Supplementary materials

What shapes the continuum of reproductive isolation?

Lessons from *Heliconius* butterflies.

Proceedings of the Royal Society, B
2017 (manuscript 20170335)
http://dx.doi.org/10.1098/rspb.2017.0335

Mérot C.[1, 2], Salazar C.[3], Merrill R. M.[4,5], Jiggins C.[4,5], Joron M.[1,6]

[1] ISYEB UMR 7205, Muséum National d'Histoire Naturelle, 45 rue Buffon, Paris
[2] IBIS, Université Laval, 1030 Avenue de la Médecine, Québec, Canada
3 Biology Program, Faculty of Natural Sciences and Mathematics. Universidad del Rosario. Carrera. 24 No 63C-69, Bogota D.C., 111221. Colombia.
4 Department of Zoology, University of Cambridge, Downing Street, Cambridge, CB2 3EJ, United Kingdom
5 Smithsonian Tropical Research Institute, MRC 0580-12, Unit 9100 Box 0948, DPO AA 34002-9998
6 Centre d'Ecologie Fonctionnelle et Evolutive, UMR 5175 CNRS - Université de Montpellier - Université Paul Valéry Montpellier - EPHE, 1919 route de Mende, 34293 Montpellier, France

1- Species studied and the continuum of divergence

Table S1: Summary of methods and references (numbers in brackets) for all species pair considered in this study

LF exp: Life-female experiment, NA: absence of quantified data, probably NS: based on unquantified observations, probably not a significant parameter.

		Pair	Location	Range overlap	Frequency of hybrids	Co-occurrence	Mate choice (Colour)	Mate choice (Male behaviour)	Mate choice (mating)	F_1 hatch rate	Larval survival	Adult survival	Mating success of F_1	F_1 Fertility
High divergence	CPxMP	*H. cydno chioneus / H. melpomene rosina*	Panama	Sympatric	0.05% [1]	[2]- Fig. S1	Models [3]	LF exp [4]	Tetrad [3] + no-choice [5]	[6]		Predation exp with models [7]	no-choice [5]	[6] **This study (Table S10)**
	CCxMC	*H. cydno cordula / H. melpomene melpomene*	Colombia	Sympatric	intermediate phenotype observed around San Cristobal but no admixed genotype [8]		Models [8]	Models [8]	no-choice + Tetrad [8]	[6]	**This study (Table S7)**			[6]
	HxMC	*H. heurippa / H. melpomene melpomene*	Colombia	Parapatric/ Sympatric	intermediate phenotype observed but no admixed genotype [8]	**This study (Fig. S1)**	Models [8]	LF exp [8]	no-choice + Tetrad [8]	[9]	**This study (Table S7)**	co-mimic with *H. melpomene*	**no-choice This study (Table S8)**	[9]
	TxM	*H. timareta thelxinoe / H. melpomene amaryllis*	Northern Peru	Parapatric/ Sympatric	0.6-1.5% of F_1; 3-5% admixed genotype [10, 11]	[11] Fig S1	Models [12]	LF exp [12]	no-choice [12]	**This study (Table S4)**	**This study (Table S4)**	co-mimic with parents	**no-choice/LF exp This study (Table S5-6)**	**This study (Table S4)**
	TfxMm	*H. timareta florencia / H. melpomene malleti*	Colombia	Parapatric/ Sympatric	about 2% [13]	**This study (Fig. S1)**	Models [14]		no-choice [13]	[14]		co-mimic with parents	**no-choice This study (Table S9)**	[14]
	CPxMG	*H. cydno chioneus / H. melpomene melpomene*	Panama/ Guiana	Allopatric	NA		Models [3]	LF exp [3]	Tetrad [3]	[6]				[6]

		Pair	Location	Range overlap	Frequency of gene flow	Habitat	Mate choice (Colour)	Mate choice (Male behaviour)	Mate choice (mating)	F_1 hatch rate	Larval survival	Adult survival	Mating success of F_1	F_1 Fertility
Intermediate divergence	CCxH	*H. cydno cordula / H. heurippa*	Colombia	Parapatric/ limited contact	no hybrids detected [8]		Models [8]	LF exp [8]	no-choice + Tetrad [8]	[9]	**This study (Table S7)**			[9]
	CxP	*H. cydno galanthus/ H. pachinus*	Costa Rica	Allopatric/ limited contact ?	7% [15]		Models [16]	LF exp [4, 16]	Tetrad [17]	NA (probably NS, see [16])	similar host-plant (probably NS, M. Kronforst)	F_1 are co-mimic with *H. cydno*	Models [16]	NA (probably NS, see [16])
	MGxMP	*H. melpomene melpomene / H. melpomene rosina*	Panama/ Guiana	Allopatric	NA		Models [3]	LF exp [3]	Tetrad [3]	[18]				[18]
Low divergence	CyxCw	*H. cydno alithea* white/yellow	Ecuador	Sympatric	unestimated: likely very high			LF exp [4, 16, 19]		NA (probably NS)	similar host-plant (probably NS)	F_1 are co-mimics with white *alithea* Predation at yellow site [20]	[19]	NA (probably NS)
	TfxTl	*H. timareta florencia / H. timareta linaresi.*	Colombia	Parapatric/ Small hybrid zone	~3% phenotypic hybrid, underestimated by Dennis-ray dominance [14]		Models [14]		No-choice [14]	[14]	similar host-plant (probably NS)		NA (probably NS)	[14]
	MaxM	*H. melpomene aglaope / H. melpomene amaryllis*	Panama/ Guiana	Parapatric/ Small hybrid zone	unestimated: likely very high. About 10% of phenotypic hybrid in the hybrid zone			Individual LF exp [3]		NA (probably NS)	similar host-plant (probably NS)		NA (probably NS)	NA (probably NS)

Table S2: Genetic divergence

Mean F_{ST} values between pairs of taxa based on different genetic markers (RAD-seq markers, AFLP and mitochondrial DNA) from the litterature [19, 21, 22]. * denote significant genetic divergence under a permutation test or no significant divergence (n.s.). Detailled methods and results can be found in original publications. The grey scale describe the continuum of divergence with the "high" category corresponding to pairs of taxa involving a representative of the melpomene-clade and the cydno-clade, and "intermediate" and "low" including pairs of taxa belonging to the same clade, respectively with and without significant genetic divergence.

