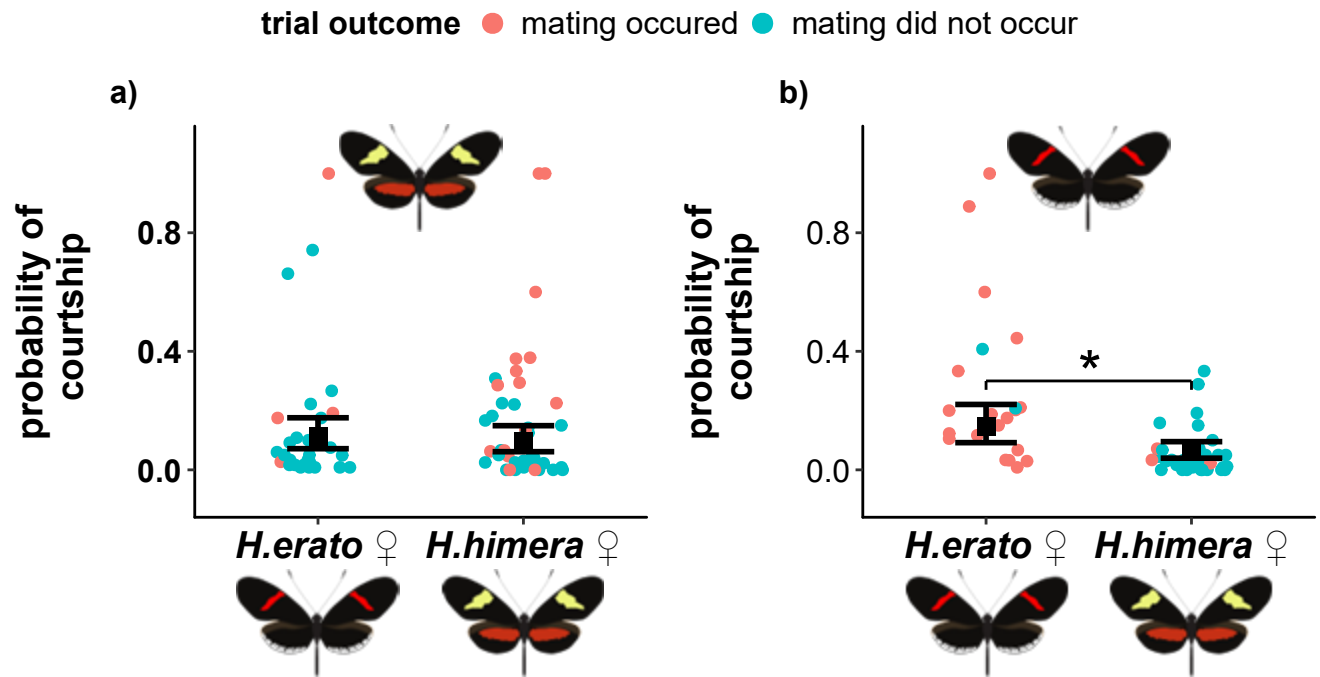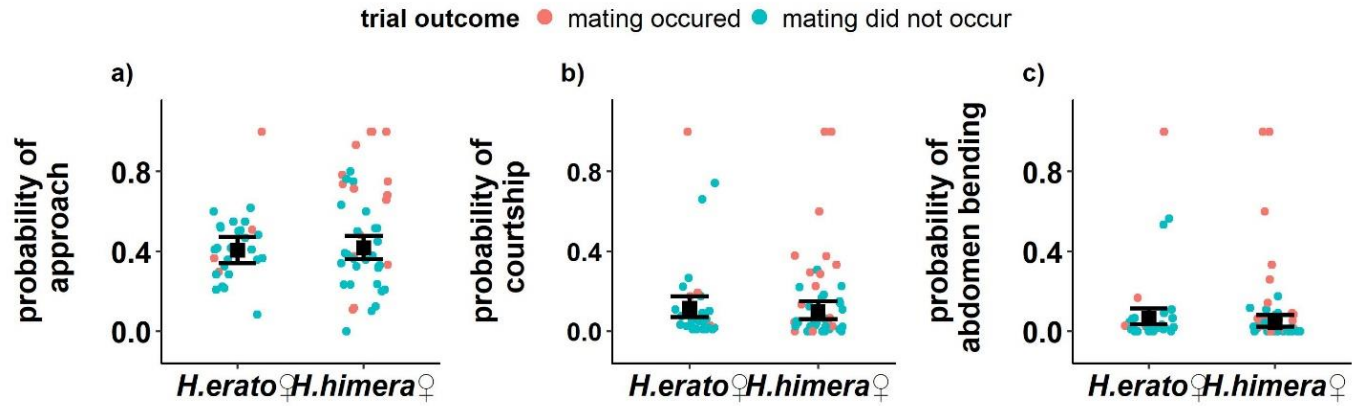
Figure 9: *H. himera* male behavior towards conspecific and heterospecific females.

a-c. Effects of female species on the courtship probability of male *H. himera* predicted by quasibinomial models. Black squares represent the point estimate and the black bars are 95% CI. Individual dots represent experimental data calculated as the number of courtship minutes males courted divided by total number of minutes.
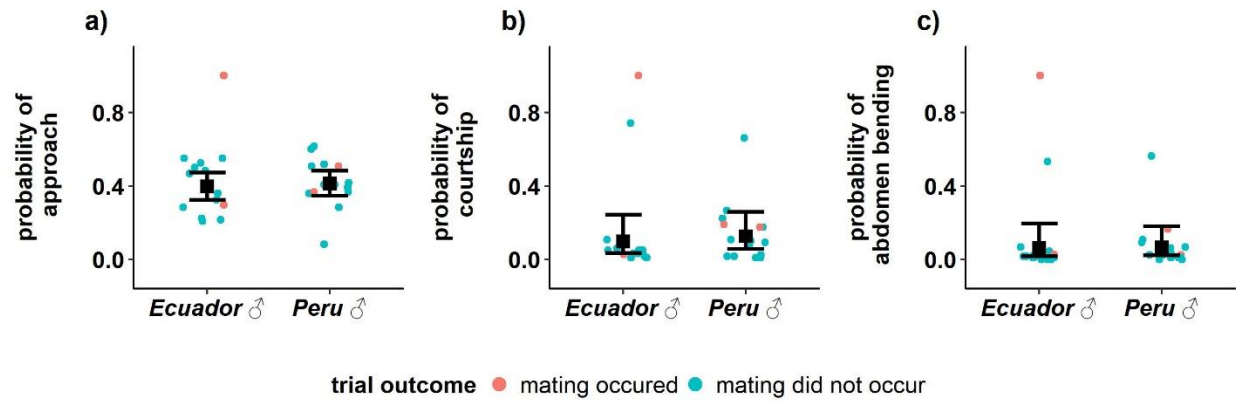
Figure 10: Behavior of *H. himera* males from Ecuador and Peru towards *H. erato* females.

A-C. Effects of male population of male *H. himera* behavior towards *H. erato* females predicted by quasibinomial models. Black squares represent the point estimate and the black bars are 95% CI. Individual dots represent experimental data calculated as the number of courtship minutes males courted divided by total number of minutes.

Table 8: Models for effect of male population on *H. himera* male behavior towards heterospecific females.

	approach	courtship	abdomen bending
(Intercept)	-0.42 (0.15)[*]	-2.22 (0.53)[***]	-2.72 (0.63)[***]
male_populationH. himera_Peru	0.06 (0.21)	0.28 (0.68)	0.07 (0.84)
Deviance	227.25	635.98	511.76
Num. obs.	29	29	29

