Sending mixed signals: convergent iridescence and divergent chemical signals in sympatric sister-species of Amazonian butterflies

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Abstract

Closely-related species are often partitioned into different geographic areas or micro-habitats as a result of competition and reproductive interference. Here, we investigate how the evolution of shared adaptive traits may shape ecological interactions and genetic divergence, and facilitate co-existence in sympatry. In closely-related *Morpho* butterfly species living in the understory of the neo-tropical rainforest, the blue iridescent coloration of the wings is likely involved in predation evasion as well as in mating recognition and courtship. These contrasted selective pressures acting on this visual trait shared between closely-related species has likely shaped their coexistence. We used spectrophotometry, behavioral experiments, visual modeling and chemical analyses applied to samples from allopatric and sympatric populations of *Morpho helenor* and *Morpho achilles* to characterize how the evolution of visual and chemical traits might favor their coexistence in sympatry: we quantified the differences in wing iridescence and tested for variations in the sexual preference for this trait between allopatric *vs.* sympatric populations. We found a strong similarity in iridescence among species in sympatry, suggesting convergence driven by predation. Although behavioral results suggest that iridescent signals could also be used as visual cues during mate choice, convergent iridescent signals may impair the visual recognition of sympatric *Morpho* species. In contrast, divergent chemical bouquets among species suggest that the visual similarity of sympatric *Morpho* species might have favored the divergence of alternative traits involved in

species recognition such as chemical cues. This study underlines how ecological interactions and trait evolution can shape species coexistence in sympatry.

Introduction

Identifying the evolutionary factors promoting diversification and co-existence of closely-related species in sympatry is a major challenge. Since the evolution of traits within species in sympatry can affect ecological interactions between individuals, it either fuels or impairs the speciation process. Closely-related species often share similar suites of traits because of their shared evolutionary history. Furthermore, in sympatry, habitat filtering excludes species with suites of traits mal-adapted to local conditions, therefore enhancing trait similarity (Chazot et al., 2014). The shared selective pressures encountered by individuals living in sympatry also often promote the evolution of locally adapted traits within species and even trait convergence among species (Muschick et al., 2012). That said, closely-related sympatric species often display divergent suites of traits because they are usually partitioned in divergent ecological niches (Berlocher & Feder, 2002). The co-existence in sympatry may also promote divergent evolution of traits in incipient species, because of competitive interactions between individuals or as a result of reinforcement (Butlin & Smadja, 2018). The evolution of traits in incipient species living in sympatry thus depends on the balance between convergence and divergence, resulting either in (i) evolutionary trade-offs of traits jointly influenced by antagonistic natural and sexual selective pressures (Ellers

& Boggs, 2003; Maan & Seehausen, 2011) or (ii) convergence in some traits and divergence in others (Le Roy, Roux, et al., 2021).

Theoretical and empirical studies have shown that sexual selection may favor the evolution of preferences for locally-adapted traits within species (Servedio, 2004; Servedio & Boughman, 2017; van Doorn et al., 2009). For instance, the predator-deterrent coloration of poison frogs is also detected and used as mating cues by females (Reynolds & Fitzpatrick, 2007). Similarly, habitat-dependent coloration of sympatric cichlid fish is also used as a visual cue for mate recognition (Seehausen et al., 2008). Yet, sexual interactions are likely to occur between individuals from closely-related species when they live in sympatry, and similar preferences for adaptive traits may thus result in substantial reproductive interference (Gröning & Hochkirch, 2008). For example, because sympatric amphibians can sometimes share the same mating cues and preferences, they often engage in misdirected mating attempts towards heterospecifics, a potentially deleterious behavior (Soni et al., 2024). In addition, hybrids, when produced, can be unfit, thus favoring the evolution of sexual preferences for species-specific cues, rather than locally-adapted traits (Maisonneuve et al., 2024). Comparing variations in adaptive traits in sympatric *vs*. allopatric populations of recently-diverged species, and testing the sexual preference for those traits can shed light on the selective processes targeting traits modulating reproductive isolation and co-existence in sympatry.

In butterflies, the evolution of wing color patterns can be influenced by both natural and sexual selection. The visual discrimination of wing color pattern has been shown to enable intraspecific recognition during courtship in many species (Costanzo & Monteiro, 2007; Li et al., 2017). However, the evolution of wing color patterns is also strongly influenced by the visual effect perceived by predators (Finkbeiner et al., 2014; Oliver et al., 2009). Whether these selective

85 pressures promote trait convergence or divergence in sympatric species might depend on their
86 relatedness: for instance, a recent study in *Papilionidae* showed multiple color pattern
87 convergences between distantly-related species living in sympatry, while divergent colorations are
88 found in closely-related species (Puissant et al., 2023). Positive selection acting on divergent traits
89 involved in species recognition could be increased because of higher reproductive interference in
90 closely related species than in distantly related taxa (Pfennig & Pfennig, 2009). In sympatric species
91 with chemical-defenses, such as *Heliconinii* butterflies, local predation pressures tend to promote
92 the convergence of similar conspicuous warning wing patterns (*i.e.* Müllerian mimicry, (Joron et
93 al., 1999; Merrill et al., 2014)). But the costs associated with hybrid production in turn favor the
94 evolution of alternative divergent mating cues in mimetic butterflies (Estrada & Jiggins, 2008), and
95 divergence in male pheromone bouquets and female attraction has been found among mimetic sister
96 species (González-Rojas et al., 2020). Similarly, the evolution of specific visual mate recognition
97 signals, limiting reproductive interference but indistinguishable by predators, can also be promoted
98 on the wings of mimetic butterflies (Llaurens et al., 2014). Thus, ecological interactions among
99 recently-diverged species living in sympatry can impact the evolution of traits involved in both
100 local adaptation and mate recognition and may trigger the evolution of alternative traits.
101 Investigating variations in traits used as mating cues in allopatric *vs*. sympatric populations of
102 sister-species can help ascertain how trait evolution might favor speciation and co-existence in
103 sympatry.
104
105 Here we focus on the evolution of mating cues in the neotropical butterfly genus *Morpho,* where
106 multiple closely-related species co-exist in sympatry (Blandin & Purser, 2013). In some *Morpho*
107 species, striking iridescent blue coloration is displayed on the dorsal side of the wings, due to
108 nanostructures on wing scales (Siddique et al., 2013). Variations in the reflected wavelength
109 depending on the angle of observation and/or illumination (*i.e.* iridescence) have been documented

in *Morpho* species (Giraldo et al., 2016). The light signal reflected by iridescent surfaces can be very directional, as hue and brightness of iridescent objects can drastically change depending on the light environment or the observer's position (Doucet & Meadows, 2009). Directional iridescent signals generated in animals could then enhance recognition by mates while remaining poorly detected by predators (Endler, 1992). For instance, in some hummingbird species, when males tilt their head in a specific direction, flashes of bright coloration can be observed in these otherwise darkly-colored birds (Simpson & McGraw, 2019). The specific directional signal produced by the iridescent feathers is a cue during courtship, suggesting that the antagonistic sexual and natural selective pressures finely shape the evolution of iridescence. Similarly, male *Hypolymnas* butterflies display abrupt shifts in UV brightness on their wings and their courtship behavior enhances the production of flashes perceived by females (White et al., 2015). How much sexual selection shapes the evolution of iridescence in sympatric *Morpho* species is currently unknown, but behavioral experiments carried out in the field in Amazonian Peru highlighted strong visually-based territorial interactions among males from sympatric species and limited species discrimination based on female coloration in males (Le Roy, Roux, et al., 2021).