		Mean F_{ST} (RAD markers) [22]	F_{ST} (AFLP) [21]	F_{ST} (mtDNA) [21]	F_{ST} (AFLP) [19]	F_{ST} (mtDNA) [19]
High divergence	***H. c. chioneus*** **(CP)** ***H. m. rosina*** **(MP)**	***0.34***	***0.23****	***0.93***		
High divergence	***H. c. cordula*** **(CC)** ***H. m. melpomene*** **(MC)**	***0.35***	***0.25****	***0.91***		
High divergence	***H. heurippa*** **(H)** ***H. m. melpomene*** **(MC)**	***0.42***	***0.35****	***0.93***		
High divergence	***H. c. chioneus*** **(CP)** ***H. m. melpomene*** **(MG)**	***0.44***	***0.29****	***0.83***		
High divergence	***H. t. thelxinoe*** **(T)** ***H. m. amaryllis*** **(M)**	***0.36***				
High divergence	***H. t. florencia*** **(Tf)** ***H. m. malleti*** **(Mm)**		***0.21****	***0.70***		
Intermediate	***H. heurippa*** **(H)** ***H. c. cordula*** **(CC)**	***0.38***	***0.35****	***0.85***		
Intermediate	***H. c. galanthus*** **(C)** ***H. pachinus*** **(P)**		***0.07****	***0.66***	***0.17****	
Intermediate	***H. m. rosina*** **(MP)** ***H. m. melpomene*** **(MG)**	***0.37***	***0.30****	***0.62***		
Low	***H. c. alithea*** **white/yellow (Cw/Cy)**				***0.001 (ns)***	***0.057 (ns)***
Low	***H. t. florencia*** **(Tf)** ***H. t. linaresi*** **(Tl)**		***0.02 (ns)***	***0.33***		
Low	***H. m. amaryllis*** **(M)** ***H. m. aglaope*** **(Ma)**	***0.16***				

2- Quantifying the strength of reproductive isolation (Methods and additional results)

Co-occurrence in sympatry

- *H. timareta thelxinoe* and *H. melpomene amaryllis* were collected at seven locations along an altitudinal gradient between 1100 and 1600m. Those were collected in North-Eastern Peru around Tarapoto, in the Alto Mayo (05°39'58"S; 77°44'35"W) and the Escalera area (06°27'28" S; 76°17'53" W) during from multiple fieldtrips (2005, 2007, 2011, 2012 and 2013). Information about collection and distribution analysis can be found in [11].

- *H. cydno chioneus* and *H. melpomene rosina* were collected along a track running through a gradient between open forest and closed-forest habitat [2]. We categorized this gradient in adjacent, 2-km wide segments.

- *H. heurippa* and *H. melpomene melpomene* were collected at six locations along an altitudinal gradient between 1150 and 1450m encompassing two areas of Colombia: the Villavicencio foothills around Buenavista (Meta; 4°10'30" N 73°40'41"W) and Chirajara (Cundinamarca; 4°12'48" N 73°47'70"W) and the area of DeleB, (Casanare; 5º 25'5"N, 72º 31'20"W).

- *H. timareta florencia* and *H. melpomene malleti* were collected at four locations along an altitudinal gradient between 673 and 1400m in the foothills of Florencia, Caqueta in Colombia, from Paraiso (01°45'02" N 75°37'55"W) to Sucre (01°48'12" N 75°39'19"W).

To estimate the reproductive isolation due the ratio of species co-occurrencce, $RI_{\text{co-occurrence}}$, we calculated the mean expected frequency of hetero-specific mating and the mean expected frequency of conspecific mating across the transect. At each location, the frequency of hetero-specific mating and the frequency of conspecific mating were define, respectively, as follow:

$$H_i = (F_{iB} \times F_{iA} + F_{iA} \times F_{iB}) \qquad C_i = (F_{iA} \times F_{iA} + F_{iB} \times F_{iB})$$

where $F_{i\,A}$ and $F_{i\,B}$ are the relative frequencies of species A and B at location i ($F_{iA} = \frac{N_{iA}}{N_{iA}+N_{iB}}$; $F_{iB} = \frac{N_{iB}}{N_{iA}+N_{iB}}$, in which $N_{i\,A}$ and $N_{i\,B}$ are the number of individual from species A and B collected at location i).

Confidence interval was drawn by bootstrapping. We sampled with replacement the same total number of individuals ($N_{i\,A}$+$N_{i\,B}$), at each location, in a pool composed of the same ratio ($N_{i\,A}$ /$N_{i\,B}$), and followed the same method to infer frequencies and calculate RI. This procedure was repeated 1000 times and the 2.5- 97.5% quantiles on the distribution of bootstrapped values of RI were taken as limits for the confidence interval.