[***]p < 0.001, [**]p < 0.01, [*]p < 0.05
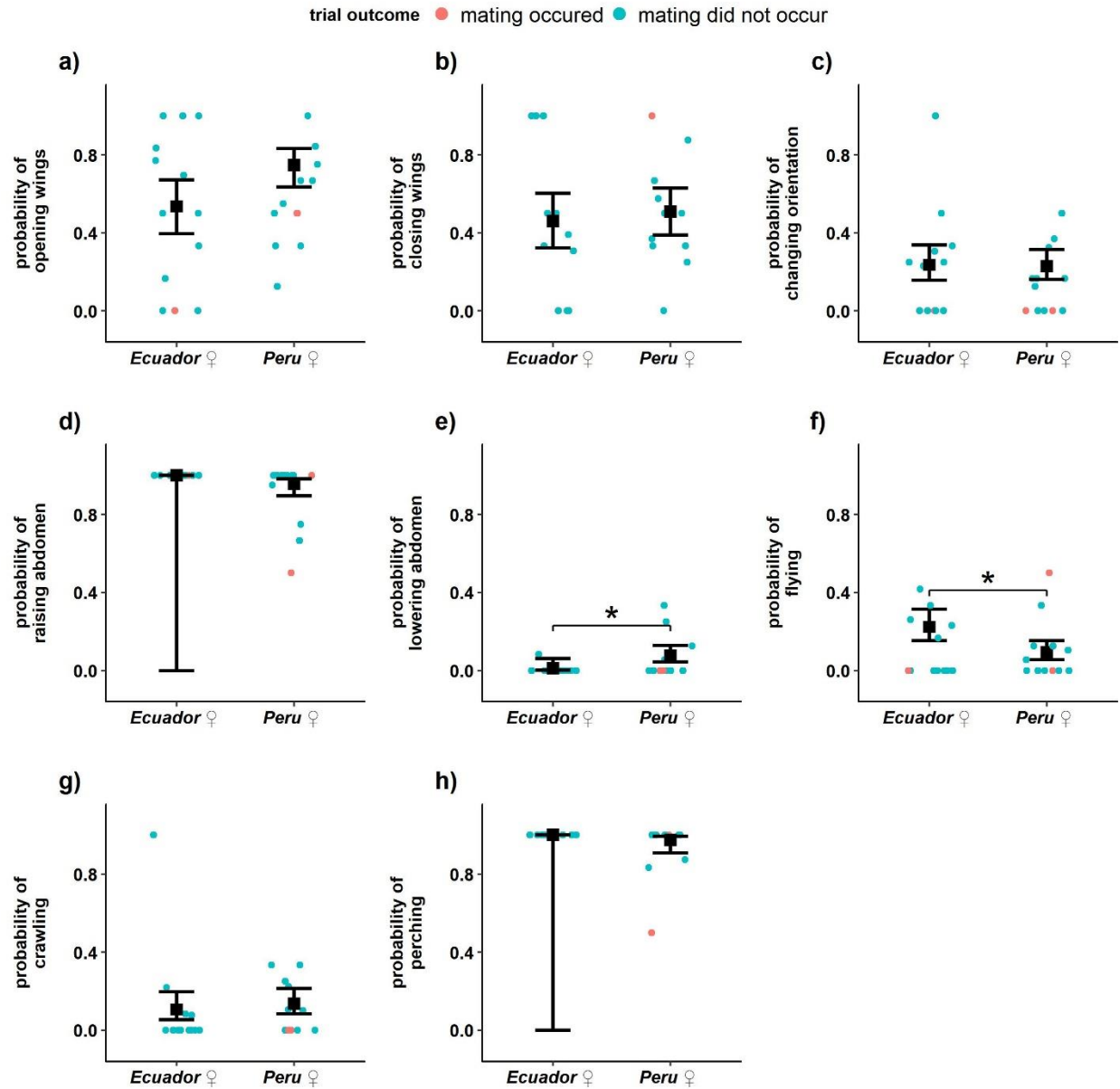
Figure 11: Responses of *H. himera* females from Ecuador and Peru to the courtship of *H. erato* males.

A-H. Effects of female population of female *H. himera* response to the courtship of *H. erato* males predicted by quasibinomial models. Black squares represent the point estimate and the black bars are 95% CI. Individual dots represent individual female responses calculated as the number of courtship minutes a female responded with a particular behavior divided by total number of courtship minutes.

Table 9: Models of *H. himera* female behavior towards courtship of male *H. erato* by female population.

	Opened Wings	Closed Wings	Changed Orientation	Abdomen lowered	Abdomen raised	Perched	Flying	Crawling
(Intercept)	0.55 (0.30)	-0.17 (0.28)	-1.18 (0.24)***	-4.43 (0.82)***	21.61 (3309.92)	21.61 (3634.89)	-1.25 (0.22)***	-2.13 (0.35)***
female_population H. himera_Peru	-0.18 (0.40)	0.20 (0.37)	-0.04 (0.32)	1.94 (0.86)*	-18.49 (3309.92)	-17.97 (3634.89)	-1.03 (0.35)***	0.28 (0.44)
Deviance	46.52	47.38	27.45	13.59	14.43	13.75	20.18	21.68
Num. obs.	25	25	25	25	25	25	25	25

***p < 0.001, **p < 0.01, *p < 0.05

Table 10: Models of *H. erato* female response to courtship of male *H. himera* by male population.

	Opened Wings	Closed Wings	Changed Orientation	Abdomen lowered	Abdomen raised	Perched	Flying	Crawling
(Intercept)	0.14 (0.28)	0.25 (0.23)	-1.08 (0.31)**	-3.12 (0.86)**	2.96 (0.67)***	3.31 (0.43)***	-2.14 (0.42)***	-2.06 (0.42)***
male_population H. himera_Peru	0.94 (0.38)*	-0.43 (0.30)	0.34 (0.39)	1.17 (0.96)	-0.43 (0.81)	2.06 (1.05)	-0.05 (0.54)	-0.07 (0.55)
Deviance	78.93	58.08	76.62	77.90	58.77	11.69	45.93	59.03
Num. obs.	29	29	29	29	29	29	29	29

***$p < 0.001$, **$p < 0.01$, *$p < 0.05$
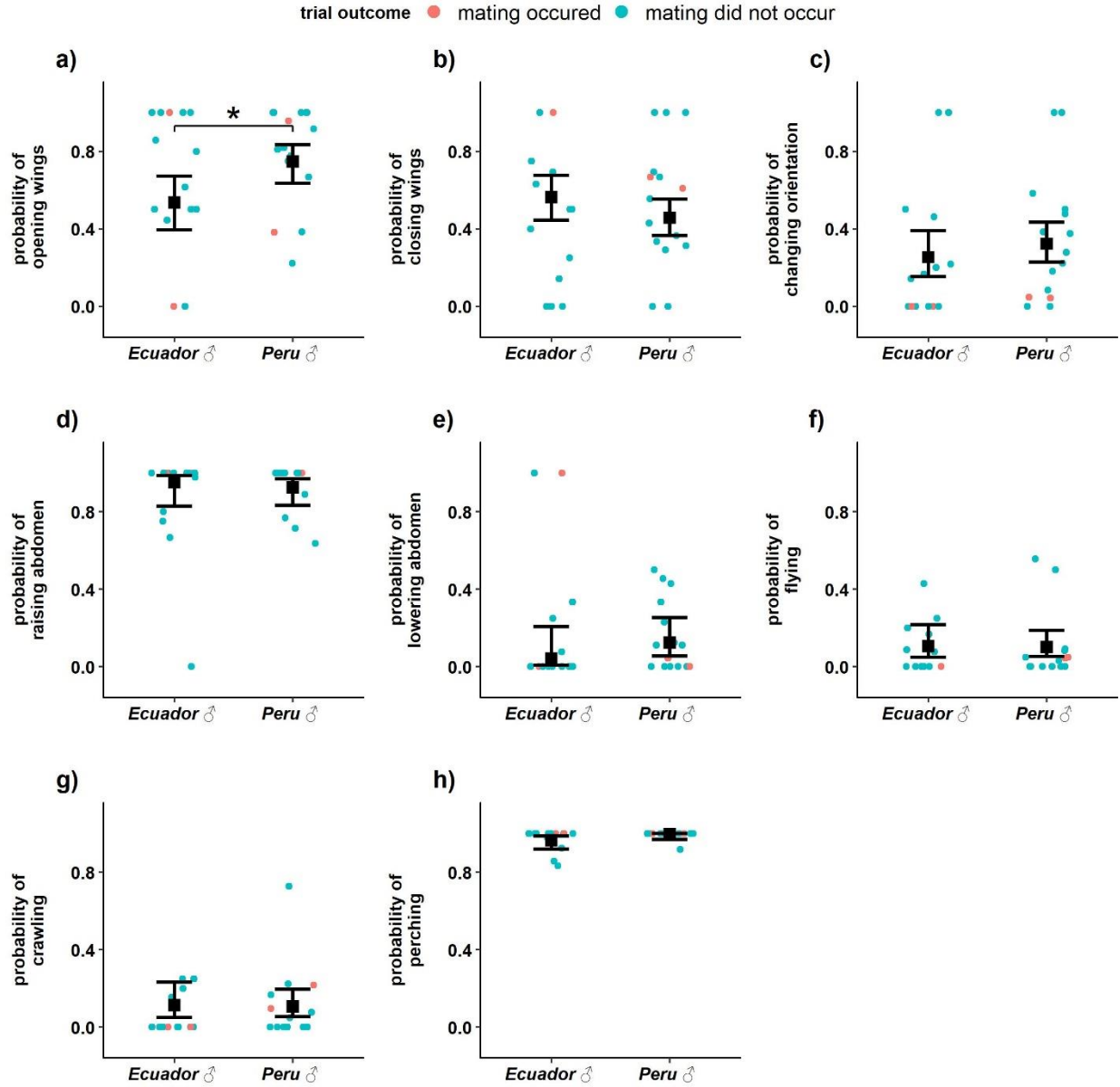
Figure 12: Responses of *H. erato* females to courtship from *H. himera* Ecuador and Peru to the courtship by *H. erato* males.