This raises questions on the key visual cues involved in mate choice, given that the iridescent blue coloration shared by closely-related species encountered within the understory is likely selectively constrained by predators. The iridescent bright blue dorsal coloration of *Morpho* wings contrasts with the brown and matte ventral side, and generates a peculiar visual effect during flight. The combination of the blue flashes produced by the alternate exposure of the bright blue *vs*. brown sides of the wings during flapping flight, in addition to erratic flight trajectories makes these *Morpho* very difficult to follow by predators (Young, 1971), potentially enhancing their evasive capabilities (Murali & Kodandaramaiah, 2020; Vieira-Silva et al., 2024). Experimental trials with evasive prey have shown that predators learn to avoid prey they repeatedly fail to catch (Páez et

al., 2021). The display of iridescent wings could thus be associated with a higher survival rate in nature because of both (i) direct effects, through successful escape of predator attacks, and (ii) indirect effects, by limiting predation attempts by birds recognizing the blue signals and refraining from attacking (Pinheiro et al., 2016). This indirect effect could promote the evolution of convergent blue patterns in sympatric species, similar to the mimicry observed in species with chemical-defenses. In line with this hypothesis, repeated local convergence in the proportion of iridescent blue *vs*. black areas on the dorsal side of the wings have been documented in the sister-species *Morpho helenor* and *Morpho achilles* living in sympatry throughout the Amazonian basin: indeed, parallel geographic variations in the width of the dorsal blue band was detected in these sympatric species (Llaurens et al., 2021). Precise quantification of variations in iridescence is now needed to assess the respective effects of selection by predators and mates, that may drive convergent *vs*. divergent evolution of iridescence in sympatric species sharing the same micro-habitats. Investigating this convergent *vs*. divergent evolution of visual cues that may act as pre-zygotic barriers to gene flow between these two species will shed light on the mechanisms allowing their genetic divergence and coexistence in sympatry.

To do so, we quantified variations of iridescence in the two sister species *M. helenor* and *M. achilles* in different geographic localities to investigate the antagonistic effects of selective pressures generated by mate recognition and shared predation. We used behavioral experiments to test for the effect of this color variation in visual signals in mate preferences. Finally, we studied variations in the volatile compounds produced by wild males and females from both species to explore the

evolution of potentially alternative traits, such as chemical cues, possibly acting as a reproductive barrier and favoring the coexistence in sympatry of closely related *Morpho* species.

Results

We investigated differences in wing iridescence, color perception and color pattern-based mate choice of two sister species of *Morpho*, *M. helenor* and *M. achilles*, which diverged ~ 3.6 Mya (Chazot et al., 2021). We relied on geographic variations of wing color patterns within *M. helenor* (Fig 1) to compare the iridescence of three different populations of *M. helenor* (subspecies *M. helenor bristowi*, *M. helenor theodorus* and *M. helenor helenor*) and test the associated visual effect using visual models.

[Fig 1: Distribution of the sister-species *M. helenor* and *M. achilles* across South America.]

The convergent iridescent signals of sympatric Morpho *species are not differentiable*

We measured the reflectance of *Morpho* wings at different angles of observation and illumination, in two different planes (referred to as proximo-distal and antero-posterior planes) following two complementary protocols (referred to as "specular" and "tilt"; see Fig 2.A and Supporting Information Text 1 for extended methods). We then extracted for each spectrum three commonly-used colorimetric variables: Hue, Brightness and Chroma (analyzed in S4 Fig).

We found that hue and brightness always vary depending on the angle of incidence of the light, as confirmed by PERMANOVAs detecting a significant effect of the "Angle" for all methods of measurements (Fig 2 and statistical analyzes in S1 Table and S5 Fig) confirming that in both species, both sexes are iridescent. Second, the brightness of *Morpho* wings is highly sexually

181 dimorphic, as the effect of sex is always significant. The brightness measured with the "tilt"
182 protocol (Fig 2.F and 2.G) shows that males tend to be brighter than females in both Ecuador and
183 French Guiana.
184
185 **[Fig 2: Differences of hue and brightness on the proximo-distal plane of *Morpho*]**
186
187 To finely appraise the complete spectrum of variation in iridescence within and among *Morpho*
188 species, we then used a multivariate approach allowing to analyze the full reflectance spectra
189 obtained from both proximo-distal and anteroposterior measurements. At the intraspecific level,
190 significant divergence of iridescence between populations was detected (Fig 3.B). In contrast,
191 iridescence did not differ between species living in sympatry (PERMANOVA, *p-value* = 0.084),
192 suggesting that iridescence converges in sympatry (Fig 3.A). In both cases, the interaction term
193 taxon*sex was non-significant, suggesting that the sexual dimorphism of iridescence was similar
194 for all taxa.
195
196 **[Fig 3: Characterization and perception of the iridescent coloration of *Morpho* butterflies.]**
197
198 We then modeled the perception of color contrast in the visual systems of *M. helenor* and UV-
199 sensitive birds respectively, and tested whether the differences of reflectance observed between
200 sexes and species at different angles of illumination could potentially be perceived by a *Morpho*
201 observer or a putative predator. As sexual dimorphism on wing reflectance was detected, we
202 measured the chromatic distances for males and females separately (Fig 3.C). As the results were
203 similar along the two directions of measurements (antero-posterior and proximo-distal), only
204 proximo-distal results are shown below (antero-posterior results in S6 Fig). *Morpho* butterflies and
205 predators can visually perceive the difference in the blue coloration between the allopatric

populations of *M. helenor*: for both *Morpho* and bird visual systems the mean chromatic contrasts are always higher than 1 JND, for both sexes and for all angles. In contrast, the mean chromatic contrasts measured between sympatric species were below 1 JND: the minute differences observed between sympatric species are thus unlikely to be discriminated either by *Morpho* or bird predators.

Evidence of mate preferences based on visual cues within species

We then performed a series of mate choice experiments to identify the visual cues used during mate choice within *M. helenor*. We specifically tested for preferences based on the differences in color measured between the two previously studied populations of *M. helenor* (*M. h. bristowi* and *M. h. theodorus*), which significantly differs in both wing pattern and iridescence.