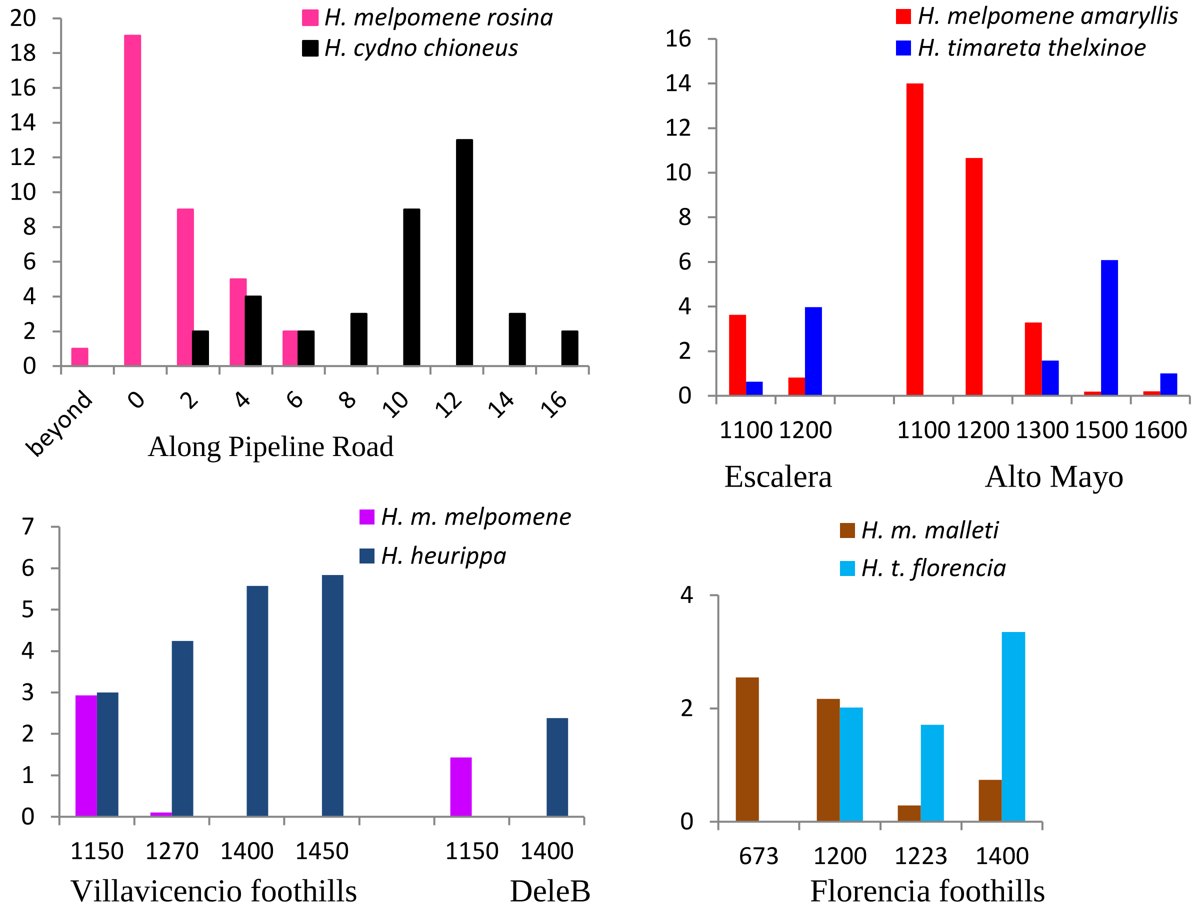

Fig. S1: Species co-occurrence.
(A) Distribution of *H. melpomene rosina* and *H. cydno chioneus* collected along the Pipeline Road (Parque Soberania, Panama,[2]). **(B)** Distribution of *H. melpomene amaryllis* and *H. timareta thelxinoe* collected along an altitudinal transect in the sympatric zone of Cordillera Escalera and Alto Mayo (Peru).[11] **(C)** Distribution of *H. melpomene melpomene* and *H. heurippa* collected along an altitudinal transect in the sympatric zone of Villavicencio foothills and at DeleB location in Casanare (Colombia). **(D)** Distribution of *H. melpomene malleti* and *H. timareta florencia* collected along an altitudinal transect in the Florencia foothills (Caqueta, Colombia).

Visual cues

Different studies have recorded the number of courtships by males of a given species towards wing models. We analysed these raw data following [3] and as described in [12] to obtain a probability P_{iA} of courting a *A*-model rather than a *B*-model for a male of species *i* by maximizing the log_e-likelihood expression given by

$$ML(P_{iA}) = \sum_j (A_{ij} \log_e(P_{iA}) + B_{ij} \log_e(1 - P_{iA}))$$

where *j* is the experimental session, A_{ij} the total number of courtships towards the *A* model within this session and B_{ij} the total number of courtships towards the *B* model. The 95% confidence intervals are equivalent to the parameter value that decreases the maximum value of ML of two units [23]. The individual contribution of colour preference to reproductive isolation was calculated with courtship probabilities as follow (for the value and its confidence interval):

$$RI_{colour\ cues\ (AxB)} = 1 - 2\times\frac{P_{AB}}{P_{AB} + P_{BB}}$$

Male choice

Data counting the number of courtship by a given male species towards a live virgin female (LF exp) were extracted from each study and analysed as described above to obtain a probability P_{iA} of courting a A female rather than a B female for a male of species i by maximizing the log_e-likelihood expression given by

$$ML(P_{iA}) = \sum_j (A_{ij} \log_e(P_{iA}) \; + \; B_{ij} \log_e(1 - P_{iA}))$$

where j is, a male monitored, A_{ij} the total number (or duration) of courtship of the male j towards the A female and B_{ij} the total number (or duration) of courtship towards the B female. Confidence intervals were estimated as describe above.

The individual contribution of male preference to reproductive isolation was calculated as follow (for the value and its confidence interval):

$$RI_{male\ choice\ (AxB)} = 1 - 2 \times \frac{P_{AB}}{P_{AB} + P_{BB}}$$

Achieved mating

The distribution of getting m_{ij} realised mating out of N_{ij} trials between males from species i and a female of species j follows a binomial law $\mathcal{B}$ (P_{ij}, N_{ij}). The probability P_{ij} of mating can be calculated by maximising the log_e-likelihood expression given by

$$ML(P_{ij}) = \; m_{ij} \log_e(P_{ij}) + \; (N_{ij} - m_{ij}) \log_e(1 - P_{ij})$$

Therefore, the value of the probability P_{ij} corresponds to the value which gives $ML'(P_{ij}) = 0$; (ML' being the derivative of ML) and can be calculated as $P_{ij} = \frac{m_{ij}}{N_{ij}}$. Confidence intervals were estimated as describe above.