A-H. Effects of male population of female *H. erato* response to the courtship of *H. himera* males predicted by quasibinomial models. Black squares represent the point estimate and the black bars are 95% CI. Individual dots represent individual female responses calculated as the number of courtship minutes a female responded with a particular behavior divided by total number of courtship minutes. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$
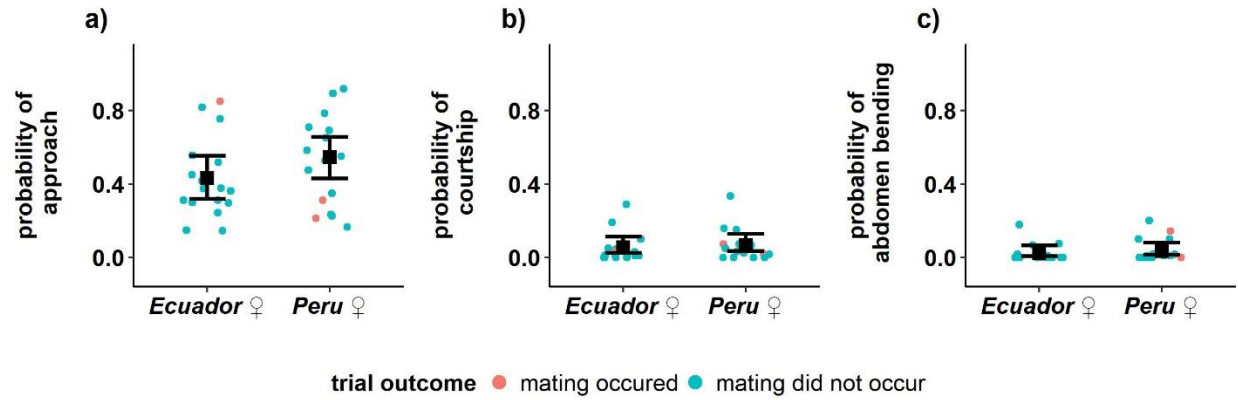
Figure 13: Behavior of *H. erato* males towards female *H. himera* from Ecuador and Peru.

A-C. Effects of female population on male *H. erato* behavior towards *H. himera* females predicted by quasibinomial models. Black squares represent the point estimate and the black bars are 95% CI. Individual dots represent experimental data calculated as the number of courtship minutes males courted divided by total number of minutes. ***p < 0.001, **p < 0.01, *p < 0.05

Table 11: Models of *H. erato* male behavior towards female *H. himera* by female population.

	approach	courtship	abdomen bending
(Intercept)	-0.27 (0.24)	-2.86 (0.39)***	-3.78 (0.54)***
female_populationH. himera_Peru	0.46 (0.33)	0.25 (0.52)	0.47 (0.69)
Deviance	733.06	313.66	235.95
Num. obs.	33	33	33

***p < 0.001, **p < 0.01, *p < 0.05
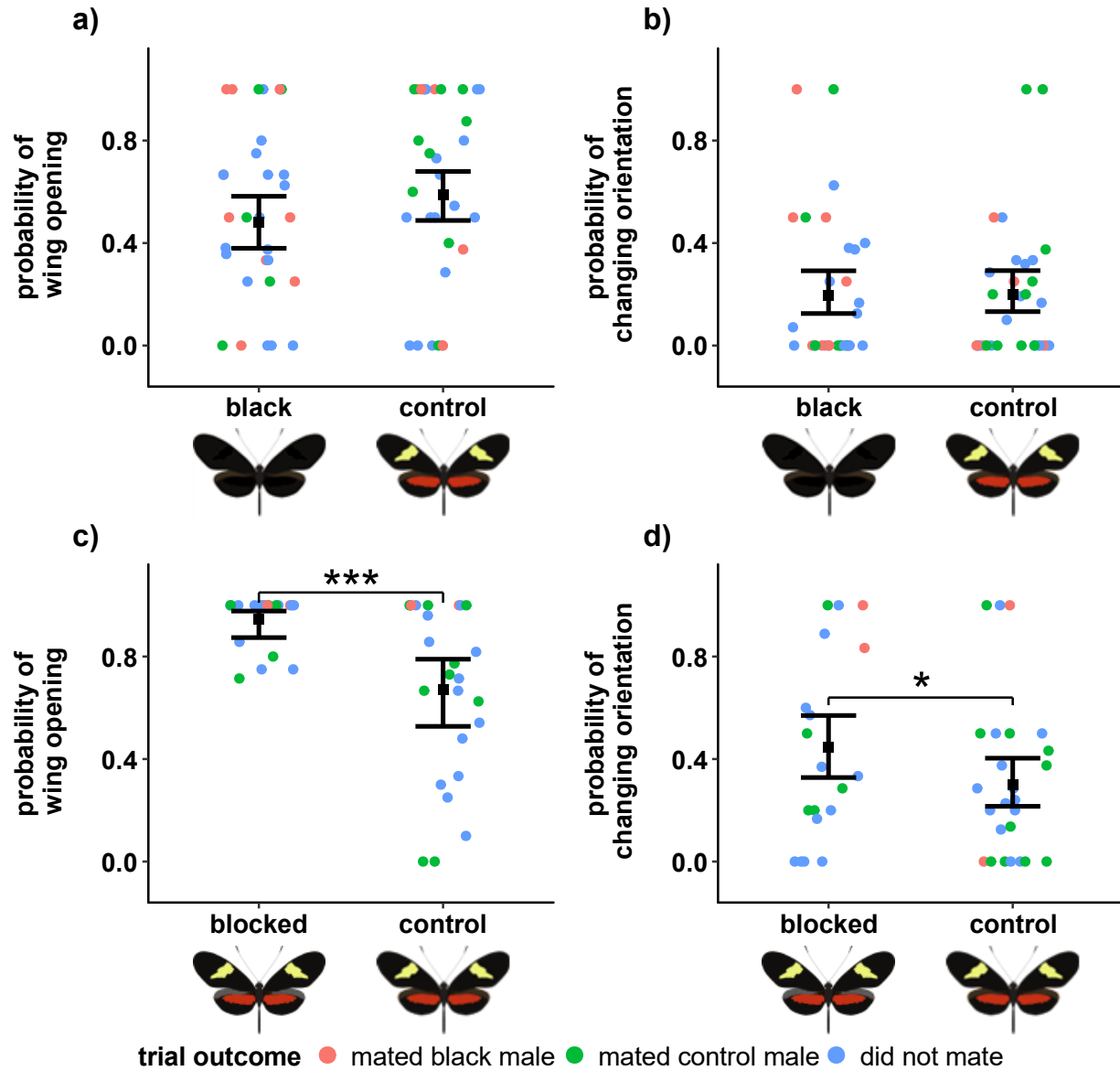
Figure 14: Female response to courtship of males with normal and altered color and pheromones.

A-B Effects of male color blocking and C-D pheromone blocking on female response to courtship in H. himera predicted by quasibinomial models. Black squares represent the point estimate and the black bars are 95% CI. Individual dots represent experimental data calculated as the number of courtship minutes where females responded with a behavior divided by the number of courtship minutes. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$

Table 12: Models of female response behaviors to courtship of males with normal and altered color pattern.

|	wing opening	wing closing	changing orientation	abdomen lowered	abdomen raised	flying	crawling	perched
(Intercept)	-0.08 (0.21)	1.21 (0.26)***	-1.42 (0.27)***	9.00 (4.18)*	-10.12 (2.94)***	-1.55 (0.25)***	-2.87 (0.52)***	216.89 (5686987.65)
malecontrol	0.43 (0.25)	-0.11 (0.30)	0.03 (0.29)	34.23 (4926398.64)	1.56 (1.42)	0.36 (0.30)	0.28 (0.47)	-205.73 (5686987.65)
AIC	171.76	156.94	136.60	19.46	35.31	139.78	105.26	12.76
BIC	178.19	163.37	143.03	25.89	41.74	146.21	111.69	19.19
Log Likelihood	-82.88	-75.47	-65.30	-6.73	-14.66	-66.89	-49.63	-3.38
Num. obs.	63	63	63	63	63	63	63	63
Num. groups: trial	36	36	36	36	36	36	36	36
Var: trial (Intercept)	0.25	0.56	0.20	45.25	53.52	0.18	1.01	98.04

***p < 0.001, **p < 0.01, *p < 0.05
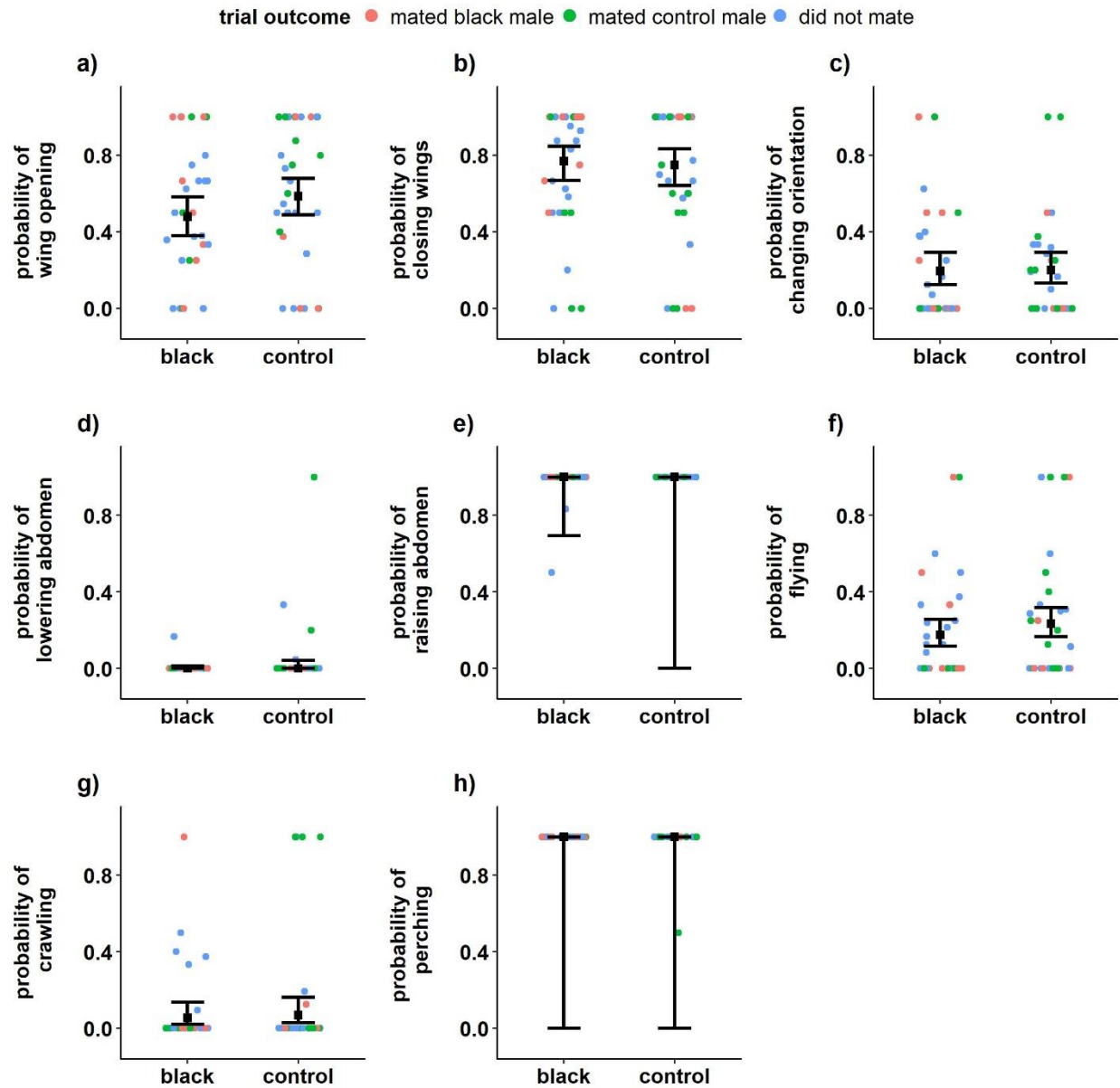
Figure 15: Female response to courtship of males with normal and altered wing color.