We first conducted a tetrad experiment using the allopatric subspecies *M. h. bristowi* and *M. h. theodorus*. The highest number of mating events occurred between the two assortative pairs (see Table 1). More than half of the matings occurred between a female and a male of the *M. h. bristowi* population. Indeed, a Chi-square test performed on the contingency table showed a significant departure from random mating (*X-squared = 4.98, p-value = 0.04*), confirmed by a Fisher exact test (*p-value = 0.04*). The time until an assortative mating (*mean* = 48 min, *sd* = 38 min) or until a

224 disassortative mating (*mean* = 48 min, *sd* = 38 min) however did not significantly differ (*T-test;*
225 *stat= -0.27, p-value = 0.79*).
226
227 **[Table 1: Number of mating events involving the different possible pairs of males and females**
228 **from the two subspecies of *M. helenor* (*bristowi vs. theodorus*) in 30 tetrad experiments**.]
229
230 **[Fig 4: Morpho male preference based on visual cues alone.]**
231
232 Different pairs of female dummies were presented to the *M. helenor* males to investigate assortative
233 preference between (sub)species (experiment 1), discrimination of the wing pattern (experiment 2)
234 and discrimination of the iridescent coloration (experiment 3) (see the experimental details in the
235 M&M section).
236
237 *Experiment 1* tested the male assortative visual preference using two dummies each harboring the
238 wings of a female of a different *M. helenor* subspecies. *M. h. theodorus* males did not show any
239 preference for any dummy female, as shown by the probability of approaches and touches that were
240 not different from the random 0.5 expectation (approaches: Wilcoxon test, *p-value=0.764*, Fig 4.A;
241 touches: Wilcoxon test, *p-value=0.287*, Fig 4.B). *Morpho helenor bristowi* males, in contrast,
242 approached and touched a con-subspecific *M. h. bristowi* dummy female significantly more often
243 than expected by chance alone (approach: Wilcoxon test, *p-value* = 0.0448, Fig 4.A; touch:
244 Wilcoxon test, *p-value* < 0.001, Fig 4.B), suggesting a preference toward the *M. h. bristowi* females
245 based on the recognition of visual cues alone.
246
247 *Experiment 2* tested male pattern-based discrimination, using two dummies harboring the wings of
248 *M. h. bristowi* females (i.e. same iridescence), but one of which was modified so as to present the

249 thin blue bands found in *M. h. theodorus* (i.e. a different pattern). *M. h. bristowi* males tended to
250 approach and touch significantly more often the wild-type *M. h. bristowi* female model as compared
251 to the modified *M. h. bristowi* female model exhibiting a *theodorus* pattern (Wilcoxon tests on
252 approaches; *p-value* = 0.0188, on touches; *p-value* < 0.001, see S7 Fig). As both female dummies
253 shared the same blue iridescence, the choice of *M. h. bristowi* males was likely influenced by the
254 difference in the pattern displayed by the dummy females, suggesting that the color pattern is used
255 by males during mate choice.
256
257 *Experiment 3* tested male color-based mate discrimination using two dummies harboring the wings
258 of females from the two subspecies (i.e. different iridescence), but those of *M. h. bristowi* were
259 modified so as to resemble *M. h. theodorus* (i.e. same pattern). *Morpho helenor theodorus* males
260 tended to approach and touch significantly more often the *M. h. theodorus* female model compared
261 to the artificially modified, theodorus-like *M. h. bristowi* female dummy (Wilcoxon test on
262 approaches; *p-value* = 0.0201, on touches; *p-value* = 0.0343, see S8 Fig). As both dummy females
263 shared the same color pattern, the preference expressed by *M. h. theodorus* males likely stems from
264 the differences in the iridescent blue color suggesting color-based discrimination in males.
265 Nevertheless, in experiment 2 and 3, modified dummies were strongly rejected by males. We
266 cannot entirely rule out that the artificial modifications performed on our dummies could generate
267 a specific visual rejection by the males.
268
269 *Visual cues are not pre-zygotic reproductive barriers between the mimetic species* M. h.
270 helenor *and* M. a. achilles
271
272 In a final experiment (experiment 4), we tested whether males from the two sympatric species from
273 French Guiana (*M. achilles* and *M. helenor*) have a visual preference toward their conspecific
274 females: two female dummies harboring the wings of females from the two species were presented

to each male. While none of the males (from either *M. helenor* or *M. achilles* species) touched the female models, they did approach them in the cages. The approach rate and the number of approaches did not significantly differ between *M. helenor* males (rate = 61%; *mean* = 4.21, *sd* = 5.34) and *M. achilles* males (rate = 66%; *mean* = 7.18, *sd* = 8.29; *binomial GLM: p-value = 0.626*), revealing similar levels of male courtship motivation. The independent analysis of the mean preference per species shows that neither *M. helenor* (*Wilcoxon test, p-value = 0.503*) nor *M. achilles* (*Wilcoxon test, p-value = 0.406*) males approached significantly more con-specific females as compared to the hetero-specific females in the cage (Fig 4.C), suggesting that the difference among the convergent color patterns and blue iridescence of female *M. helenor* and *M. achilles* is not perceived by males.

Divergent chemical profiles observed in males between the mimetic species M. helenor *and* M. achilles

We finally investigated the volatile chemical compounds extracted from the genitalia of males and females from the mimetic sympatric species *M. helenor* and *M. achilles*. The nMDS analyses performed (Fig 5) suggest a strong divergence in the chemical profiles between males of the two species, especially for C16 to C30 compounds. This result contrasts with the strong similarity of chemical profiles found between the females of these two species in these longer-chain compounds (S9 Fig).

The PERMANOVA of the chemical spectra of *M. h. helenor* and *M. a. achilles* genitalia revealed a significant effect of sex, species (*p-value* < *0.001* on both the C8 to C16 and the C16 to C30 datasets, see Fig 5) and a significant interaction between the two variables. When analyzing the results for each sex separately (post-hoc), we found a significant effect of the species variable (*PERMANOVA, p-value=0.001* for males and *p-value* =0.049 for females in the C8 to C16 compounds, *PERMANOVA, p-value=0.001* for males and *p-value = 0.048* for females in the C16

to C30 compounds), suggesting a substantial divergence between species in chemical bouquets, especially marked in males.

Next, we performed an Indicator Value Analysis to identify the different compounds significantly associated with each species, separating males and females (Table S2). Among all the annotated compounds, we found traces of beta-ocimene, previously identified as a butterfly pheromone. Interestingly, different proportions of this compound were found on the genitalia of *M. helenor* and *M. achilles* males.

[Fig 5: Divergent chemical compounds found on the genitalia of sympatric *Morpho* males.]

Discussion

Convergent iridescence between sympatric Morpho *butterflies suggests a prominent role of predation on the evolution of iridescence*

Our study revealed a striking similarity in the iridescent coloration of the blue patches in the sympatric species of *M. helenor* and *M. achilles* in French Guiana, in sharp contrast with the divergent iridescence observed between allopatric populations of *M. helenor* sampled in different geographic areas. Together with the convergent evolution of the blue band width previously detected in these sympatric species (Llaurens et al., 2021), this observation is consistent with there being a primary role of visual predation on the evolution of wing coloration in *Morpho* (Chouteau et al., 2016; Stuckert et al., 2014). The results of the visual models showing that these convergent iridescent signals are likely undistinguishable by avian predators further supports there being positive selection generated by predators promoting mimicry in these sympatric species. These results suggest that predation pressure can strongly impact the evolution of iridescence, and are