In no choice experiment, P_{AB} represents a probability of mating rather than avoiding mating. Without isolation, the expected probability of hetero-specific mating would be the same as the probability of conspecific mating. Therefore, we used the second expression of the index *RI*.

$$RI_{barrier1} = 1 - \frac{Observed\ hybridization_{\ (with\ only\ barrier1)}}{Expected\ hybridization_{\ (with\ only\ barrier1)}}$$

and calculate the individual contribution of mating to reproductive isolation as follows (for the value and its confidence interval):

$$RI_{mating\ (AxB)} = 1 - \frac{P_{\ AB}}{P_{\ BB}}$$

In the tetrads experiment, P_{AB} represents a probability of mating a species over the other species. Therefore, the expected probability of heterospecific mating is 0.5, which is equal to $\frac{H+C}{2}$ so *RI* was calculated using the first expression (for the value and its confidence interval).

$$RI_{mating\ (AxB)} = 1 - 2\times\frac{P_{AB}}{P_{AB} + P_{BB}}$$

One should be aware that the two methods might bias the estimation in opposite directions. No-choice experiment might over-estimate hetero-specific mating rate due to the high density of males within the cage. On the contrary, tetrad experiments might under-estimate hetero-specific matings because it stops at the first mating and therefore evaluate the relative speed of conspecific and hetero-specific mating.

Hatch rate and hybrid sterility

Fertility was defined as the hatch rate, the percentage of egg hatched over the total number of eggs laid. Confidence interval were drawn as standard binomial proportion confidence interval.

The individual contribution of hybrid sterility to reproductive isolation was calculated as follow (for the value and its confident interval):

$$RI_{hybrid\ fertility\ (AxB)} = 1 - 2\times\frac{Fertility_{AB}}{Fertility_{AB} + Fertility_{BB}}$$

in which $Fertility_{AB} = \frac{Fertility_{males\ AB} + Fertility_{females\ AB}}{2}$ because the sex-ratio was never found to be significantly different from 0.5.

Hybrid adult fitness

Then, the contribution of adult survival to reproductive isolation was calculated as follow (for the value and its confidence interval):

$$RI_{adult\ survival\ (AxB)} = 1 - 2\times\frac{Survival_{AB}}{Survival_{AB} + Survival_{pure\ parents}}$$

The contribution of adult mating success (with each parental species separately) to reproductive isolation was calculated as follow (for the value and its confidence interval):

$$RI_{mating\ success\ (AxB)} = 1 - 2\times\frac{Mating\ success\ _{AB}}{Mating\ success\ _{AB} + Mating\ success\ _{pure\ parent}}$$

in which $Mating\ success_{AB} = \frac{Mating\ success_{males\ AB} + Mating\ success_{females\ AB}}{2}$.

Total reproductive isolation

For some pairs of taxa, we could calculate a cumulative degree of isolation by considering the contribution of each barrier to total reproductive isolation in a sequential order (following [24, 25]). We assumed that larval survival is not a significant isolating barrier and that co-mimetic hybrids do not suffer from higher predation.

Between *H. c. chioneus* and *H. m. rosina*, total RI reaches 100% [confidence intervals: 0.99-1], fitting the estimated frequency of 0.05% of F_1 on the field [1]. For co-mimetic pairs, total RI is just slightly lower, with 98% [confidence intervals: 0.93-0.99] between *H. t. florencia* and *H. m. malleti* and 97% [confidence intervals: 0.89-0.99] between *H. t. thelxinoe* and *H. m. amaryllis*, also fitting the frequency of F_1 in the sympatric area (0.6-4 %,[10, 11]).

Table S3: **Strength of reproductive isolation and confidence intervals associated with each barrier to gene flow**

RI ranges from -1 to 1 (full isolation) with 0 corresponding to no isolation (non-significant barrier and a probability of gene flow of 0.5, *e.g.* random mating, etc). For each pair of species, the two lines correspond to the two possible directions of heterospecific mating with the female/mother given first. Barriers that could not be estimated are not shown. Dash indicates unestimated barriers that are likely non-significant. Numbers in bold are significant isolating barriers.