A-H. Effects of male color blocking on female response to courtship in H. himera predicted by quasibinomial models. Black squares represent the point estimate and the black bars are 95% CI. Individual dots represent experimental data calculated as the number of courtship minutes where females responded with a behavior divided by the number of courtship minutes. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$
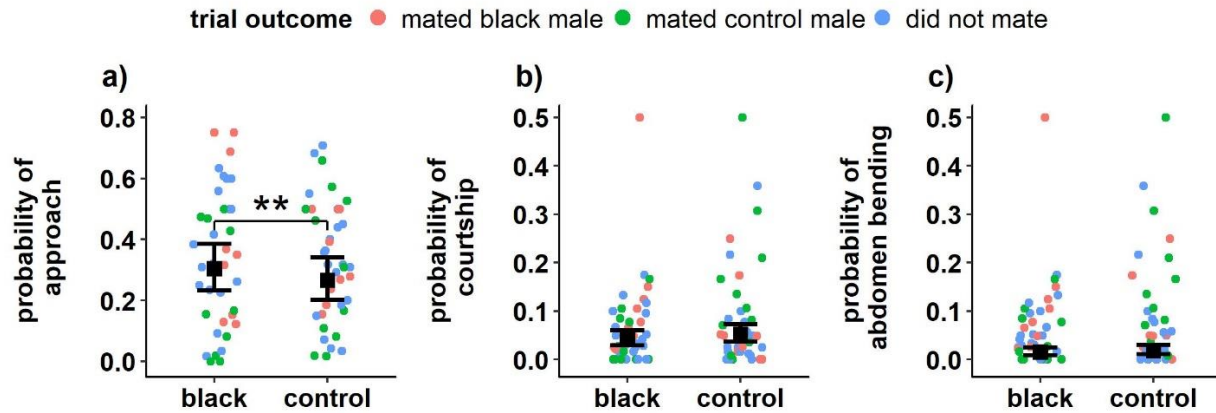
Figure 16: Male behavior by treatment in color blocking trials

A-C Effects of male treatment in color blocking trials on male behavior towards females predicted by quasibinomial models. Black squares represent the point estimate and the black bars are 95% CI. Individual dots represent experimental data calculated as the number of courtship minutes divided by total number of minutes. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$

Table 13: Models of male behaviors by treatment in color blocking trials.

	approach	courtship	abdomen bending
(Intercept)	-0.83 (0.18)***	-3.12 (0.19)***	-4.16 (0.26)***
malecontrol	-0.19 (0.06)**	0.21 (0.12)	0.19 (0.19)
AIC	755.02	402.76	305.80
BIC	762.09	409.83	312.87
Log Likelihood	-374.51	-198.38	-149.90
Num. obs.	78	78	78
Num. groups: trial	38	38	38
Var: trial (Intercept)	1.14	0.95	1.40

***p < 0.001, **p < 0.01, *p < 0.05

Table 14: Models of female response behaviors to courtship of males with normal and blocked androconial pheromones

|	wing opening	wing closing	changing orientation	abdomen lowered	abdomen raised	flying	crawling	perched
(Intercept)	2.85 (0.47)***	0.44 (0.34)	-0.22 (0.25)	2.75 (0.64)***	-5.60 (1.29)***	-0.66 (0.25)**	-0.60 (0.22)**	4.55 (1.58)**
malecontrol	-2.14 (0.43)***	0.73 (0.28)**	-0.62 (0.26)*	-0.41 (0.38)	1.82 (1.14)	-0.78 (0.28)**	-0.75 (0.25)**	1.16 (0.69)
AIC	121.47	155.05	156.46	124.47	55.86	149.53	138.87	70.48
BIC	126.82	160.40	161.81	129.82	61.21	154.89	144.22	75.84
Log Likelihood	-57.74	-74.52	-75.23	-59.24	-24.93	-71.77	-66.43	-32.24
Num. obs.	44	44	44	44	44	44	44	44
Num. groups: trial	27	27	27	27	27	27	27	27
Var: trial (Intercept)	1.21	1.44	0.53	4.93	2.22	0.42	0.17	7.85

***$p < 0.001$, **$p < 0.01$, *$p < 0.05$
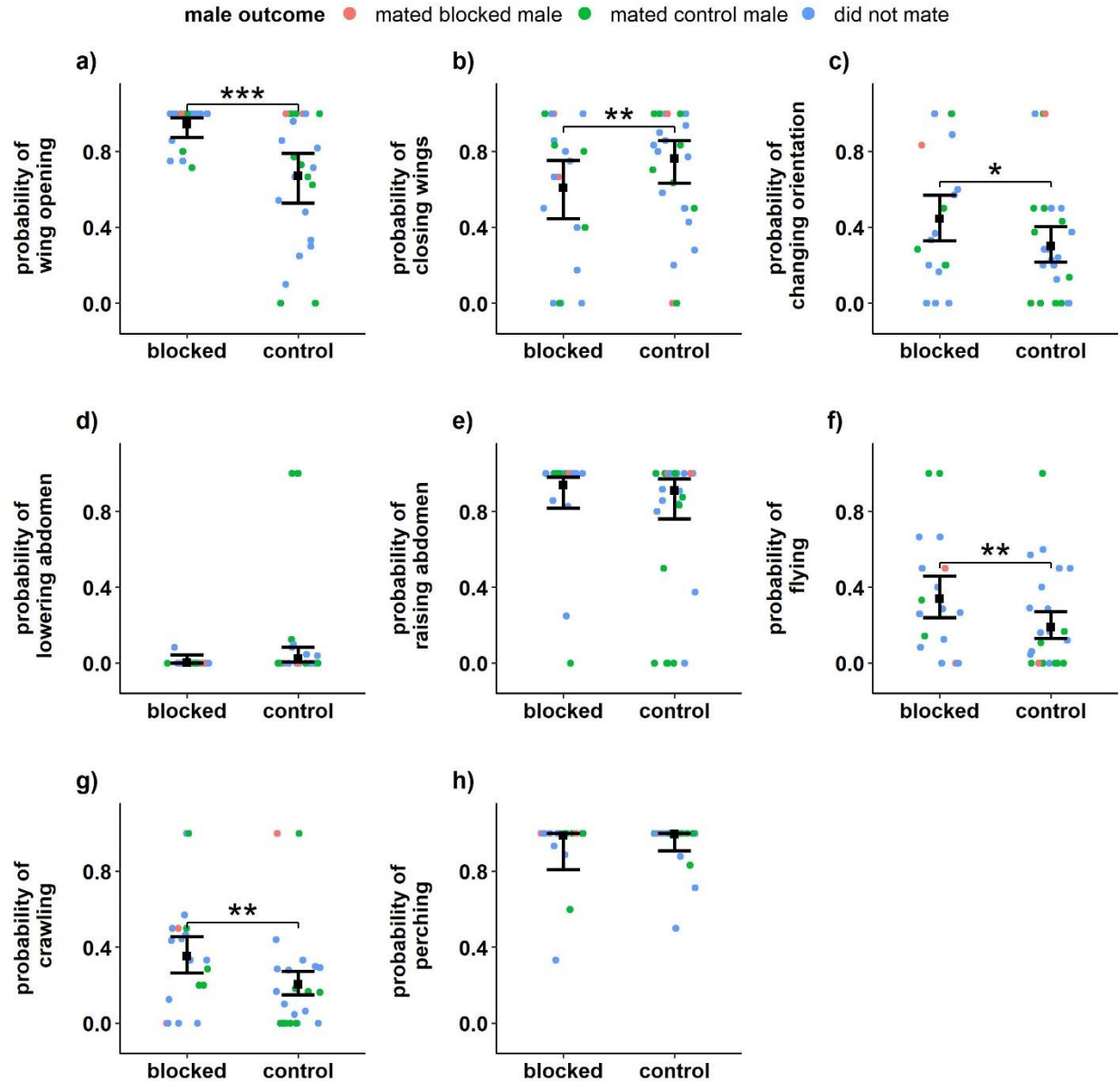
Figure 17: Female response to courtship of males with normal and blocked pheromones.