326 further supported by the lack of directionality for the iridescent signal generated by *Morpho* wings.
327 Directionality in iridescent signals is expected when sexually selected traits incur a predation cost
328 due to their conspicuousness: directionality might enhance signaling to mates while remaining
329 poorly detected by predators (Endler, 1992). Our study shows that *M. helenor* and *M. achilles* wings
330 display a gradual variation of hue and brightness when changing the illumination/observation
331 angles contrary to some other iridescent butterfly species (e.g. *Hypolimnas bolina*; (White et al.,
332 2015)). The lack of signal directionality observed in these *Morpho* species thus suggests that the
333 iridescent signal is likely on display for both potential mates and predators to see. These gradual
334 changes in hue and brightness could also enhance the confusing effect generated by the alternate
335 exposure of the iridescent and brown side of their wings during erratic flapping flight (Le Roy,
336 Amadori, et al., 2021; Young, 1971).
337 Overall, the lack of directionality and the gradually changing aspect of *Morpho* iridescent signals
338 do not point at any private iridescent signal. Instead, these visual effects are consistent with the
339 emission of a confusing visual signal that increases the chances of escaping predators. The
340 convergence of visual signals could have been favored because of the indirect advantage that results
341 in a reduction in attacks from predators that have experienced and learned to avoid the escape
342 capabilities of these prey, consistent with the hypothesis of escape mimicry (Pinheiro & Freitas,
343 2014; Ruxton et al., 2004).
344 Interestingly, our reflectance measurements showed that males are generally brighter than females
345 in both species, implying that the flashes generated by males during flight could be more
346 conspicuous. Field experiments have highlighted the territorial behavior of males found patrolling
347 along rivers and displaying competitive interactions with other males (Le Roy, Roux, et al., 2021).
348 This flight behavior strongly differs from female behavior, which are much less frequently
349 observed in the wild and typically spend more time hidden in the understory (Young, 1973).
350 Considering that these ecological niche differences between males and females could result in

351 different predation pressures in contrasted light conditions (open river banks *vs*. cluttered
352 understory), iridescent signals could have evolved differently in the two sexes, consistent with a
353 similar effect of divergent micro-habitats of males and females in both species (Allen et al., 2011;
354 Reimchen & Nosil, 2004).
355 Altogether, our results show that natural selection exerted by predators may have favored the
356 evolution of mimetic iridescent color patterns, as well as sexual dimorphism in sympatric *Morpho*
357 species.
358
359 *Evolution of visual and olfactory cues in mimetic sister-species living in sympatry*
360
361 Our tetrad and male mate choice experiments in *M. helenor* butterflies clearly show assortative
362 mating based on wing coloration. This result is consistent with the high attraction generated by blue
363 coloration in males, as well as the preference of males for local *vs*. exotic wing color patterns found
364 in previous field behavioral experiments carried out in Amazonian Peru (Le Roy, Roux, et al.,
365 2021). Our male mate choice experiments suggest that both the wing patterning and the iridescent
366 properties of the blue patch could have an effect on male preference. Furthermore, visual modeling
367 also suggests that the differences of iridescent coloration between the populations of the same
368 species can be perceived by mates. Coloration and color perception are key in mate recognition in
369 many taxa (marine invertebrates (Baldwin & Johnsen, 2009), birds (Caro et al., 2021), mammals
370 (Waitt et al., 2003)) but patterns can also inform mate choice (Houde, 1987; Pérez-Rodríguez et
371 al., 2017). In jumping spiders, both pattern and coloration are important cues used during mate
372 choice (Zhou et al., 2021). Similarly, in *Heliconius* butterflies, both pigmentary coloration and wing
373 pattern are used in mate choice, although the preference is hierarchical and coloration is the most
374 important cue (Finkbeiner et al., 2014). Further experiments are needed to disentangle the relative

375 effects of iridescent coloration and wing pattern in mate preference in *Morpho* butterflies, but our
376 results show that iridescent color patterns can be used as mate recognition cues in *M. helenor*.
377
378 Interestingly, intraspecific variations in courtship motivation were detected in our behavioral
379 experiments within the species *M. helenor*. Individuals from the western areas of the Andes
380 displayed high courtship motivation while butterflies from the Amazonian area, where *M. helenor*
381 coexists with *M. achilles,* showed a strong decrease in responses to our visual dummies. The joint
382 convergence of the color pattern and blue iridescence between *M. helenor* and *M. achilles* in
383 Amazonia probably makes visual cues poor species recognition signals: visual modeling even
384 suggests that *M. helenor* cannot perceive the color difference between the two. This is further
385 supported by our male choice experiment showing that although males are attracted by blue
386 iridescent colors and approach each female, they do not preferentially interact with their conspecific
387 female dummies. Selection on visual preference might be relaxed in Amazonian areas where the
388 two sister species are found in sympatry: the shared predator communities may strongly favor
389 convergent evolution of dorsal iridescent color pattern, making visual signals poor species
390 recognition cue.
391
392 The convergent evolution of iridescence in sympatric species might thus promote divergent
393 evolution of alternative mating cues. By quantifying chemical compounds found on the genitalia
394 of males and females sampled in Amazonian French Guiana, we did indeed find a strong divergence
395 in the chemical profiles of sympatric *M. helenor* and *M. achilles,* especially in males. High male
396 chemical divergence in sympatric species suggests the evolution of female mate choice based on
397 olfactory discrimination (Mérot et al., 2015). Although all compounds were not identified, some of
398 those that were have previously been identified as pheromone compounds used during male/female
399 interactions in butterflies. In particular, beta-ocimene was found on the genitalia of males and is

known to contribute to mate choice in courtship (Li et al., 2017) or as an anti-aphrodisiac used by *Heliconius* males to repel other males after mating with a female (Schulz et al., 2008). Additionally, we found strong divergence between males for heavier chemical compounds (C16-C30). Because cuticular hydrocarbons can be involved in mate discrimination during courtship (Ômura et al., 2020), diverging non-volatile compounds could also be used as a discriminating cue. Although we did not perform mate choices to test for the discrimination of olfactory cue by females, divergence of odorant cues could be due to reproductive interference between sympatric species (Bacquet et al., 2015; Dyer et al., 2014). Altogether, our results therefore strongly suggest that the convergent evolution of iridescent wing pattern between the sympatric *M. helenor* and *M. achilles* in Amazonia, likely promoted by shared predation pressures, have relaxed selection on visually-based preferences and may have favored the evolution of divergent olfactory cues.

Conclusions

Overall, our study shows that local ecological interactions could result in iridescent color patterns and mate preferences evolving separately in closely-related sister species. We find evidence of converging iridescent patterns in sympatry suggesting that predation could play a major role in the evolution of iridescence, by favoring the emergence of evasive mimicry in a similar manner as the Müllerian mimicry of chemically-defended species (Pinheiro et al., 2016). There are nevertheless striking differences between the two types of mimicry: while a wide diversity of conspicuous color patterns are involved in Müllerian mimicry (MALLET & GILBERT, 1995), the variation of iridescent patterns involved in evasive mimicry might be more tightly constrained, because iridescence itself directly participates in survival via visual confusion (Murali & Kodandaramaiah, 2020). Moreover, switches in color pattern is generally considered as a magic trait facilitating speciation in chemically defended species (Merrill et al., 2012), and Müllerian mimicry frequently

occurs among distantly-related species (Puissant et al., 2023). Here we observe the co-existence of *Morpho* sister-species displaying convergent color patterns, suggesting that speciation can be promoted by the evolution of traits other than visual such as olfactory chemical cues, or by the specialization in other ecological temporal niches (Le Roy, Roux, et al., 2021).

Altogether, this study shows how convergent trait evolution might lead to mutualistic interactions enhancing species coexistence (Boussens-Dumon & Llaurens, 2021), and alter how selection acts on other traits. It illustrates how the feedback between trait evolution and ecological interactions can fuel speciation and co-existence in sympatry.

Materials and Methods

I. Study system

We investigated trait variations in the sister-species *M. achilles* and *M. helenor*, that occur in sympatry in the Amazonian basin (Fig 1), with two main sampling schemes, focusing on intraspecific and interspecific variations respectively.