♀ X ♂	Co-occurrence	Mating	F_1 egg	F_1 larva	F_1 adult	F_1 mating with #1	F_1 mating with #2	F_1 fertility
CPxMP	**0.74** [0.71;0.90]	**1** [0.91;1]	-0.03 [-0.06;0]		**0.35** [0.33;0.36]	**0.2** [0.09;0.22]	**0.52** [0.36;0.67]	**0.32** [0.27;0.37]
MPxCP	**0.74** [0.71;0.90]	**1** [0.91;1]	-		**0.35** [0.33;0.36]			**0.15** [0.09;0.21]
CCxMC		**0.82** [0.69;0.92]	-0.05 [-0.37; 0.12]	-0.03 [-0.24;0.18]				**0.29** [0.01;0.54]
MCxCC		**0.88** [0.78;0.95]	0.06 [ ]	0.39 [0.22;0.53]				**0.18** [0;0.33]
HxMC	**0.91** [0.87;0.96]	**0.93** [0.84;0.98]	-0.07 [-0.33; 0.26]	0.06 [-0.18;0.32]		**0.44** [0.16;0.76]	**0.29** [0.15;0.71]	**0.27** [0.02;0.51]
MCxH	**0.91** [0.87;0.96]	**0.9** [0.78;0.97]	-0.12 [-0.42;0.36]	-0.18 [-0.38;0.05]		**0.75** [0.08;0.98]	**0.2** [0.07;0.76]	0.05 [-0.19;0.30]
CPxMG	*no overlap*	**0.78** [0.36;0.95]	0.02 [-0.09;0.03]					**0.48** [0.36;0.61]
MGxCP	*no overlap*	**1** [0.80;1]	-0.02 [ ]					**0.34** [0.31;0.37]
TxM	**0.63** [0.55;0.75]	**0.86** [0.72;0.94]	0.09 [-0.18;0.48]	0.02 [-0.20;0.18]	-	**0.48** [0.42;0.81]	-0.02 [-0.04;0.25]	**0.33** [0.25;0.40]
MxT	**0.63** [0.55;0.75]	**0.85** [0.66;0.95]	0.05 [-0.08;0.17]	0.12 [-0.30;0.58]	-	**0.87** [0.69;0.97]	0.06 [0;0.17]	**0.16** [0.07;0.24]
TfxMm	**0.48** [0.41;0.56]	**0.9** [0.81;0.96]	-		-			**0.33** [0.17;0.44]
MmxTf	**0.48** [0.41;0.56]	**0.96** [0.89;0.99]	0.23 [-0.24;0.83]		-	**0.52** [0.28;0.75]	**1** [0.72;1]	**0.19** [-0.17;0.61]
CCxH		**0.56** [0.36;0.75]	0.18 [-0.06;0.45]	0.02 [-0.16;0.21]				0 [-0.13;0.11]
HxCC		**0.98** [0.90;1]	0.06 [-0.21;0.41]	0.16 [-0.13;0.53]				0.07 [-0.21;0.44]
CxP		**0.83** [0.35;0.99]	-	-		-0.06 [-0.07;-0.04]	**0.94** [0.76;0.99]	-
PxC		**1** [0.6;1]	-	-		-0.06 [-0.07;-0.04]	**0.94** [0.76;0.99]	-
MGxMP	*no overlap*	**1** [0.69;1]	-0.01 [-0.06;0.03]	-				0.07 [-0.05;0.21]
MPxMG	*no overlap*	**0.48** [0.04;0.81]	0.04 [-0.06;0.14]	-				**0.32** [0.28;0.34]
CyxCw		**0.26** [0.17;0.35]	-	-	**0.18** [0.12;0.27]	-	**0.26** [0.17;0.35]	-
CwxCy		**0.07** [-0.02;0.16]	-	-	**0.18** [0.12;0.27]	-	**0.26** [0.17;0.35]	-
TfxTl		**0.02** [0;0.14]	0 [-0.15;0.13]	-				0.09 [-0.09;0.25]
TfxTl		**0.48** [0.21;0.75]	0.12 [-0.07; 0.30]	-				0.09 [-0.08;0.26]
MaxM		**0.4** [0.11;0.66]	-	-				-
MxMa		0 [-0.28;0.32]	-	-				-

3- New data: Survival, fertility and mating success for *H. timareta thelxinoe* x *H. melpomene amaryllis* hybrids

Methods:

- Fertility and survival

Females of *H. melpomene amaryllis, H. timareta thelxinoe* and hybrid females were provided young shoots of *Passiflora menispermifolia, P. triloba, P. edulis, P. riparia, P. seratodigitata, P.oestredii*. We collected the eggs every two days. Females from crosses or laying backcross eggs were kept individually. Most pure females were kept in large "stock" cages and some of them were isolated. Fertility of hybrid males and females was estimated as the hatching rate of a cross between a hybrid and a pure parent. Fertility was analysed using a GLM with a quasi-binomial distribution, to model for over-dispersion of those data, and a logit-link function with the library lme4 in R [26]. The binomial response variable was the number of hatched eggs, the binomial total was the total number of eggs and the predictor was the type of crosses (heterospecific *vs.* conspecific).

After recording hatching, larvae were raised in individual plastic containers for the first instars. Then, they were gathered by family group in a larger box. They were fed ad libitum on young shoot of *Passiflora* sp.

All larvae with a *H. t. thelxinoe* mother (*H. t. thelxinoe,* F_1 from *TxM,* and backcrosses to *H. t. thelxinoe*) were fed on *P. granadilla riparia,* chosen by most *H. t. thelxinoe* females in our insectaries. All larvae with a *H. m. amaryllis* mother (*H. m. amaryllis,* F_1 from *MxT* and backcrosses to *H. m. amaryllis)* were given *P. triloba,* host-plant of *H. m. amaryllis.*

Survival rate was calculated for each family as the proportion of larvae growing until imago. Association between larval survival rate and identity of the parent (hybrid *vs.* pure parent) was analysed as hatching rate, using a GLM with a quasibinomial distribution with lme4 in R [26].

- Hybrid mating success
  - Females

In large insectaries, a virgin female was released singly during 48h in the presence of ten mature males, either ten *H. melpomene* or ten *H. timareta.* Mating was checked every hour between 6AM and 6PM. To ensure that no unobserved mating had occurred, male spermatophore presence in the genital tracts of the female was controlled in the evening of the first and second day and at the end of the experiment. For each experiment, a group of males was randomly composed from a stock of 20 to 50 mature males. The stock was continuously renewed as new mature males became available.

We analysed the probability of mating and calculated confidence interval as described in [5]. Briefly, the distribution of getting m_{ij} realized mating out of N_{ij} trials between males of species *i* and a female of species *j* follows a binomial law $\mathcal{B}(P_{ij}, N_{ij})$. The probability P_{ij} of mating can be calculated by maximising the log_e-likelihood expression given by:

$$ML(P_{ij}) = m_{ij}\log_e(P_{ij}) + (N_{ij} - m_{ij})\log_e(1 - P_{ij})$$

Therefore the value of the probability P_{ij} corresponds to the value which gives $ML'(P_{ij}) = 0$ (ML' being the derivative of ML) and can be calculated as $P_{ij}^* = \frac{m_{ij}}{N_{ij}}$. The 95% confidence intervals are equivalent to the parameter value that decreases the maximum value of ML by two units [23].

