

LRH: Davison, McMillan, Griffin, Jiggins and Mallet

RRH: Behavior and physiology in two *Heliconius* sp.

**Behavioral and Physiological Differences Between Two Parapatric
Heliconius Species¹**

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ABSTRACT

The behavior and physiology of two parapatric sibling species, *Heliconius erato cyrbia* Godt. and *H. himera* Hew., were investigated to assess whether environmental adaptation enables stable morphological, genetic, and ecological differences to exist in the face of hybridization. Morning and evening activity, egg production, and larval developmental time of *H. himera* and *H. erato* in insectaries were recorded, using individuals collected in allopatry and from sympatry, in a hybrid zone where the species overlap. Studies were performed at ambient conditions within the natural range of *H. himera*. *H. himera* was considerably more active than *H. erato*, flying earlier in the morning and later in the evening, even when both species were collected in sympatry. Similarly, *H. himera* laid more eggs, and the hatched larvae developed more rapidly. The results suggest that physiological constraints are an important selective force which may have been important in speciation, and which counteracts hybridization in the maintenance of the *H. himera* / *H. erato* contact zone. Ecological selection, arising from adaptation to low temperatures, may help explain the competitive exclusion of *H. erato* by *H. himera* in the drier, cooler montane habitat favored by the latter species.

RESUMEN

Se investiga la conducta y fisiología de dos especies gemelas parapátricas, *Heliconius erato cyrbia* Godt. y *H. himera* Hew., a fin de dilucidar como la adaptación al ambiente puede contribuir a diferencias morfológicas, genéticas y ecológicas a pesar de la hibridación de las mismas. Se registró la actividad matinal y vespertina, la producción de huevos y tiempo de desarrollo larvario de *H. himera* y *H. erato* en insectarios utilizando individuos recolectados en alopatria y en zonas de hibridación donde ambas especies se solapan. Los estudios se realizaron en condiciones ambientales dentro del rango natural de *H. himera*. *H. himera* es

considerablemente más activa que *H. erato* volando desde mas temprano por la mañana y hasta más tarde al atardecer, incluso cuando ambas especies se recolectan en zonas de simpatria. Igualmente *H. himera* pone mayor número de huevos, y el desarrollo larvario es mas rápido. Los resultados sugieren que restricciones fisiológicas pueden actuar a modo de fuerza selectiva importante que contrarreste la hibridación en el mantenimiento de *H. himera* y *H. erato* en zonas de solapamiento. Una selección ecológica en relación a una adaptación a bajas temperaturas puede ayudar a explicar la exclusión competitiva de *H. erato* por *H. himera* en habitats montanos más frios y secos que favorecen a esta última especie.

Key words: Lepidoptera; competitive exclusion; hybridization; hybrid zone; parapatry; speciation.

SPECIATION IS MOST USEFULLY STUDIED BY investigating intermediate cases, races or species that maintain morphological, genetic, and ecological differences despite hybridization (Endler 1977, Szymura & Barton 1986, 1991, Barton & Hewitt 1989, Hewitt 1989, Harrison 1993). Reproductive differences between sibling species have been intensively studied (Coyne & Orr 1989), but much less is known about the role of ecological adaptation in speciation. Parapatric contact zones between geographic races and species are very common, especially in the tropics (MacArthur 1972, Brown 1979, Barton & Hewitt 1989, Harrison 1993). Bull (1991) reports that many coincide with ecotones, which suggests that ecological factors are important in maintaining species differences. Species differences can be maintained in the face of liberal gene flow, implying that ecological differences can stabilize adaptive peaks (Grant & Grant 1992). In this study we assess the extent of ecological differentiation between two butterfly sibling species, *Heliconius erato cyrba* Godt. and *Heliconius himera* Hew., and their hybrids (Descimon & Mast de Maeght 1984).