M. helenor populations of Ecuador: Pupae from the Ecuadorian populations of *M. h. bristowi* and *M. h. theodorus* were purchased from a breeding farm located in Ecuador (Quinta De Goulaine, https://quintadegoulaine.com/es/papillons.php). These two subspecies of *M. helenor* are found in western *vs*. eastern areas of the ecuadorian Andes respectively. These commercially-bought pupae were then raised in insectaries at STRI in Gamboa, Panama, between January and March 2023.

Sympatric species of French Guiana: *M. h. helenor* and *M. a achilles* individuals were wild-caught and raised in insectaries between July and September 2023 in the Amazonian forest of the Kaw Mountains (GPS coordinates: 4.57, -52.21), French Guiana, France.

449

450 # II. Quantification and characterization of coloration and iridescence
451 of *Morpho* butterfly wings

452

453 ## A. Measures of wing reflectance

454

455 We investigated the variations in iridescence in *M. h. bristowi*, *M. h. theodorus* (from Ecuador), *M.*
456 *h. helenor* and *M. a. achilles* (from French Guiana), using 10 females and 10 males of each. We
457 estimated the iridescence of the blue patches of the dorsal side of the wings by performing a series
458 of reflectance measurements on wings positioned on a flat surface, following a template to ensure
459 that the wing orientation remained the same across all measurements. Reflectance spectra captured
460 at different angles were recorded on the right forewing, at a specific point located in the wing zone
461 defined by the M3 and Cu1 veins according to the nymphalid ground plan (see (Martin & Reed,
462 2010)). Wing reflectance was measured using a spectrophotometer (AvaSpec-ULS2048CL-EVO-
463 RS 200-1100nm, Avantes) coupled with a deuterium halogen light source (AvaLight-DH-S-BAL,
464 Avantes) for 21 combinations of illumination and observation angles, covering the proximo-distal
465 and anteroposterior planes of the wing (Fig 2.A, see Supporting Information Text 1 for extended
466 methods).
467 These measurements were repeated 3 times for all individuals to estimate the repeatability of the
468 reflectance measurements at each angle using the R package *rptR* 0.9.22 (Stoffel et al., 2017). Since
469 no difference was detected between replicates for any of the tested angles (S12 Fig), the mean of
470 these 3 measurements at each angle for each individual was used in the subsequent analyses.

471

472 ## B. Statistical analysis of iridescence

473

Each illumination/observation position produced a reflectance spectrum whose characteristics (*i.e.* distribution of reflectance) define the optical properties of the illuminated surface. We focused our analyses on the wavelength range [300, 700 nm], relevant for butterfly and bird observers. All reflectance spectra were analyzed using the R package *pavo2* 2.9.0 (Maia et al., 2019).

a. Extraction and analysis of colorimetric variables

To identify the main optical effect produced by the wing at each angle, we extracted three commonly-used colorimetric variables from each spectrum, using the *summary()* function in *pavo2*: namely Hue (color in the common meaning of the term - measured as the wavelength at the maximum reflectance), Brightness (average proportion of reflected light - measured by the mean reflectance over each spectra) and Chroma (also referred to as saturation, it characterizes the color's purity - measured as the ratio of the reflectance range of each spectra by the brightness). Because the normality and heteroscedasticity assumptions were not met for the distributions of values in these three parameters, we relied on permutation-based ANOVAs (*vegan 2.6.4* package, (Oksanen et al., 2001)) to test the effect of sex, angle of measurement, locality and species on these three colorimetric variables. To specifically test whether differences in iridescence patterns were observed among species or localities, we estimated the interaction between the effects of *Morpho* populations and of the angle of illumination.

b. Global iridescence analysis

As iridescence broadly refers to any change in reflectance in relation to illumination and observation angle (Doucet & Meadows, 2009), we characterized the iridescence of each individual using all complete spectra obtained at all angular positions (400 wavelengths x 21 angular

positions). We applied a Principal Component Analysis (PCA) to this dataset, where each dot corresponds to the iridescence of an individual wing and thereby showing the similarities and differences in iridescence among groups. The samples from Ecuador and French Guiana were analyzed separately to test for more precise differences in iridescence between allopatric and sympatric *Morpho* butterfly populations. The coordinates of the 10 first PCs (representing 98% of the explained variance) were then extracted and a PERMANOVA was performed to test for the effect of sex, locality and species on iridescence patterns.

III. Modeling how *Morpho* butterflies and avian predators perceive *M. helenor*

To investigate whether the variations observed among the different spectra could be perceived by different observers, we then used the R package *pavo2* 2.9.0 (Maia et al., 2019) to model both avian and butterfly visual capabilities. We used the avian UV-sensitive vision model implemented in *pavo* ("*avg.uv*") as a proxy for predator vision and specifically built a model of *M. helenor* vision, using the spectral sensitivity measured in (Pirih et al., 2022). We built an hexachromatic visual model using the *sensmodel* function, using 345, 446, 495, 508, 570 and 602 nm as the maximum of absorbance in our six simulated cones respectively (S10 Fig). The *vismodel* function was used to compute the quantum catch of each receptor in the *Morpho* vision model under standard daylight illumination for each spectrum. Finally, assuming that the discrimination of colors is limited by photoreceptor noise (Receptor-Noise Limited model, (Vorobyev et al., 2001)), we estimated the distance between colored stimuli as perceived by Morphos using the *bootcoldist* function. We used the default options (*weber* = 0.1, *weber.achro* = 0.1) and set the photoreceptor densities for each cones used in the visual model to 1. The resulting chromatic distances (*dS*) and confidence interval were then used to test how much the visual capacities of *Morpho* butterflies could allow the discrimination of wing reflectance depending on sex, localities and species, at each angle.

525 Similarly, we used the avian UV sensitive visual model implemented in *pavo* to test the
526 discrimination of *Morpho* wing reflectance by predators. We used $dS \geq 1$ *Just Noticeable Difference*
527 as the threshold of discrimination.
528

529 # IV. Mate choice experiments

530

531 All mate choice experiments took place in cages of similar dimensions (3m x 3m x 2m). All *Morpho*
532 butterflies were fed 30 minutes before the beginning of the experiments taking place between 8am
533 and 12am.
534

535 ## a. Testing for mate discrimination within *M. helenor*

536

537 To study the discrimination of mating partners within the species *M. helenor*, tetrad experiments
538 were performed between *M. h. bristowi* and *M. h. theodorus*. For each experiment, one virgin
539 female and one mature male of both *M. h. bristowi* and *M. h. theodorus* were placed within the
540 same cage. Their starting positions in each corner of the cage were randomized. The first mating
541 occurring out of the four possibilities was recorded and the experiment was stopped. Experiments
542 could also be ended when no mating occurred within 5 hours of trial. We carried out N = 33 trials
543 to estimate whether the first mating observed preferentially involved assortative pairs, due to either
544 male and/or female mate preference, of which N=30 trials actually resulted in assortative or
545 disassortative matings. We statistically tested for departure from the random mating expectation
546 using a *chi*-square test of independence, confirmed with a Fisher's exact test.
547

548 Note that *M. achilles* is not commercially bred and wild females are usually all mated, preventing
549 experiments on virgin females in this species, and thus restricting experiments on virgin females in
550 the commercially-bred species *M. helenor*.
551

552 **b. Identifying specific visual cues used during mate choice by *M.***
553 ***helenor* males**
554