- Males (see also [12])

In experimental cages of 2x1x2m, 3 to 5 mature males (>8 days old) were marked on the wing patch so as to be individually identified when flying or courting. Experiments were performed under sunny conditions between 9AM and 4PM. Males were presented a young virgin female of one species (*H. timareta thelxinoe*) for ten minutes and then a young virgin female of the other species (*H. melpomene amaryllis*) for ten minutes again. Females were matched by age and were under five days old. Each male was monitored individually and repeated the experiment at least 5 times with different female pairs, randomizing the presentation order and on a different day. Mating was recorded when it occurred, but male and female were separated gently and quickly.

Results:

- F_1 hatch rate:

F_1 hybrid broods show no significant reduction of hatch rate (p=0.58).

- F_1 larval survival:

For the *H. t. thelxinoe/H. m. amaryllis* experiment, hybrid larvae were fed on the plant corresponding to their mother's oviposition preference, *i.e.* the plant on which they could have hatched. Under those conditions, *MxT* hybrid survival rate is slightly lower than pure broods but the difference is not significant (Table S4, p=0.23). Similarly, no significant effect on survival was observed in the second generation (Table S4).

However, we can note that earlier attempts of feeding of *H. m. amaryllis* and some hybrids (back-crosses towards *H. m. amaryllis*) with *P. edulis* or *P. granadilla* (well-accepted by *H. t. thelxinoe*) led to reduced growth and higher mortality rate.

- F_1 fertility:

F_1 hybrid males between *H. t. thelxinoe* and *H. m. amaryllis* show no reduction of hatch rate compare to control broods (Table S4, p=0.58).

F_1 hybrid females with a *H. t. thelxinoe* mother (*TxM* females) showed complete sterility: they tended to lay smaller eggs and none of the 102 eggs hatched (Table S4).

F_1 hybrid females (*MxT*) have a lower sterility compared to pure broods (Table S4, p<0.001) but the pattern of fertility is not uniform (dispersion =10.04) . We tested 16 females from 3 different *MxT* hybrid families. Seven of them were fully-fertile with a hatch rate about 0.7-0.9, similar to pure broods, (p=0.58), three had a lower fertility than pure brood, with hatch rate reaching 0.1 to 0.3, (p<0.001) and six were sterile. Among sterile females, four laid eggs which never hatched (0 out of 121 eggs) and two never laid any eggs despite spending over 14 days in a cage with access to young shoots of several *Passifloras* and pollen.

Table S4: Hatch rate, survival rate, sex-ratio for control, F1 and backcross broods between *H. melpomene amaryllis* (M) and *H. timareta thelxinoe* (T). (mean ± sd)

Brood	***Genotype***	***Mother***	***Father***	***N broods***	***N eggs***	***N adults***	***Hatch rate***	***Survival rate***	***Mean sex-ratio (Males/Total)***
control	MxM	M	M	10	241	49	0.88±0.12	0.24±0.15	0.53±0.26
control	TxT	T	T	4	147	16	0.88±0.13	0.20±0.25	0.60
control	MxM	M	M	stock	977	179	0.89	0.21	0.44
control	TxT	T	T	stock	680	127	0.85	0.22	0.55
F_1	TxM	T	M	2	158	28	0.73±0.28	0.21	0.71
F_1	MxT	M	T	7	574	68	0.80±0.15	0.17±0.12	0.40±0.08
Bx	pure x (TxM)	M/T	(TxM)	3	268	38	0.89±0.09	0.23±0.18	0.61±0.10
Bx	pure x (MxT)	M/T	(MxT)	15	808	172	0.92±0.06	0.21±0.16	0.47±0.13
Bx sterile	(TxM)x pure	(TxM)	M/T	4	102	0	**0.00 ***	na	na
Bx partially-fertile	(MxT)x pure	(MxT)	M/T	16	582	43	**0.36±0.39***	0.15±0.16	0.53±0.16
→ details:									
Bx fully-fertile	(MxT)x pure	(MxT)	M/T	7	327	43	0.81±0.13	0.17±0.16	0.53±0.16
Bx partially-f	(MxT)x pure	(MxT)	M/T	3	134	0	**0.21±0.14 ***	0.00	na
Bx sterile	(MxT)x pure	(MxT)	M/T	4	121	0	**0.00 ***	na	na
Bx sterile	(MxT)x pure	(MxT)	M/T	2	**0***	0	**na**	na	na

- F_1 mating success:

Table S5: Probability of mating in no-choice experiments for F_1 female hybrids between *H. melpomene amaryllis* (M) and *H. timareta thelxinoe* (T). [Confidence interval]
Controls are from [12]

		Female	*Male*	*N Trials*	*Mating probability*
Mating success with H. melpomene	***Control***	M	M	19	0.89 [0.71-0.98]
	F_1 females	TxM	M	2	1 [0.37-1]
		MxT	M	15	0.87 [0.64-0.98]
Mating success with H. timareta	***Control***	T	T	15	0.93 [0.73-0.99]
	F_1 females	TxM	T	2	1 [0.37-1]
		MxT	T	12	0.25 [0.07-0.53]

Table S6: Probability of mating for F_1 male hybrids in a total of 5 experiments of 10 minutes with *H. melpomene amaryllis* (M) female. [Confidence interval]

		Female	*Male*	*N Trials*	*Mating probability*
Mating success with H. melpomene	***Control***	M	M	60	0.33 [0.22-0.46]
	F_1 males	M	TxM	22	0.32 [0.15-0.53]
		M	MxT	22	0.27 [0.12-0.48]
	Back-crosses males	M	MxF_1 or (MxT)xM	60	0.47[0.34-0.60]

We decided to attribute a null mating success to males F_1 with *H. t. thelxinoe* females because this type of back-crosses never occurred naturally despite numerous trial experiments, neither in the 10-minute behavioural experiments nor in longer trials (pers. obs.).