A-H. Effects of male pheromone blocking on female response to courtship in H. himera predicted by quasibinomial models. Black squares represent the point estimate and the black bars are 95% CI. Individual dots represent experimental data calculated as the number of courtship minutes where females responded with a behavior divided by the number of courtship minutes. ***p < 0.001, **p < 0.01, *p < 0.05

**Table 15:** Models of male behaviors with normal and blocked androconial pheromones.

	approach	courtship	abdomen bending
(Intercept)	-2.98 (0.29)***	-3.23 (0.27)***	-3.83 (0.31)***
malecontrol	0.49 (0.10)***	0.61 (0.11)***	0.07 (0.16)
AIC	409.51	424.06	303.79
BIC	415.89	430.44	310.17
Log Likelihood	-201.76	-209.03	-148.89
Num. obs.	62	62	62
Num. groups: trial	31	31	31
Var: trial (Intercept)	2.21	1.70	2.00

***$p < 0.001$, **$p < 0.01$, *$p < 0.05$
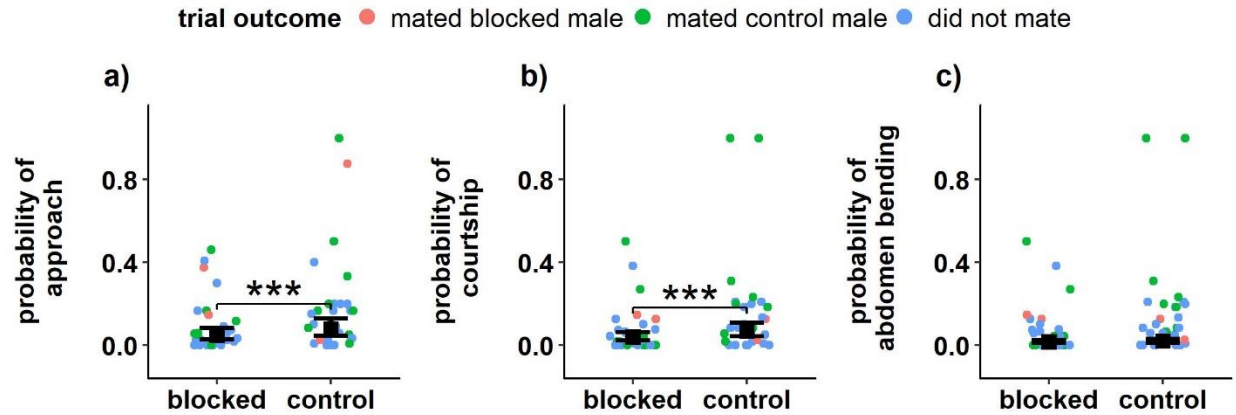
Figure 18: Male behavior by treatment in pheromone blocking trials

A-C Effects of male treatment in pheromone blocking trials on male behavior towards females predicted by quasibinomial models. Black squares represent the point estimate and the black bars are 95% CI. Individual dots represent experimental data calculated as the number of courtship minutes divided by total number of minutes. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$
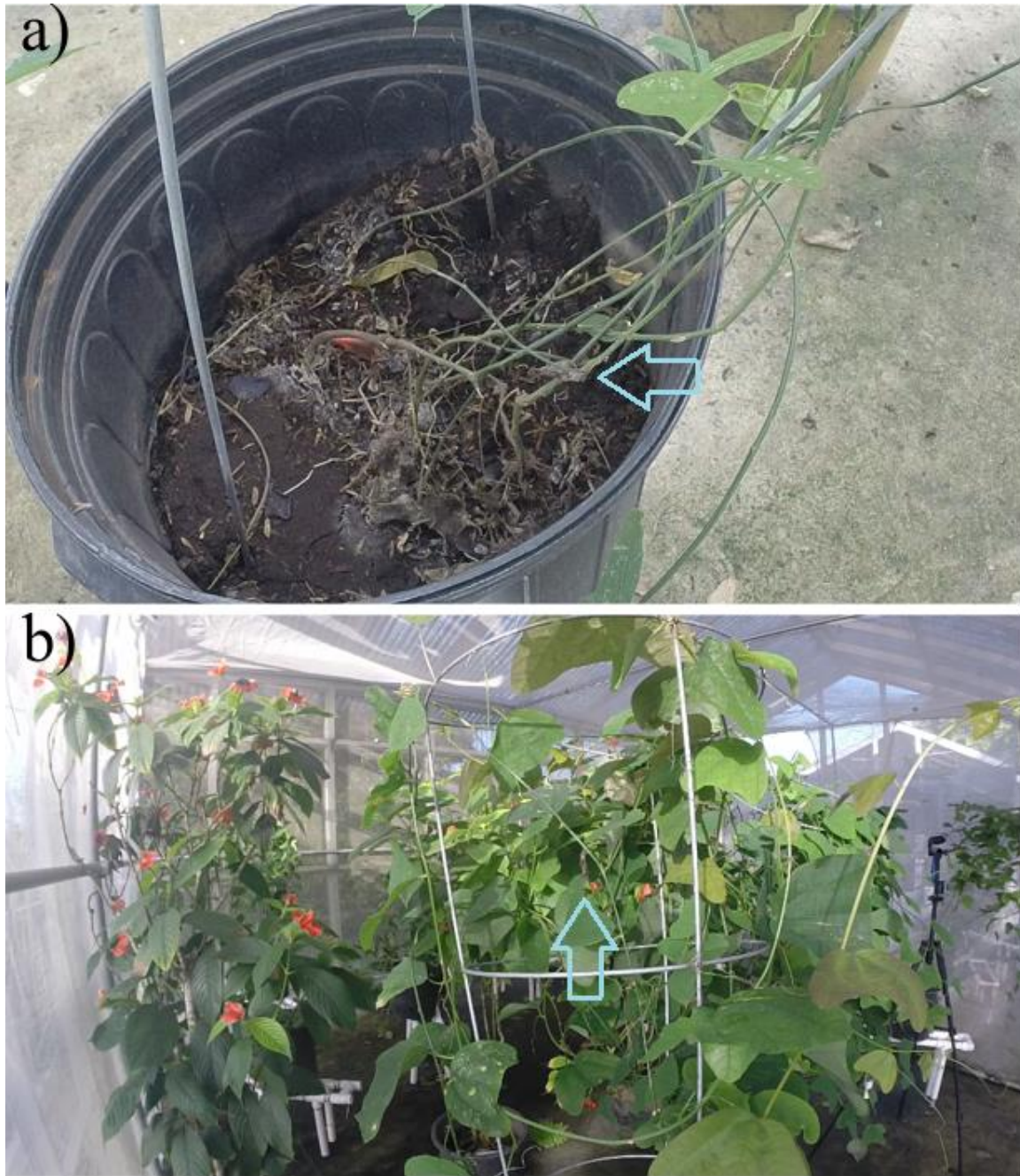
Figure 19: Typical pupation site selected by female larvae