555 To investigate the visual preference of *M. helenor* males for the dorsal color pattern of females, we
556 performed several choice experiments using dummy butterflies built with actual female wings. To
557 ensure that preferences were not triggered by olfactory cues, all female wings used to build the
558 artificial dummies were washed with hexane before any experiment. The details of the experimental
559 design and specific visual cues tested are described in Figure 6.
560

561 First, we tested male preference for the visual appearance of *M. h. bristowi* and *M. h. theodorus*
562 females (Experiment 1). Since *M. h. bristow*i and *M. h. theodorus* females differ both in terms of
563 color patterns (width of blue band) and iridescence, we then conducted experiments to isolate these
564 two components.
565 We specifically tested the effect of the proportion of blue to black areas (Experiment 2) by
566 presenting males *M. h. bristowi* to female dummies harboring *M. h. bristowi* wings, except one was
567 modified with black ink to resemble the narrow blue band on *M. h. theodorus* female wings.
568 We finally tested the effect of the blue iridescent band within the same black and blue pattern
569 (Experiment 3) by presenting *M. h. theodorus* males to a *M. h. theodorus* female with a narrow
570 blue band, and to the modified *M. h. bristowi* female dummy with a narrow band. The wing pattern
571 modifications were performed using a black permanent marker.

To ensure that ink odor would not bias the behavior of *Morpho* males, all dummies used in experiments 2 and 3 contained traces of black marker (either on the modified bands or on the ventral side of the wings of natural females).

In all experiments, two female dummies were hanging from the ceiling of the cage, 1.5 m apart, and presented to a live male. The male was released in the experimental cage in front of the two female dummies and was free to interact with them for 10 minutes. The positions of the female models were swapped 5 minutes into each trial to avoid potential position-related bias. Two types of male behaviors were recorded. Each time a male flew within a 40 cm radius from a female model, an "approach" was recorded. Each time a male touched a female dummy, a "touch" was recorded. The number of "approaches" and "touches" toward each female dummy per trial was used to determine male preference. Each male performed this experiment 3 times on 3 different days to account for potential individual variations in mate preference.

c. Testing whether visual cues can act as a pre-zygotic barrier between *M. h. helenor* and *M. a. achilles*

In order to investigate the importance of color vision in species recognition, we then performed a male choice experiment with *M. h. helenor* and *M. a. achilles* (experiment 4 in Figure 6) in French Guiana, using natural wings of females *M. h. helenor* and *M. a. achilles* as artificial butterflies following the exact same protocol.

[**Figure 6: Experimental design of the male choice experiments**]

a. Statistical analyses

Comparing male mating motivation

Because we noticed behavioral differences between the species and subspecies of *Morpho* during the course of the experiments 1-4, we attempted to account for variations in male mating motivation. The approach and touch rates of males, *i.e.* their willingness to approach and touch (respectively) any female dummies during each trial, was defined as the proportion of trials where the males approached and touched (respectively) either female dummies at least once. The approach rate and touch rate of *M. h. bristowi*, *M. h. theodorus, M. h. helenor* and *M. a. achilles* males were calculated, and a binomial generalized linear model (GLM) was used to test whether the approach or touch rate was significantly different between males from different populations. All statistical analyses were performed with the R package *glmmTMB* (Brooks et al., 2017). The normality, homogeneity of variance and overdispersion tests for the GLMs were performed using the R package *DHARMa* (Hartig, 2022). We controlled for potential individual variations by using the male ID and trial number as a random effect in all the tests.

Visually-based male mate preferences

We then tested for male preferential attraction for different dummy females. The mean proportion of approaches and touches toward each female dummy was calculated over the four types of trials performed by each male. Non-parametric Wilcoxon tests were used to test whether the mean probability of approaching or touching a dummy 1/dummy 2 was significantly different from the 0.5 proportion expected under random mating. A significant male preference towards either dummy was detected when the mean probability was significantly above 0.5.

V. Pheromone analysis for sympatric species

622 ## a. Chemical analyses

623

624 We investigated variations in chemical compounds between the two sympatric species *M. helenor*
625 and *M. achilles* sampled in French Guiana. The male and female genitalia of wild caught *M. h.*
626 *helenor* and and *M. a. achilles* individuals (from 9 up to 12 per sex per species) were dissected and
627 extracted in 20µL of dichloromethane, containing 5µL of octadecane as an internal standard. Two
628 sets of chromatography were performed in order to extract either the light compounds (all volatile
629 carbonate compounds below C16) or the heavier ones (all carbonate compounds above C16) found
630 on the genitalia of those Morphos.

631 The chemical compounds of those samples were analyzed by gas chromatography and time-of-
632 flight mass spectrophotometry (GC-ToF-MS) using a 8890 GC system (Agilent, Santa Clara, USA)
633 coupled to a PEGASUS BT mass spectrophotometer (LECO, Saint Joseph, USA). The connected
634 capillary column was a Rxi-5ms (30 m x 0.25 mm, df = 0.25 µm, Restek, Bellefonte USA) and
635 helium was used as carrier gas at 1 mL min^{-1} constant flow rate. 1 µL of each sample was injected
636 in a GC split/splitless injector at 300 °C. Two temperature programs were used: (i) the analysis of
637 the C8-C16 compounds started at 40 °C maintained for 1 min, then increased at 210°C at 6 °C min^{-}
638 ^{1} rate, finally increased to 340 °C at a 10 °C min^{-1}, and (ii) the analysis of the C16-C30 compounds
639 started at 70 °C maintained for 1 min, then increased to 150 °C at 30 °C min^{-1} rate and finally
640 increased to 340 °C at a 6 °C min^{-1} rate. Mass spectra were recorded with a 70eV electron impact
641 energy. Mixtures of alkanes C8 to C19 and C16 to C44 (ASTM® D5442 C16-C44 Qualitative
642 Retention Time Mix, Supelco, Bellefonte, USA) were injected under the same conditions to be
643 used as external standards.

644 The chromatograms were converted in netCDF format and operated using *MZmine 2.43* for
645 compound detection and area integration. To this aim, chromatograms were cropped before 4 min
646 and after 27 min, and baseline-corrected using the Rolling ball algorithm (Kneen & Annegarn,

647 1996). Mass detection was performed using the centroid mass detector, and chromatograms of each
648 mass were constructed using the ADAP algorithm (Myers et al., 2017; Pluskal et al., 2010).
649 Chromatograms were deconvoluted into individual peaks using the local minimum search
650 algorithm and aligned on a master peak list with the RANSAC algorithm. Finally, the Peak Finder
651 gap filling algorithm was used to search for missing peaks, and a matrix compiling the area under
652 each peak detected for each butterfly sample was extracted. This matrix was purged from siloxane
653 like compounds as well as compounds present in less than 10% of the samples. The areas of the
654 blanks were subtracted to the samples, and the final data were used for statistical analysis. Mass
655 spectra were then visualized using the ChromaTOF® software, compared to MZmine-selected
656 ones, in order to manually annotate the chemical compounds. Annotation were based on scan
657 comparison with the NIST2017 database, and linear retention indexes (LRI) calculated from the
658 alkane mixtures with free database (https://pubchem.ncbi.nlm.nih.gov/) and literature (Adams,
659 2017).
660

661 ## b. Statistical analyses of the chemical compounds

662

663 In order to quantify variations in chemical compounds detected in the different sample, we
664 performed a nonmetric multidimensional scaling (NMDS) ordination using the Bray-Curtis
665 dissimilarity calculation. By considering the amount of each compound found in our samples as a
666 variable, we performed a permutation MANOVA with the *adonis2()* function found in the *vegan 2*
667 package on R. We tested whether the sex or the species had a significant effect on the chemical
668 bouquet produced by their genitalia.