4- New data: Survival and mating success for *H. heurippa/ H. melpomene melpomene/ H. cydno cordula* hybrids

Larvae were raised in individual plastic containers for the first instars. Then, they were gathered by family group in a larger box. *H. heurippa*, *H. c. cordula* and *H. m. melpomene* and their F_1 hybrids were fed ad libitum on young shoot of *Passiflora oesterdii*, which is used as oviposition plants by all three species [9]. Survival rate was calculated for each family as the proportion of larvae growing until imago. Association between larval survival rate and identity of the parent (hybrid *vs.* pure parent) was analysed as hatching rate, using a GLM with a quasibinomial distribution with lme4 in R [26]

Hybrid ability of mating has been investigated with no-choice experiment as described in [8] and analysed with likelihood methods as described earlier and in [12].

- F_1 survival

For *H. heurippa/H. m. melpomene/H. c. cordula*, hybrids were all raised on the same plant, *P. oesterdii*, used by the three species in the wild. Therefore, differences in survival rate was not expected nor observed (Table S7, H/M: p=0.22; C/M: p=0.92; H/C: p=0.33)

Table S7: Survival rate for control, F_1 and backcross broods between *H. melpomene melpomene* (MC), *H. cydno cordula* (CC) and *H. heurippa* (H). (mean ± sd)

Brood	*Genotype*	*Mother*	*Father*	*N broods*	*N adults*	*Survival rate*
control	CCxCC	CC	CC	27	336	0.62±0.30
control	MCxMC	MC	MC	24	224	0.51±0.26
control	HxH	H	H	22	241	0.68±0.26
F_1	CCxH	CC	H	21	233	0.62±0.28
F_1	HxCC	H	CC	5	46	0.47±0.27
F_1	CCXMC	CC	MC	14	143	0.61±0.28
F_1	MCxCC	MC	CC	2	25	0.25±0.29
F_1	HxMC	H	MC	7	63	0.53±0.23
F_1	MCxH	MC	H	2	78	0.89±0.16
Bx	75% CC / 25% H			19	143	0.59±0.25
Bx	75% H / 25% CC			11	96	0.62±0.26
Bx	75% CC / 25% MC			72	601	0.55±0.30
Bx	75% MC / 25% CC			9	94	0.57±0.35
Bx	75% MC / 25% H			9	121	0.59±0.30
Bx	75% H / 25% MC			9	46	0.46±0.20

- F_1 mating success

Table S8: Probability of mating in no-choice experiments for F_1 hybrids between *H. melpomene melpomene* (MC) and *H. heurippa* (H). [Confidence interval]

		Female	Male	N Trials	Mating probability
Mating success with H. melpomene	*Control*	M	M	17	1 [0.89-1]
	F_1 females	HxM	M	12	0.42 [0.17-0.7]
		MxH	M	10	0.6 [0.3-0.86]
	F_1 males	M	HxM	2	1 [0.35-1]
		M	MxH	1	1 [0.13-1]
Mating success with H. heurippa	*Control*	H	H	22	1 [0.91-1]
	F_1 females	HxM	H	9	0.44 [0.16-0.75]
		MxH	H	2	0.5 [0.04-0.96]
	F_1 males	H	HxM	6	0.67 [0.27-0.94]
		H	MxH	1	0 [0-0.87]
No-choice experiments (Mavarez et al, 2006 [8])		H	M	55	0.07 [0.02-0.16]
		M	H	40	0.1 [0.03-0.22]

5- New data: Mating success for *H. melpomene malleti* X *H. timareta florencia* hybrids

Hybrid ability of mating has been investigated with no-choice experiment as described in [8] and analysed with likelihood methods as described earlier and in [12].

Table S9: Probability of mating in no-choice experiments for F_1 hybrids between *H. melpomene malleti* (Mm) and *H. timareta florencia* (Tf). [Confidence interval]

	Female	Male	N Trials	Mating probability
Mating success with H. melpomene malleti	Mm	Mm	35	0.86 [0.72-0.95]
	MmxTf	Mm	23	0 [0-0.083]
	Mm	MmxTf	8	0 [0-0.221]
Mating success with H. timareta florencia	Tf	Tf	45	0.91 [0.80-0.97]
	MmxTf	Tf	24	0.38 [0.20-0.58]
	Tf	MmxTf	10	0.2 [0.04-0.50]

6- New data: Fertility for *H. melpomene rosina* X *H. cydno chioneus* hybrids

Four hybrid females from the same family (*H. melpomene rosina* mother X *H. cydno chioneus* father) were provided young shoots of *Passiflora sp* in captivity. Eggs were collected individually in plastic cup and hatch rate was recorded.

Partial fertility was observed, with a low hatch rate compared to pure individuals. Given the small sample size, no statistics were drawn on those data. However, additional observations (unquantified) on several hybrids females from other families (same direction of cross) also support the observed partial fertility.

Table S10: Hatch rate for control and F_1 females between *H. melpomene rosina* (MP) and *H. cydno chioneus* (CP). (mean ± sd)

	Brood	***Genotype***	***Mother***	***Father***	***N broods***	***N eggs***	***Hatch rate***
From (Naisbit et al, 2002 [6])	***control***	MPxMP	MP	MP	22	943	0.95±0.05
	control	CPxCP	CP	CP	16	820	0.86±0.15
	Bx sterile	(CPxMP)x pure	(CPxMP)	MP/CP	25	209	**0.00**
New data	***Bx partially-fertile***	(MPxCP)x pure	(MPxCP)	MP/CP	4	149	**0.32±0.08**