Arrows indicate the location of the pupae of a) *H. himera* and b) *H. erato cyrbia.*
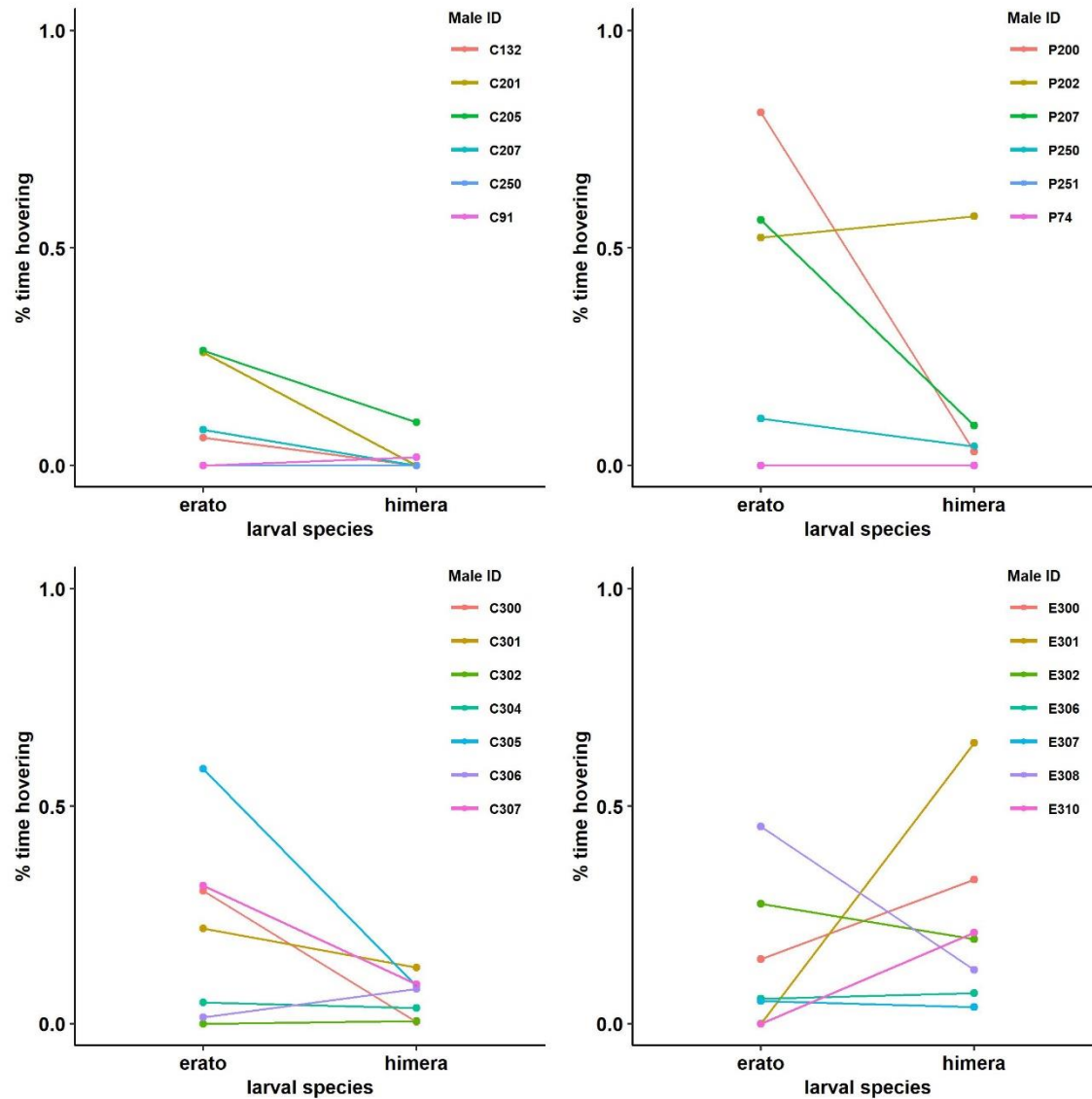
Figure 20: Percent of time spent hovering larvae by species

Top left: *H.erato* males in experiment 1 in which *H.himera* males and female larva were from Peru population. Top right: *H.himera* in experiment 1. Bottom left: *H.erato* males in experiment 2 in which *H.himera* males and female larva were from Ecuador population. Bottom right: *H.himera* in experiment 2. Each point represents the percent of time that an individual male spent hovering (total time of behavior divided by total time of all observations), the line segments merely connect points corresponding to the same individual. Only males with at least 5 days of observation with larvae of both species shown.

Figure 21: Percent of time spent hovering and sitting on pupae by species

Top left: *H.erato* males in experiment 1in which *H.himera* males and female larva were from Peru population. Top right: *H.himera* in experiment 1. Bottom left: *H.erato* males in experiment 2 in which *H.himera* males and female larva were from Ecuador population. Bottom right: *H.himera* in experiment 2. Each point represents the percent of time that an individual male spent hovering or sitting (total time of behavior divided by total time of all observations), the line segments merely connect points corresponding to the same individual. Only males with at least 5 days of observation with larvae of both species shown. The Y-axes are not identical across plots.

Table 16: Mating outcomes for females that emerged during pupal mating experiment.

Pupal mating was defined as any mating that occurred before the female flew from the pupa and adult mating any mating thereafter. *Three *H. himera* females remained unmated after two days in the cage.

Female Species	Total mated *H. erato*♂	Pupal matings *H. erato*♂	Adult *matings H. erato*♂	Total mated *H. himera* ♂	Pupal *H. himera* ♂	Adult *H. himera* ♂
H. erato	**5**	2	3	**5**	4	1
*H. himera**	**1**	1	0	**6**	2	4

Table 17: Comparison of candidate models for male approach of larva.

Model	K	AICc	Delta AICc	AICc weight	log-Likelihood
larval feeding and male size	5	1047.10	0.00	0.71	-518.52
larval feeding	4	1050.68	3.58	0.12	-521.32
Male species * larva_species * population	10	1051.48	4.37	0.08	-515.62
male size	4	1052.23	5.12	0.06	-522.09
density	4	1054.82	7.72	0.02	-523.39
null	3	1055.89	8.78	0.01	-524.93
larva species	4	1057.59	10.48	0.00	-524.77
male species	4	1057.90	10.80	0.00	-524.93
Male species + larva_species	5	1059.61	12.50	0.00	-524.77
Male species * larva_species	6	1060.62	13.52	0.00	-524.27

Table header spanning row: **Model selection based on AICc**

Table 18: Best candidate model for male approach to larvae.

	Model 1
(Intercept)	-18.12 (2.74)***
crawlerNO	0.38 (0.17)*
FW_average	1.75 (0.67)**
log(Total_Time_Partial)	1.40 (0.20)***
AIC	1047.04
BIC	1071.31
Log Likelihood	-518.52
Num. obs.	948
Num. groups: MALE_ID	46
Var: MALE_ID (Intercept)	0.22

***$p < 0.001$, **$p < 0.01$, *$p < 0.05$
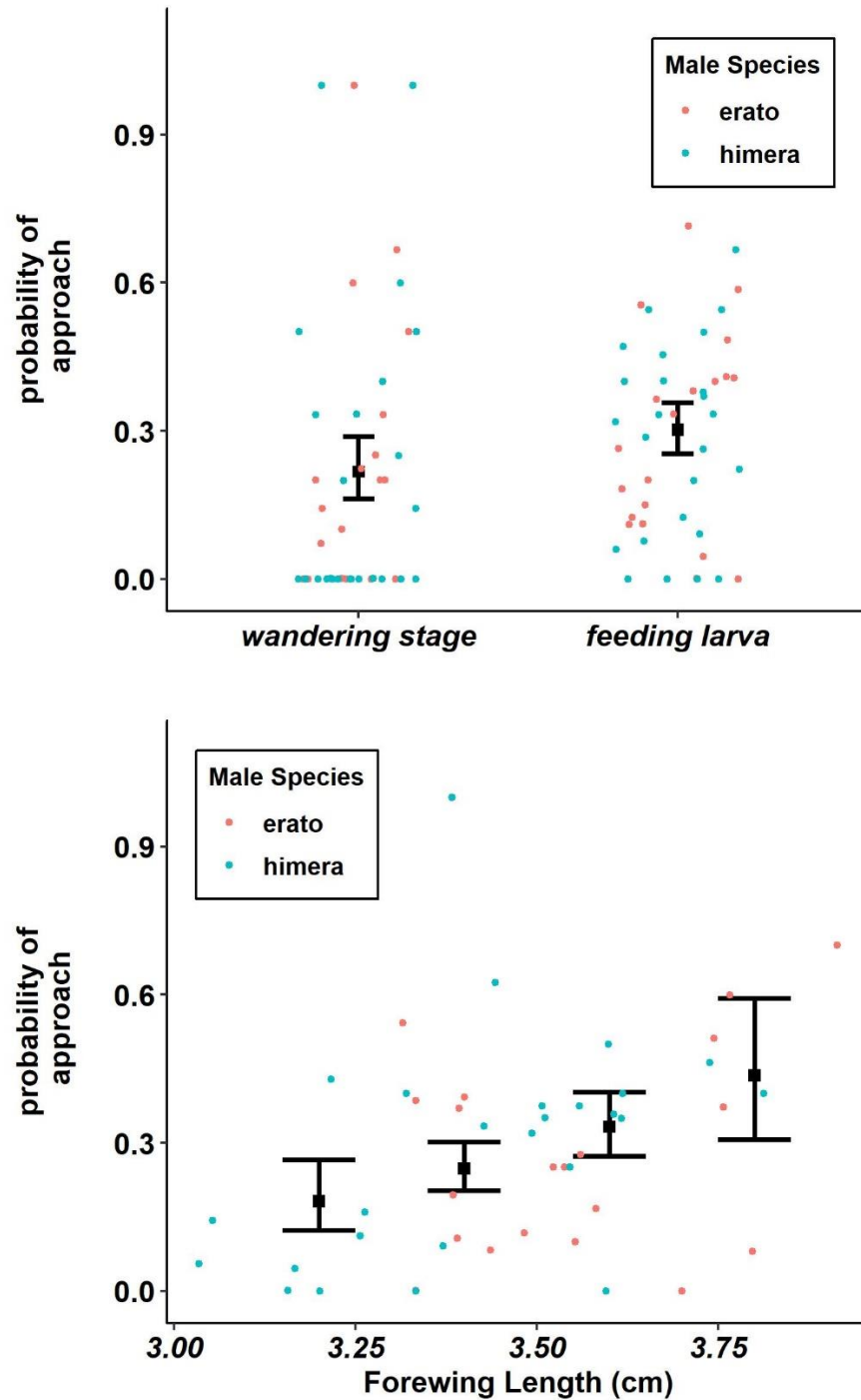
Figure 22: Effects of male size and larval feeding on male approach to larvae