Heliconius erato is common in light gaps and second growth forest from sea level to 1500m throughout much of the Neotropics. Wing pattern variation within the unpalatable *H. erato* is extensive and there are more than 30 different racial types, each a Müllerian mimic of a similar race of *H. melpomene* (Brown 1981, Brower 1994). Parapatric races often differ strikingly in wing patterns, but mate randomly in contact zones composed largely of hybrids (Mallet 1993). Müllerian mimicry between species and narrow boundaries between color pattern races of *H. erato* are maintained by frequency-dependent predation against rare warning color variants (Turner 1971, Benson 1982, Mallet 1986, 1993, Mallet *et al.* 1990).

Heliconius himera replaces its sibling species *H. erato* in dry forest and thorn scrub of southern Ecuador and north-east Peru (Brown 1979, Sheppard *et al.* 1985). Contact zones between *H. himera* and three distinct color pattern races of *H. erato* have been located (Jiggins *et al.* 1996). In the Río Puyango drainage (Loja and El Oro provinces of Ecuador) a

relatively abrupt transition occurs over approximately 5km from *H. erato cyrbia* to *H. himera*. Unlike the contact zones between races of *H. erato*, hybrids between *H. himera* and *H. erato* are much rarer than the pure species, making up only 8-10% of the population (Jiggins *et al.* 1996). These hybrids survive well, and mate freely with either parental type (McMillan *et al.* 1997); however, the transition between *H. erato* and *H. himera* is considerably narrower than in previously studied contact zones between color pattern races of *H. erato*, which implies stronger selection (Jiggins *et al.* 1996, Mallet *et al.* 1998). Frequency dependent selection by predators against rare hybrid phenotypes, similar to the mimetic selection known from inter-racial hybrid zones of *H. erato* (Turner 1971, Benson 1982, Mallet 1986, 1993, Mallet *et al.* 1990), is probably important in the *erato* / *himera* contact zone, but the larvae of the two species use identical larval hostplants in the genus *Passiflora*, and adults do not differ in oviposition preference (Jiggins *et al.* 1997a). Genetic differences between *H. himera* and *H. erato* are stable and there is little evidence of introgression at color pattern loci, allozyme markers, or mtDNA (Jiggins *et al.* 1997b). Extensive crosses have failed to show any hybrid inviability or sterility, and the scarcity of hybrids relative to pure forms is mainly caused by strong assortative mating, balanced by some unidentified form of ecological selection (McMillan *et al.* 1997, Mallet *et al.* 1998). The transition between *H. erato* and *H. himera* coincides very closely with a steep ecotone between wet forest and dry thorn scrub (Jiggins *et al.* 1996), and it therefore seems likely that adaptive environmental differences between the species (other than frequency dependent predation or host-plant adaptation) play a role in maintaining species differences and the position of the hybrid zone.

This paper describes experiments to investigate potential behavioral and ecological differences between *H. erato*, *H. himera* and their hybrids which could explain the narrow contact zones with little introgression, their position on an ecotone, and also the divergence

that led to speciation. Specifically, we examine differences in morning and evening activity, egg production, and larval developmental time.

METHODS

SOURCES OF BUTTERFLIES.- Butterflies were collected in Loja and El Oro provinces, southwest Ecuador (Jiggins *et al.* 1996). *Heliconius erato* were captured in two wet forest sites near Balsas and Piñas (altitude ~800-1000m), while *Heliconius himera* were collected from dry forest near Vilcabamba (altitude ~1500-1600m). These sites were far from the hybrid zone, i.e. allopatric. In addition, individuals of both species were collected in sympatry within the narrow contact zone. This region, near Chaguarpamba (altitude ~1000m), marks a rapid transition between wet and dry forest habitat types (Jiggins *et al.* 1996).