We also performed an Indicator Value Analysis to identify the chemical compounds significantly associated to our different *Morpho* samples with the *mutlipatt* function from the *indicspecies* package in R using the IndVal index from (Dufrêne & Legendre, 1997).

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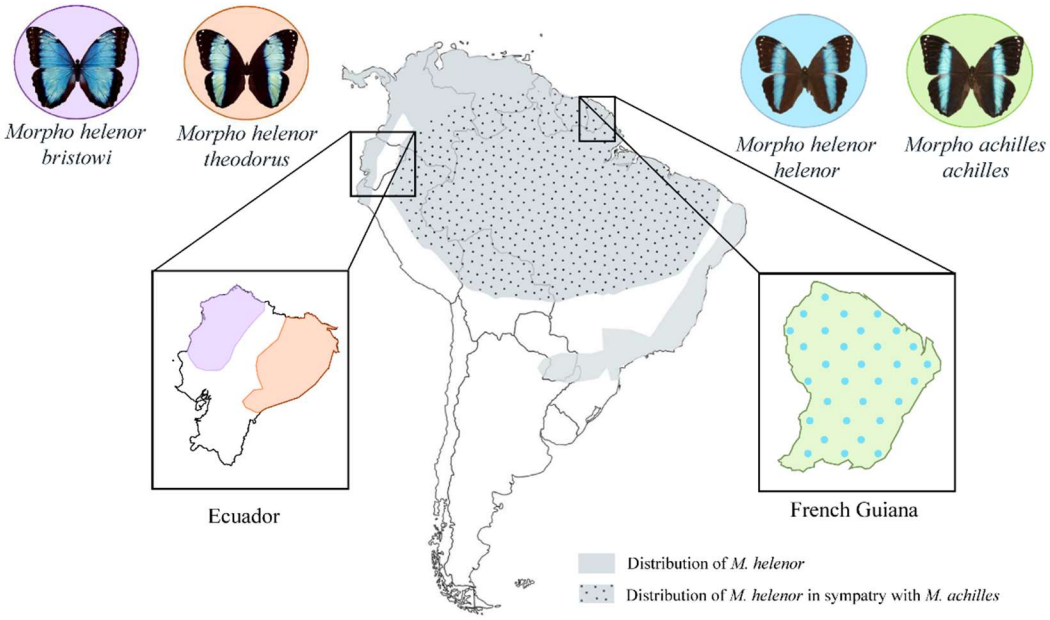
933

934 # Figures and Tables

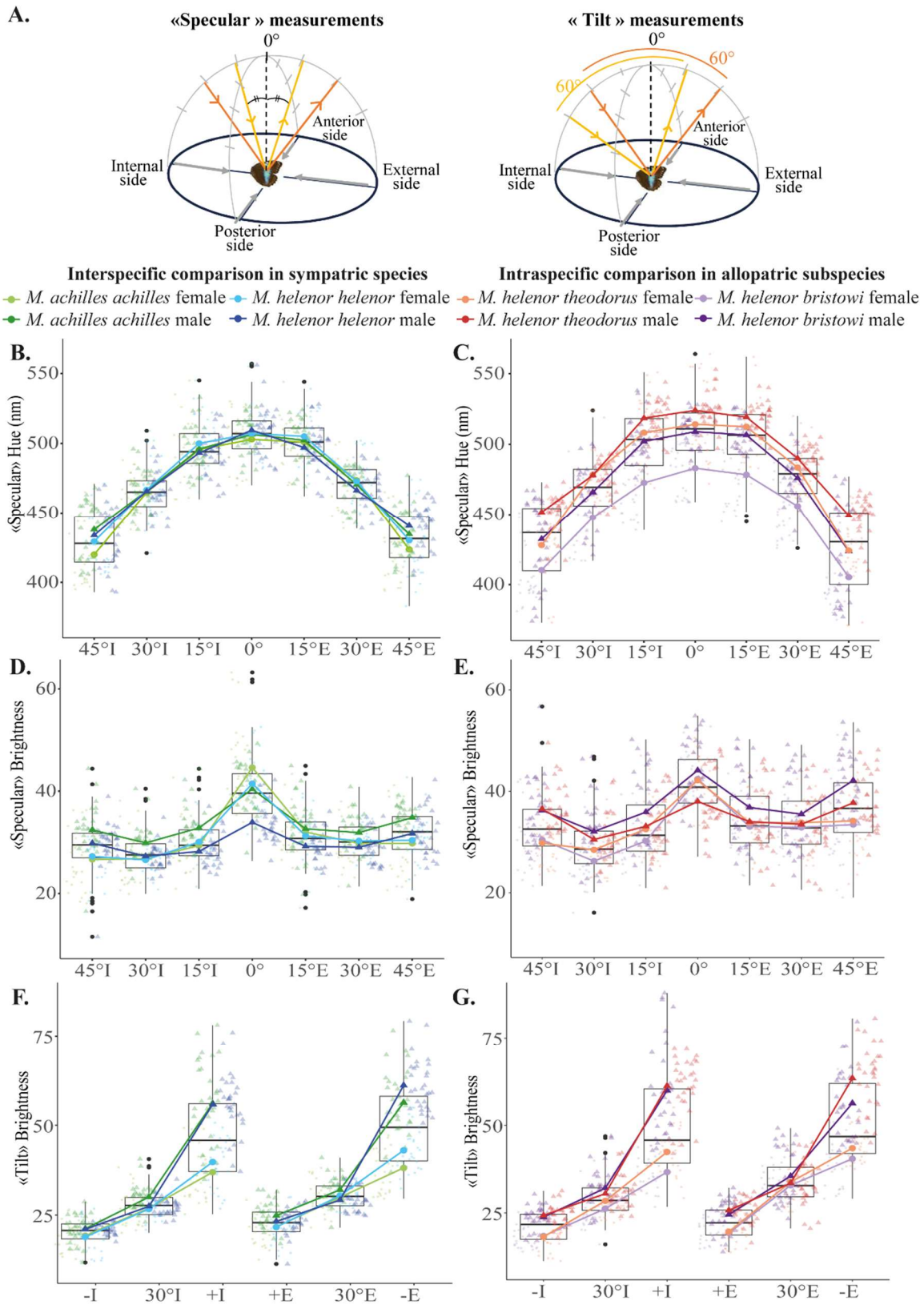


935

936 **Fig 1: Distribution of the sister-species *M. helenor* and *M. achilles* across South America**. The
937 gray area represents the whole distribution of *M. helenor*, and the dotted area indicates the localities
938 where *M. helenor* is in sympatry with *M. achilles*. Note that the different subspecies of *M. helenor*
939 are found in different localities and display substantial variations in the proportion of black *vs*. blue
940 areas on the dorsal sides of their wings. For example, *M. h. bristowi* and *M. h. theodorus* are
941 allopatric in Ecuador, with their respective distribution separated by the Andes such that they are
942 never in contact. While *M. h. bristowi* is found on the Pacific side of the country and has a wide
943 blue band on the dorsal side of its wings, *M. h. theodorus* is found in Western Amazonia and
944 displays a narrow blue band on the dorsal side of its wings. However, *M. helenor* is sympatric with
945 *M. achilles* throughout the Amazonian rainforest. In French Guiana, the subspecies *M. helenor*

946 *helenor* and *M. achilles achilles* display convergent dorsal color patterns with thin blue bands

947 (Llaurens et al., 2021).