Effects of male size and larval feeding on male approach to pupae based on binomial glmm. Black squares represent the point estimate and the black bars are 95% CI. The colored dots represent the number of observations where approaches occurred divide by total number of observations.
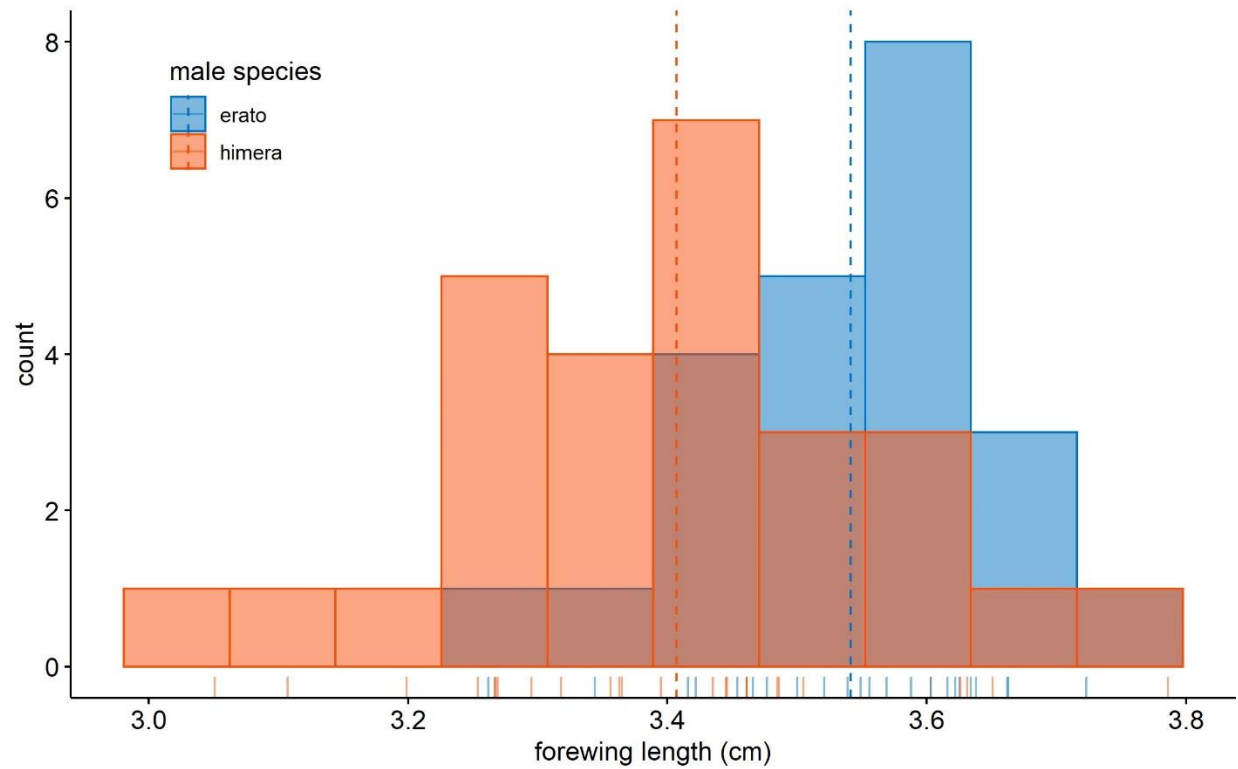
Figure 23: Histogram of male forewing length by species for pupal mating experiment

Frequency of experimental males within each size category by species. Dashed vertical lines are species means. Small vertical lines along X-axis represent an individual male's forewing length measurement.

Table 19: Comparison of candidate models for proportion of time male spent hovering larva.

Model selection based on AICc					
Model	**K**	**AICc**	**Delta AICc**	**AICc weight**	**log-Likelihood**
male species + larva species	5	-2315.63	0.00	0.58	1162.93
male species * larva species	5	-2314.06	1.57	0.26	1162.14
larva species	3	-2311.58	4.05	0.08	1158.83
male species * larva species * population	9	-2311.51	4.12	0.07	1165.09
male species	3	-2304.42	11.21	0.00	1155.25
null	2	-2300.84	14.79	0.00	1152.44
larval feeding	3	-2300.47	15.16	0.00	1153.28
male size	3	-2299.88	15.76	0.00	1152.98
larval feeding and male size	4	-2299.35	16.29	0.00	1153.75
density	3	-2298.86	16.77	0.00	1152.48

Table 20: Best candidate model for the proportion of time males spent around larvae.

	Model 1
(Intercept)	-4.98 (0.10)***
MALE_SPECIEShimera	0.25 (0.10)*
larva_specieshimera	-0.38 (0.10)***
Precision: (phi)	100.08 (10.36)***
Pseudo R^2	0.11
Log Likelihood	1161.75
Num. obs.	278

***$p < 0.001$, **$p < 0.01$, *$p < 0.05$

Figure 24: Effect of male and larval species on the time males spent around larvae

Effects of male and pupal species on male approach to pupae based on binomial glmm. Black squares represent the point estimate and the black bars are 95% CI. The colored dots represent the proportion of time for each individual male, calculated as the total amount of time spent divided by total observation times when male approached larvae.

Table 21: Comparison of candidate models for male approach of pupa

Model selection based on AICc					
Model	**K**	**AICc**	**Delta AICc**	**AICc weight**	**log-Likelihood**
male species * pupa species	6	1332.96	0.00	0.50	-660.45
pupa species	4	1334.81	1.85	0.20	-663.39
male species + pupa species	5	1335.10	2.14	0.17	-662.53
male species * pupa species*stage of pupa	10	1336.39	3.43	0.09	-658.13
male species * pupa species*population	10	1337.71	4.75	0.05	-658.79
null	3	1377.21	44.26	0.00	-685.60
male species	4	1377.41	44.46	0.00	-684.69
emerged well	4	1377.48	44.52	0.00	-684.73
male observed pupa as larva	4	1378.67	45.71	0.00	-685.32
stage	4	1378.95	45.99	0.00	-685.46
male size	4	1379.04	46.09	0.00	-685.51
density	4	1379.17	46.22	0.00	-685.57

Table 22: Best candidate model for male approach to pupae.

	Model 1
(Intercept)	-5.87 (1.04)***
MALE_SPECIEShimera	-0.10 (0.21)
larva_specieshimera	-0.71 (0.22)**
log(Total_Time_Partial)	0.58 (0.13)***
MALE_SPECIEShimera:larva_specieshimera	-0.61 (0.35)
AIC	1334.98
BIC	1378.13
Log Likelihood	-659.49
Num. obs.	1626
Num. groups: MALE_ID	49
Var: MALE_ID (Intercept)	0.22
Var: MALE_ID larva_specieshimera	0.27
Cov: MALE_ID (Intercept) larva_specieshimera	-0.05