THE INSECTARIES.- After capture, butterflies were placed in envelopes and transported to Vilcabamba (30km south of Loja). During transit butterflies were fed on 10% sugar solution every 2-3 hours. Survival using this strategy was high. Butterflies were allowed to acclimatize in outdoor insectaries for 2-3 days prior to data collection. Each insectary was partitioned into six 1x1x2m³ cages that housed one or two butterflies (Jiggins *et al.* 1997a, McMillan *et al.* 1997). Individual cages were provided with ample artificial nectar, pollen resources, and larval food-plants. Artificial nectar consisted of a sugar solution displayed in small red and yellow plastic cups. Sugar solution was checked daily and replenished as necessary. In addition, fresh nectar and pollen resources (critical for egg production) were provided by cut *Lantana* sp. flowers. *Passiflora rubra* and *P. punctata*, the larval hosts of both *erato* and *himera* (Jiggins *et al.* 1996, 1997a) were provided for oviposition. Eggs were collected daily and larvae were raised individually in small plastic pots to prevent cannibalism. Developing larvae were fed ample new growth of *P. rubra* and *P. punctata* (Jiggins *et al.* 1997a). Larval containers were kept at ambient shade temperature, cleaned daily, and new food was added as needed. A small twig taped to the top of each pot provided a suitable pupation site. Pupae were then housed together by brood in larger plastic cages until emergence. Sex and date of

egg collection, pupation and eclosion were recorded for each individual. We avoided inbreeding by crossing only unrelated individuals (McMillan *et al.* 1997).

BEHAVIORAL OBSERVATIONS.- Within the outdoor insectaries we monitored butterflies for five behavioral criteria: 1) roosting- butterfly hanging in inverted position, wings together; 2) perching- butterfly at rest, upright, wings together; 3) sunning- butterfly at rest, upright, wings open at ~180°, facing sun; 4) flying; and 5) feeding. Clustered cages allowed the activity of up to 18 butterflies to be recorded simultaneously. Observations were carried out every 10 minutes from 07:10 to 11:00, and from 16:00 to 18:30 between October to December 1994. Except in unusual circumstances, butterflies were still roosting at 07:10 (sunrise was at ~06:00 to 06:20), and by 18:30 (sunset was at ~18:00) all had roosted again for the night.

The times of four behavioral events were recorded: 1) time of departure from the roost in the morning; 2) morning activity - time of second observation of flying or feeding. The *second* time of flying, rather than the time the butterfly actually left the roost, was used to indicate the earliest point in the day when adults were consistently active. Adults frequently left the roost, but then perched on the ground or sides of the cage for some time before flying again. In contrast, after the “second flying time” adults remained more or less continuously active; 3) evening activity - latest time that the butterflies were actively flying and feeding. Again, we used the time of the second to last observation period when adults were flying or feeding because *Heliconius* frequently remain perched for some time in the late afternoon, before slowly fluttering about and flying into the roost; 4) the time when adults roosted.

Most observations were carried out on females of the two species collected from allopatry in either wet forest (*H. erato*) or dry forest (*H. himera*) biotopes, but a smaller group

of males collected from the same locations, and a group of males of both species collected in sympatry from the Chaguarpamba contact zone were also included. Unfortunately, females were rare in the hybrid zone so studies with sympatric individuals could be done only with males.

PAIRWISE DESIGN.- Adult behavior and larval developmental time were clearly sensitive to temporal fluctuations in temperature, solar intensity, and rainfall. We therefore compared only individuals kept together and experiencing the same ambient conditions. For the behavioral observations, five types of pairwise comparison were made: 1) female *himera* against female *erato* (allopatric); 2) female allopatric *himera* against female F1; 3) female allopatric *erato* against female F1 (in both 2 and 3 the F1 was the offspring of a mating between *himera* and *erato* individuals collected from allopatric populations); 4) male *himera* against male *erato* (allopatric); 5) male *himera* against male *erato* (sympatric). Where possible individuals were only used in a single pairwise comparison; however, because of a lack of material some F1 butterflies were compared to *himera* and *erato* adults previously used in inter-specific comparisons.