References

[1] Mallet, J. 2007 Hybrid speciation. *Nature* **446**, 279-283.
[2] Estrada, C. & Jiggins, C.D. 2002 Patterns of pollen feeding and habitat preference among *Heliconius* species. *Ecol. Entomol.* **27**, 448-456.
[3] Jiggins, C.D., Naisbit, R.E., Coe, R.L. & Mallet, J. 2001 Reproductive isolation caused by colour pattern mimicry. *Nature* **411**, 302-305.
[4] Merrill, R.M., Gompert, Z., Dembeck, L.M., Kronforst, M.R., McMillan, W.O. & Jiggins, C.D. 2011 Mate preference across the speciation continuum in a clade of mimetic butterflies. *Evolution* **65**, 1489-1500. (doi:10.1111/j.1558-5646.2010.01216.x).
[5] Naisbit, R.E., Jiggins, C.D. & Mallet, J. 2001 Disruptive sexual selection against hybrids contribute to speciation between *Heliconius cydno* and *Heliconius melpomene*. *Proc. R. Soc. B-Biol. Sci.* **268**, 1849-1854.
[6] Naisbit, R.E., Jiggins, C.D., Linares, M., Salazar, C. & Mallet, J. 2002 Hybrid sterility, Haldane's rule and speciation in *H. cydno* and *H. melpomene*. *Genetics* **161**, 1517-1526.
[7] 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. *Proc. R. Soc. B-Biol. Sci.* **279**, 4907-4913. (doi:10.1098/rspb.2012.1968).
[8] Mavarez, J., Salazar, C.A., Bermingham, E., Salcedo, C., Jiggins, C.D. & Linares, M. 2006 Speciation by hybridization in *Heliconius* butterflies. *Nature* **441**, 868-871.
[9] Salazar, C.A., Jiggins, C.D., Arias, C.F., Tobler, A., Bermingham, E. & Linares, M. 2005 Hybrid incompatibility is consistent with a hybrid origin of *Heliconius heurippa* Hewitson from its close relatives, *Heliconius cydno* Doubleday and *Heliconius melpomene* Linnaeus. *J. Evol. Biol.* **18**, 247-256.

[10] Mérot, C., Mavarez, J., Evin, A., Dasmahapatra, K., Mallet, J., Lamas, G. & Joron, M. 2013 Genetic differentiation without mimicry shift in a pair of hybridizing Heliconius species (Lepidoptera : Nymphalidae). *Biol. J. Linn. Soc.* **109**, 830-847.
[11] Mérot, C., Poul, Y.L., Théry, M. & Joron, M. 2016 Refining mimicry: phenotypic variation tracks the local optimum. *J. Anim. Ecol.* **85**.
[12] Mérot, C., Frérot, B., Leppik, E. & Joron, M. 2015 Beyond magic traits: Multimodal mating cues in *Heliconius* butterflies. . *Evolution* **69**, 2891-2904.
[13] Giraldo, N., Salazar, C., Jiggins, C.D., Birmingham, E. & Linares, M. 2008 Two sisters in the same dress: *Heliconius* cryptic species. *BMC Evol. Biol.* **8 324**.
[14] Sanchez, A., Pardo-Diaz, C., Munoz, A., Jiggins, C., Salazar, C. & Linares, M. 2015 An introgressed wing patterning gene acts as a mating cue. *Evolution*.
[15] Kronforst, M.R., Young, L.G., Blume, L.M. & Gilbert, L.E. 2006 Multilocus analyses of admixture and introgression among hybridizing *Heliconius* butterflies. *Evolution* **60**, 1254-1268. (doi:10.1554/06-005.1).
[16] Kronforst, M.R., toung, 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. *Proc. Natl. Acad. Sci. USA* **103**, 6575-6580.
[17] Kronforst, M.R., Young, L.G. & Gilbert, L.E. 2007 Reinforcement of mate preference among hybridizing Heliconius butterflies. *J. Evol. Biol.* **20**, 278-285. (doi:10.1111/j.1420-9101.2006.01198.x).
[18] Jiggins, C.D., Linares, M., Naisbit, R.E., Salazar, C., Yang, Z.H. & Mallet, J. 2001 Sex-linked hybrid sterility in a butterfly. *Evolution* **55**, 1631-1638.
[19] Chamberlain, N.L., Hill, R.I., Kapan, D.D., Gilbert, L.E. & Kronforst, M.R. 2009 Polymorphic Butterfly reveals the missing link in ecological speciation. *Science* **326**, 847-850.
[20] Kapan, D.D. 2001 Three-butterfly system provides a field test of müllerian mimicry. *Nature* **409**, 338-340.
[21] Arias, C.F., Salazar, C., Rosales, C., Kronforst, M.R., Linares, M., Bermingham, E. & McMillan, W.O. 2014 Phylogeography of *Heliconius cydno* and its closest relatives: disentangling their origin and diversification. *Mol. Ecol.* **23**, 4137-4152. (doi:10.1111/mec.12844).
[22] Nadeau, N.J., Martin, S.H., Kozak, K.M., Salazar, C., Dasmahapatra, K.K., Davey, J.W., Baxter, S.W., Blaxter, M.L., Mallet, J. & Jiggins, C.D. 2013 Genome-wide patterns of divergence and gene flow across a butterfly radiation. *Mol. Ecol.* **22**, 814-826. (doi:10.1111/j.1365-294X.2012.05730.x).
[23] Edwards, A.W.F. 1972 *Likelihood*, (Cambridge Univ. Press, Cambridge, 1972).
[24] Coyne, J.A. & Orr, H.A. 2004 In *Speciation.* (Sunderland, MA: Sinauer Associates.
[25] Ramsey, J., Bradshaw, H.D. & Schemske, D.W. 2003 Components of reproductive isolation between the monkeyflowers Mimulus lewisii and M-cardinalis (Phrymaceae). *Evolution* **57**, 1520-1534.
[26] Bates, D., Maechler, M., Bolker, B. & Walker, S. 2013 lme4: Linear mixed-effects models using Eigen and S4. R package version 1.0-4. In *Accessed online* (