***$p < 0.001$, **$p < 0.01$, *$p < 0.05$

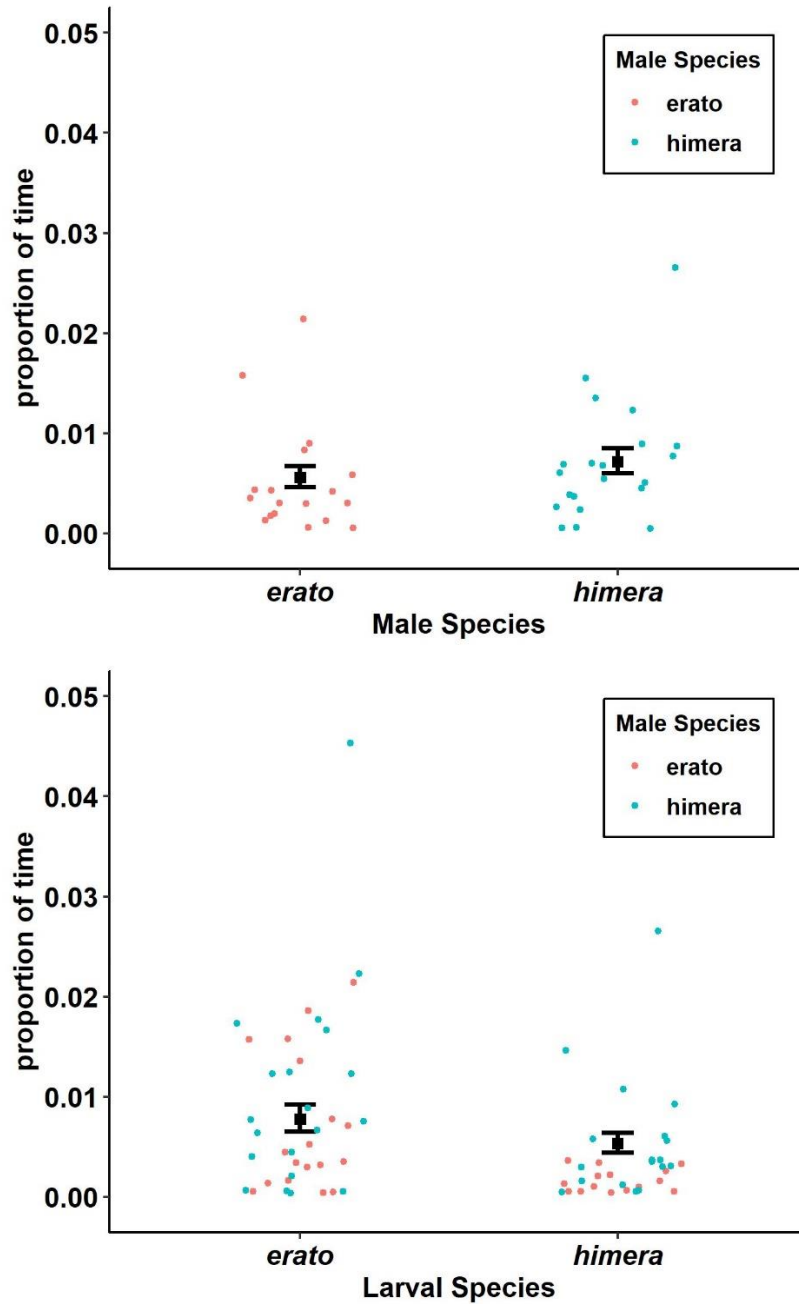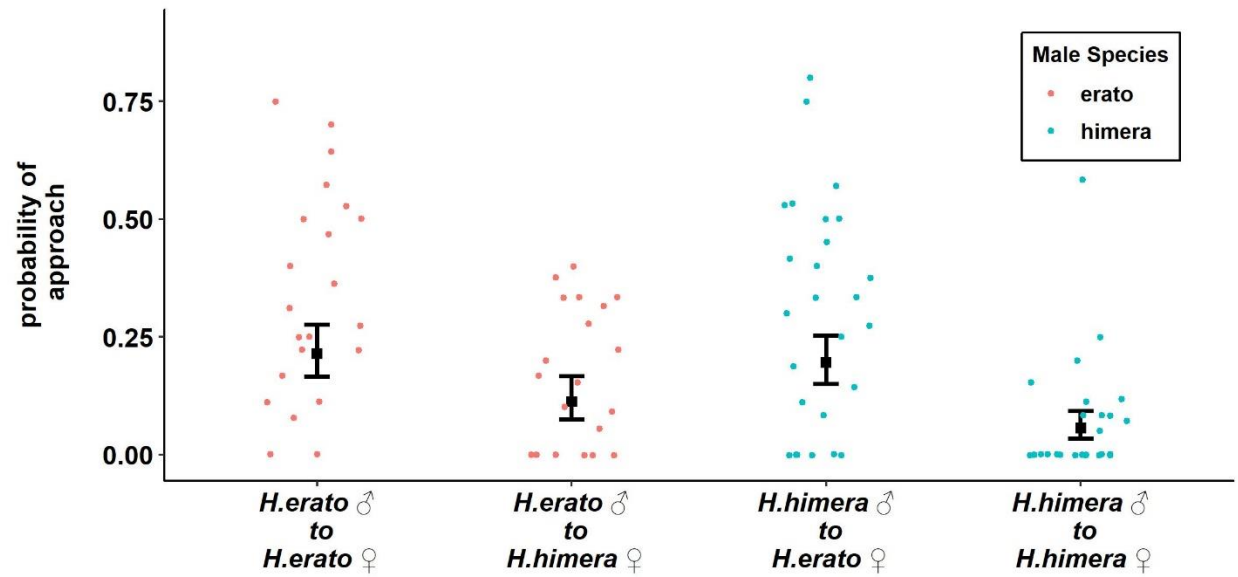**Figure 25:** Effect of male and larval species on male approach to pupae

Effects of male and pupal species on male approach to pupae based on binomial glmm. Black squares represent the point estimate and the black bars are 95% CI. The colored dots represent the probability of approach for each individual male, calculated as the total number of larva male approached divided by the total number of pupae male experienced.

Table 23: Comparison of candidate models for proportion of time males spent hovering pupa.

Model	K	AICc	Delta AICc	AICc weight	log-Likelihood
Model selection based on AICc					
null	2	-1942.43	0.00	0.24	973.24
male observed pupa as larva	3	-1941.37	1.06	0.14	973.73
emerged well	3	-1940.92	1.51	0.11	973.51
stage	3	-1940.52	1.92	0.09	973.31
density	3	-1940.52	1.92	0.09	973.31
male species	3	-1940.48	1.95	0.09	973.29
male size	3	-1940.43	2.00	0.09	973.26
larva species	3	-1940.39	2.04	0.09	973.24
male and larva species without male preference	4	-1938.43	4.00	0.03	973.30
male preference	5	-1936.74	5.69	0.01	973.49
male preference by age of pupa	9	-1930.87	11.56	0.00	974.81
male preference by population	9	-1928.65	13.78	0.00	973.69

Table 24: Outcomes of male-male interaction by species.

Counts of the outcomes of male-male interactions by the species of males. Absolute counts are not directly comparable between rows, as male-male interactions could only occur when one male was already hovering or sitting a larva or pupa and thus observation time varied. Description of behaviors: Displaced = approaching male caused resident male to leave, and approaching male followed him away from larva or pupa; Replaced= approaching male caused resident male to leave, and approaching male remained at larva or pupa; Not displaced= resident male did not leave when male approached, and approaching male flew away; Joined= resident male did not leave when male approached, and both males continued hovering larva or pupa.

Approaching Male	Resident male	Displaced	Replaced	Total-resident left	Not displaced	Joined	Total-resident stayed
H. erato	*H. erato*	30	6	**36**	18	0	**18**
H. himera	*H. erato*	13	1	**14**	9	5	**14**
H. himera	*H. himera*	18	0	**18**	23	2	**25**
H. erato	*H. himera*	16	1	**17**	13	3	**16**

Table 25: Candidate model comparisons for the frequency of male-male interactions. "male 1" refers to first male at larva or pupa and Male 2 is the male which approached male 1.

Model	K	AICc	Delta AICc	AICc weight	log-Likelihood
Model selection based on AICc					
male 1 species * male 2 species + stage of female	6	571.11	0.00	1.00	-279.46
male 1 + male 2 species + stage of female	5	596.29	25.18	0.00	-293.08
male 1 * male 2 species	5	602.89	31.78	0.00	-296.38
male 2 species	3	625.53	54.42	0.00	-309.74
male 1 + male 2 species	4	626.41	55.29	0.00	-309.16
stage of female	3	637.68	66.57	0.00	-315.82
null	2	669.70	98.59	0.00	-332.84
male 1 species	3	671.56	100.44	0.00	-332.75

Table 26: Best candidate model of the rate of male-male interactions.

The term 'Male2_species' denotes the species of the male approaching. The term 'Male1_species' denotes the species of the resident male which was approached.

	candidate model
(Intercept)	-4.52 (0.31)***
Male1_specieshimera	-2.22 (0.28)***
Male2_species himera	-1.56 (0.43)***
stagepupa	-1.26 (0.22)***
Male1_specieshimera: Male2_species himera	2.11 (0.42)***
AIC	570.93
BIC	595.69
Log Likelihood	-279.46
Num. obs.	458
Num. groups: MALE_ID	48
Var: MALE_ID (Intercept)	0.87

***$p < 0.001$, **$p < 0.01$, *$p < 0.05$

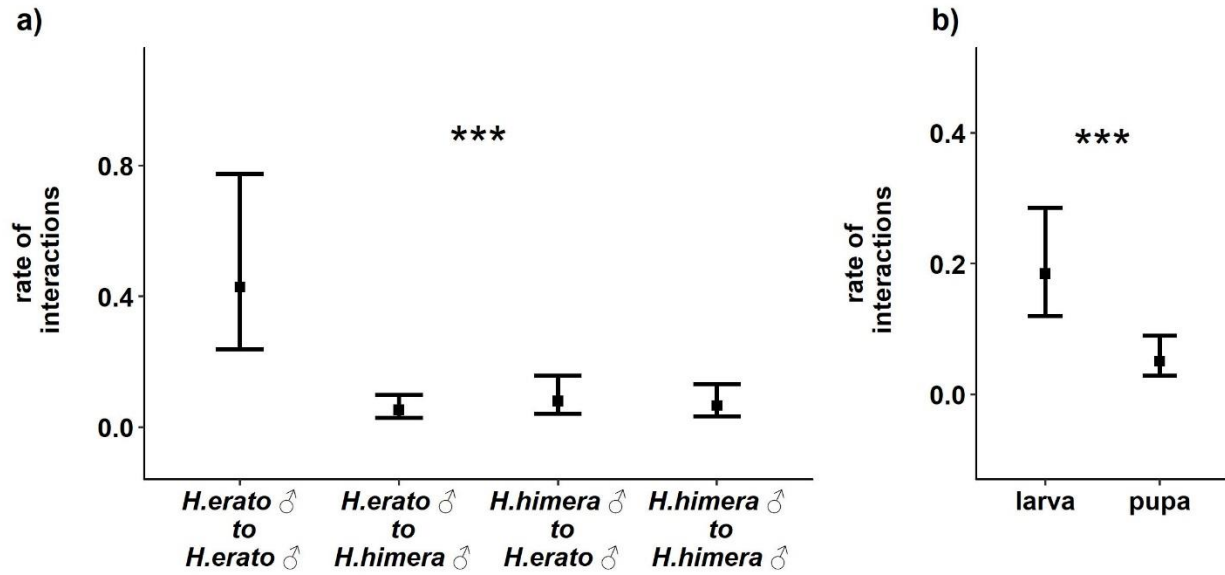**Figure 26:** Predictors of the frequency of male-male interaction.

In Figure a) the effect of male species on the rate (count offset by the log(time)) of male-male interactions. In the x-axis, the male who is doing the approaching is listed first, followed by the species of the male being approached. In figure b) the effect of the female being a larva or pupa on the rate of male-male approaches. Black squares represent the point estimate and the black bars are 95% CI. [***]$p < 0.001$, [**]$p < 0.01$, [*]$p < 0.05$