ANALYSIS.- The difference in time between the first and second individual of a pair (*himera* / *erato*, *himera* / F1 etc.) completing one of the four behavioral events was measured for each day. An arbitrary ranking was used - *H. himera* > hybrid F1 > *H. erato* - so a positive difference in time indicated that highest ranking individual carried out that activity first. In most pairwise trials, activities were recorded over more than one day. If this was the case, the mean of the difference in paired timed observations between any two individuals over a number of days was used as the pairwise time difference. The mean pairwise time difference and 95% confidence limits (students *t* - distribution) of the sample mean were calculated for each set of comparisons to test for significant deviation from zero.

RESULTS

BEHAVIORAL ANALYSIS.- Butterflies appeared to acclimatize rapidly to our insectaries, and soon were flying and feeding during the day, and roosting from small twigs during the night. *Heliconius himera*, *H. erato*, and F1s left the roost at similar times during the morning (Table 1; Fig. 1a). However, in pairwise comparisons, both male and female *H. erato* were active significantly later in the morning than *H. himera* (Fig. 1b). Female *H. himera* actively flew and fed on average 40 minutes earlier than *H. erato* (Fig. 1b). Differences between males were even more pronounced, with allopatric male *H. himera* flying 90 minutes earlier than male *H. erato* (Fig. 1b). Female F1s were similar to *H. himera* in morning activity. A difference in morning activity between *H. himera* and *H. erato* was also evident (although less pronounced) among males collected in sympatry in the Chaguarpamba contact zone (Fig. 1b).

In the evening, allopatric female *H. himera* roosted 30 to 40 minutes earlier than *H. erato*, but ceased flying and feeding 25 minutes later than *H. erato* females (Fig. 2c-d; Table 1). For males, no significant differences were observed in the evening flying and feeding times, but both allopatric and sympatric *H. himera* males again tended to roost on average 40 minutes earlier. Again, F1 females were active in the evening like *H. himera*, but they did not differ significantly in roosting time. The direction of the cross (i.e. male *himera* x female *erato* or *vice versa*) did not affect morning and evening activity of hybrids (data not shown).

EGG LAYING AND DEVELOPMENTAL RATE.- Differences in daytime activity between *H. himera* and *H. erato* were also reflected in rates of egg production. In these pairwise experiments, females were allowed to lay eggs for between 2 weeks and 1 month and laid on average 50 eggs over the observational period (McMillan *et al.* 1997). In 19 pairwise comparisons (12 reared and 7 wild-caught pairs), *H. himera* laid significantly more eggs per day than *H. erato*

(mean difference = 0.74 eggs/day; $t = -2.66$; $p = 0.016$; $df = 18$). As expected, the number of eggs laid per day in hybrid crosses depended largely on the genotype of the mother.

Heliconius himera females mated by *H. erato* males laid fewer (but not significantly so) eggs per day than conspecific mated *H. himera* (mean difference = 0.1 eggs/day; $t = -0.33$; $p = 0.753$; $df = 7$). *H. erato* females mated by *H. himera* males showed significantly reduced egg production relative to *H. himera* females mated by conspecifics (mean difference = 0.5 eggs/day; $t = -2.47$; $p = 0.031$; $df = 11$).

There were also differences in egg to adult development time between *H. himera* and *H. erato*. Using a pairwise comparison between broods raised together, *H. erato* took on average 1.3 days longer to develop into an adult than *H. himera* ($t = 12.9$; $p = 0.0001$; $df = 11$). However, significant differences were not detected without this pairwise comparison, probably because of the effect of changing weather conditions throughout the study [mean egg to adult developmental time 29.1 ± 0.7 days for *H. himera* ($n = 26$), and 29.9 ± 1.0 days for *H. erato* ($n = 12$)]. Developmental time for larvae from both directions of hybrid crosses were similar and took significantly longer to develop than *H. himera* x *H. himera* crosses [1.2 days longer for offspring of *H. himera* females mated by *H. erato* males ($t = 4.32$; $p = 0.005$; $df = 6$) and 0.8 days longer for the offspring of matings between *H. erato* females and *H. himera* males ($t = 3.08$; $p = 0.011$; $df = 11$)].

DISCUSSION

In *Heliconius*, closely related pairs of species usually differ strongly in their ecology. For example, the species most closely related to *H. erato*, such as *H. himera*, *H. clysonymus*, *H. charithonia*, *H. hermathena*, *H. hortense*, *H. telesiphe*, and *H. hecalesia* are all found in more arid or montane environments than *H. erato*. In contrast, races within *H. erato* differ far less from each other ecologically (Benson 1982, Mallet 1993). This contrasting pattern in the habitat associations of races and species suggests that adaptation to different biotopes could play a crucial role in the transition from color pattern races to species. Subsequently, parapatric distributions may be maintained along ecotones because habitat adaptation geographically restricts distinct species by competitive exclusion, until eventually ecological divergence (particularly the evolution of host plant ecology) permits coexistence. However, few attempts have been made to investigate how speciation might result from adaptive differences in ecology, physiology or behavior. We have here performed experiments to investigate the ecological parameters by which *H. himera* and *H. erato* must have diverged in order to acquire enough differences to form sharp parapatric contact zones with little introgression, but not enough to invade the other's range. By excluding classical intrinsic hybrid inviability and sterility as causes of the lack of introgression, we were aware that this selection must be largely ecological, but the precise mode of selection was previously unclear (Jiggins *et al.* 1996, 1997b, McMillan *et al.* 1997, Mallet *et al.* 1998).

In Vilcabamba, *H. himera* was considerably more active than *H. erato*. It flew both earlier in the morning and later in the evening (excepting the males in the evening for which there was a lack of data), despite the fact that adults leave the roost at similar times in the morning, and *H. himera* roosting earlier in the evening. Physiological differences are perhaps expected

between individuals collected from different localities and altitudes, simply due to local adaptation, but this is not be the sole explanation as both morning behavior and evening roosting differences were maintained between *H. himera* and *H. erato* collected in sympatry. F1 hybrid behavior was either intermediate or somewhat like that of *H. himera*.

Egg laying and developmental rates of *H. himera* and *H. erato* closely corresponded to differences in flying, feeding, and roosting. The more active *H. himera* laid more eggs than *H. erato*, and egg-adult development time was 1-2 days less in *H. himera* than in *H. erato*. Hybrids were again intermediate.

While the selection against hybrids and the narrowness of the hybrid zone can be explained in part by predator selection against hybrid colour patterns, the strong correlation between wet / dry ecotone and the contact zone in three separate contacts between *H. erato* and *H. himera* in Ecuador and Peru suggests that the ecological differences we have found are adaptive (Descimon & Mast de Maeght 1984, König 1986, Mallet 1993, Jiggins *et al.* 1996). Our results show that the species differ in the amount of time spent foraging during the morning and afternoon, the number of eggs laid, and their consequent development rate. These traits must directly impact the relative fitness of individuals. Butterflies which forage more will be able to collect more pollen, and thus produce more eggs (Dunlap-Pianka *et al.* 1977, Boggs 1981). In addition, more active females will be able to search for scarce, cryptic (even mimetic) new shoots of their host plants, and lay more eggs on them (Gilbert 1975). Males will be able to forage more effectively for newly eclosing females (Deinert *et al.* 1994). Finally, rapidly developing larvae will outcompete slower ones, especially since new growth of the host *Passiflora* may be limiting, and larvae are cannibalistic (Gilbert 1975).

Just what are the differences between the habitats which might cause the observed differences in the butterflies? The experiments were carried out in the dry habitat of *H. himera* near the upper limit of the altitudinal range for *H. erato*. The higher altitude and dryer climate in the range of *H. himera* results in cooler average temperatures and greater diurnal temperature fluctuation (Jiggins *et al.* 1996). Every increase in altitude of 100 metres coincides with a temperature decrease of 0.6° to 1.0°C in the Neotropics (MacArthur 1972). In our study, between October and November 1994 temperatures at 1500m in Vilcabamba varied considerably between a daytime maximum of >30°C and a night-time minimum of 11°C. In contrast lowland wet forest rarely falls below 20°C at night and average daily temperature fluctuation may be as low as 4°C (Wallace 1853, Mabberly 1992) . Therefore the most probable explanation is that *H. himera* is physiologically adapted to lower temperatures early and late in the day. This is entirely concordant with the faster development time of *H. himera* larvae in Vilcabamba, particularly since it is difficult to imagine other factors which could have such an adverse affect in the relatively controlled, humid environment of our larval pots. The existence of performance differences in larvae protected from direct solar radiation implies that the effect is not a result of differences in the ability to absorb heat. Other species of butterflies are able to use dark areas of their wings and bodies to heat up more rapidly during basking, and this has a direct effect on the flight capacity (Watt 1968, Douglas & Guala 1978). As the temperature rises in the early morning, *H. himera* becomes active faster than *H. erato*, and remains active later during cooling in the evening. Increased foraging results in more eggs laid and shorter development times of the larvae in the cold resistant species.

Because larval host plant adaptation plays no role in *H. himera* / *H. erato* speciation, and because overlapping species of *Heliconius* almost always differ in their food-plant choice, competition for hosts probably prevents the coexistence of the two species (Jiggins *et al.* 1997a). *H. himera* may forage more sustainedly for pollen-donor plants, larval host plants and mates in dry areas, which could result in the competitive exclusion of *H. erato* from the cooler, drier upland forests. The converse experiment to test whether *H. erato* has the competitive advantage in warm, wet habitats was, unfortunately, not logistically possible. We predict that *H. erato* can forage better in the less sunny but more equable, humid conditions, with lower diurnal temperature fluctuations, of the rainforests and cloudforests where it occurs. The competitive switch between the species across the transition between wet and dry forest could lead to a very sudden changeover between the species even if the ecotone were fairly broad (Bull 1991). However, the hybrid zone between the two species may not necessarily be a result of mutual competitive exclusion. Neither may be able to successfully reproduce in the range of the other, or else a single species may exclude the other.

One of the most interesting aspects of this system for understanding speciation is that these two species can maintain their genetic integrity in the face of hybridization. A number of previous studies have shown that even in cases where there is no evidence for intrinsic genetic incompatibilities between taxa, disruptive environmental selection can eliminate intermediate phenotypes (Grant & Grant 1989, 1992, Schluter 1993). In association with partial assortative mating, this disruptive ecological selection can maintain two distinct genotypic clusters in sympatry. This represents an important intermediate stage in the speciation process. Our example is slightly different to those studied by Schluter and Grant & Grant as the two species and the two niches are parapatric, rather than broadly sympatric. The ecotone itself, where intermediate phenotypes might perhaps be ecologically favored,

may be too narrow relative to dispersal, and the rare intermediate phenotypes too strongly selected against via Müllerian mimicry, to cause a spreading of the contact zone.

This study contributes evidence to the hypothesis that speciation and parapatry in *Heliconius* is strongly associated with divergence in habitat adaptation (Mallet 1993). It provides evidence for behavioral differences between *H. himera* and *H. erato* which must translate into large fitness differences in the field. The results provide a partial explanation for a switch in competitive advantage between the species in the center of the wet / dry ecotone, and may also provide a key as to how the gene pools of these two species remain distinct through selection against hybrids.

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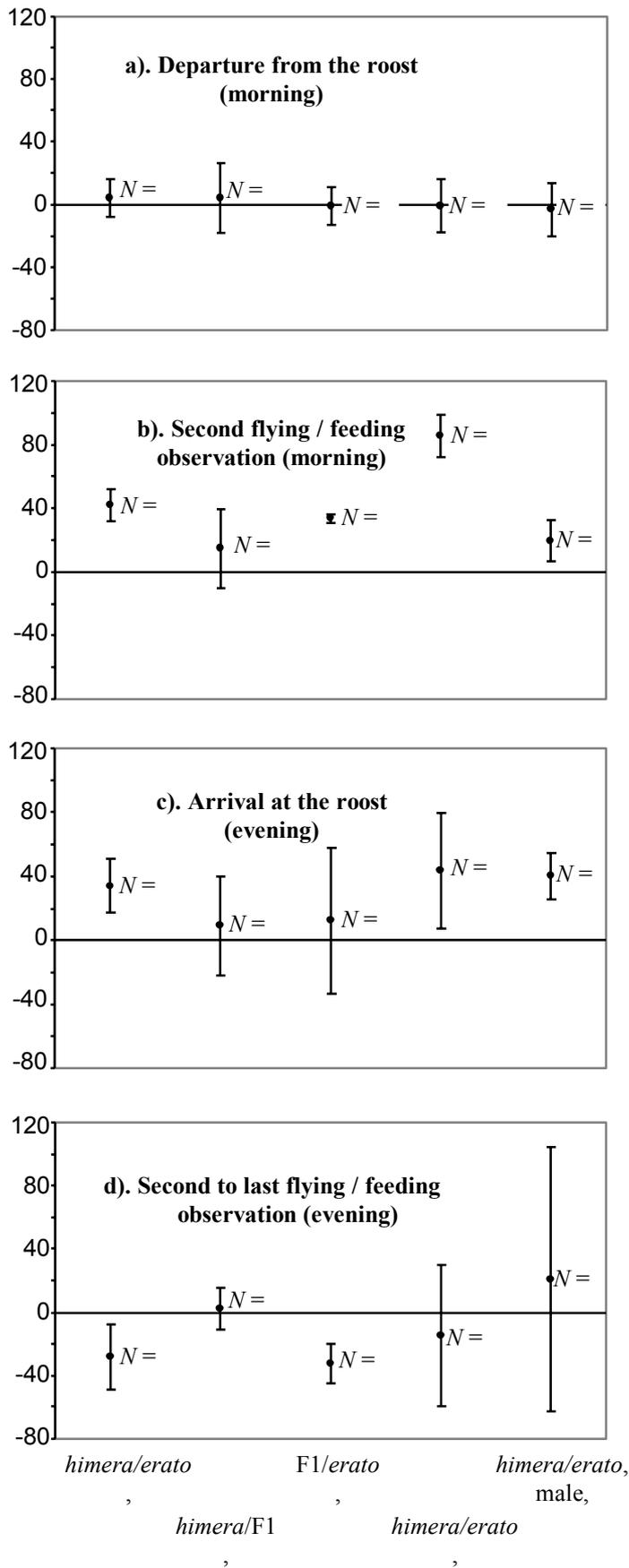
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Figure 1a-d Pairwise comparisons between *Heliconius himera*, *H. erato cyrbia*, and hybrid F1s for the four behavioral categories. All butterflies were of allopatric origin except where stated. A positive Δt value shows that the highest ranking species carried out that activity first (ranking *H. himera* > hybrid F1 > *H. erato*). The bars indicate \pm 95% confidence limits.



Paired comparison

Table 1. Estimated mean times of departure from the roost, morning / evening activity, and arrival at the roost for allopatric *H. himera* and *H. erato* (including data from males and females). These behavioral events are defined in the methods. Values given are the mean time \pm SE in minutes (sample size in parentheses).

	Departure from Roost	Active in Morning	Active in Evening	Arrival at Roost
<i>H. himera</i>	0753 h \pm 0:04 (22)	0841 h \pm 0:04 (24)	1712 h \pm 0:06 (14)	1718 h \pm 0:06 (21)
<i>H. erato</i>	0755 h \pm 0:04 (22)	0937 h \pm 0:04 (24)	1645 h \pm 0:05 (14)	1749 h \pm 0:08 (21)