A.
«Specular » measurements
0°
Anterior side
Internal side
External side
Posterior side
« Tilt » measurements
0°
60°
60°
Anterior side
Internal side
External side
Posterior side
Interspecific comparison in sympatric species
M. achilles achilles female
M. helenor helenor female
M. achilles achilles male
M. helenor helenor male
Intraspecific comparison in allopatric subspecies
M. helenor theodorus female
M. helenor bristowi female
M. helenor theodorus male
M. helenor bristowi male
B.
C.
«Specular» Hue (nm)
550
500
450
400
45°I 30°I 15°I 0° 15°E 30°E 45°E
D.
E.
«Specular» Brightness
60
40
20
45°I 30°I 15°I 0° 15°E 30°E 45°E
F.
G.
«Tilt» Brightness
75
50
25
-I 30°I +I +E 30°E -E

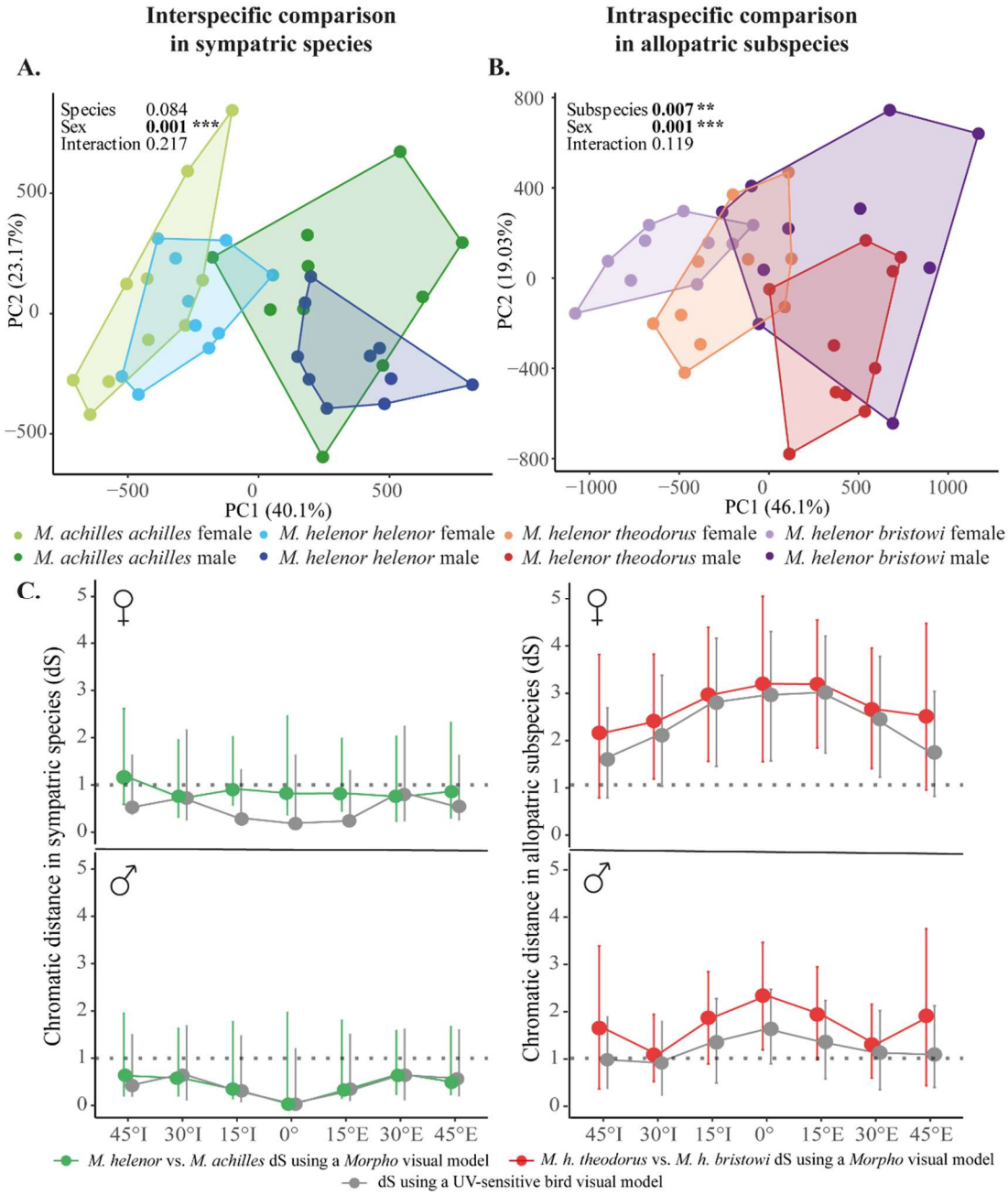
948

949 **Fig 2: Differences of hue and brightness on the proximo-distal plane of *Morpho* wings** (A)
950 Illustration of the two protocols used to assess the differences in *Morpho* wing reflectance. The
951 "Specular" set-up allows for the quantification of the wing color variations, while the "tilt" set-up
952 can be used to quantify brightness variation for each *Morpho* wing (see Appendix for extended
953 methods). Variations of hue (B and C) and brightness (D and E) calculated from the wing
954 reflectance measured with the "Specular" set-up, and variations of brightness calculated from the
955 wing reflectance measured from the "tilt" set-up (F and G). Those hue and brightness parameters
956 were calculated for the sympatric *M. h. helenor* and *M. a. achilles* (first column in green and blue)
957 and for the allopatric *M. h. theodorus* and *M. h. bristowi* (second column in orange and purple) on
958 the proximal-plane plane of their wings (I= illumination on the internal side of the wings, E=

959 illumination on the external side of the wings). See S1 Table for the PERMANOVA analyses

960 describing those graphs.

961



962

963 **Fig 3: Characterization and perception of the iridescent coloration of *Morpho* butterflies.** (A)

964 and (B): PCAs showing the variation in iridescence for both sexes, (A) in the two sympatric species

965 from French Guiana (*M. h. helenor* vs. *M. a. achilles*) and (B) in the two allopatric Ecuadorian
966 subspecies of *M. helenor (M. h. theodorus vs*. *M. h. bristowi*). Each point represents the global
967 signal of iridescence of each individual, corresponding to the 21 complete reflectance spectra
968 obtained from the 21 tested angles of illumination. The results of the PERMANOVA are shown on
969 the top left corner of each graph. (C) The chromatic distances (*i.e.* the visual discrimination rate by
970 a visual model) of the wing reflectance measured with the "Specular" set-up on the proximo-distal
971 plane. Visual modeling was used to calculate the chromatic contrast of blue coloration between
972 allopatric *M. helenor* subspecies (red) and between the two sympatric sister-species *M. helenor* and
973 *M. achilles* (green), as perceived by a *Morpho* visual system for every angle of illumination
974 measured on the proximo-distal plane. The chromatic contrast likely perceived by UV-sensitive
975 birds is shown in gray. Chromatic contrast of the female wings (top) and male wings (bottom). The

threshold of discrimination is shown by the dotted line and set to 1 *Just Noticeable Difference* (*JND*).

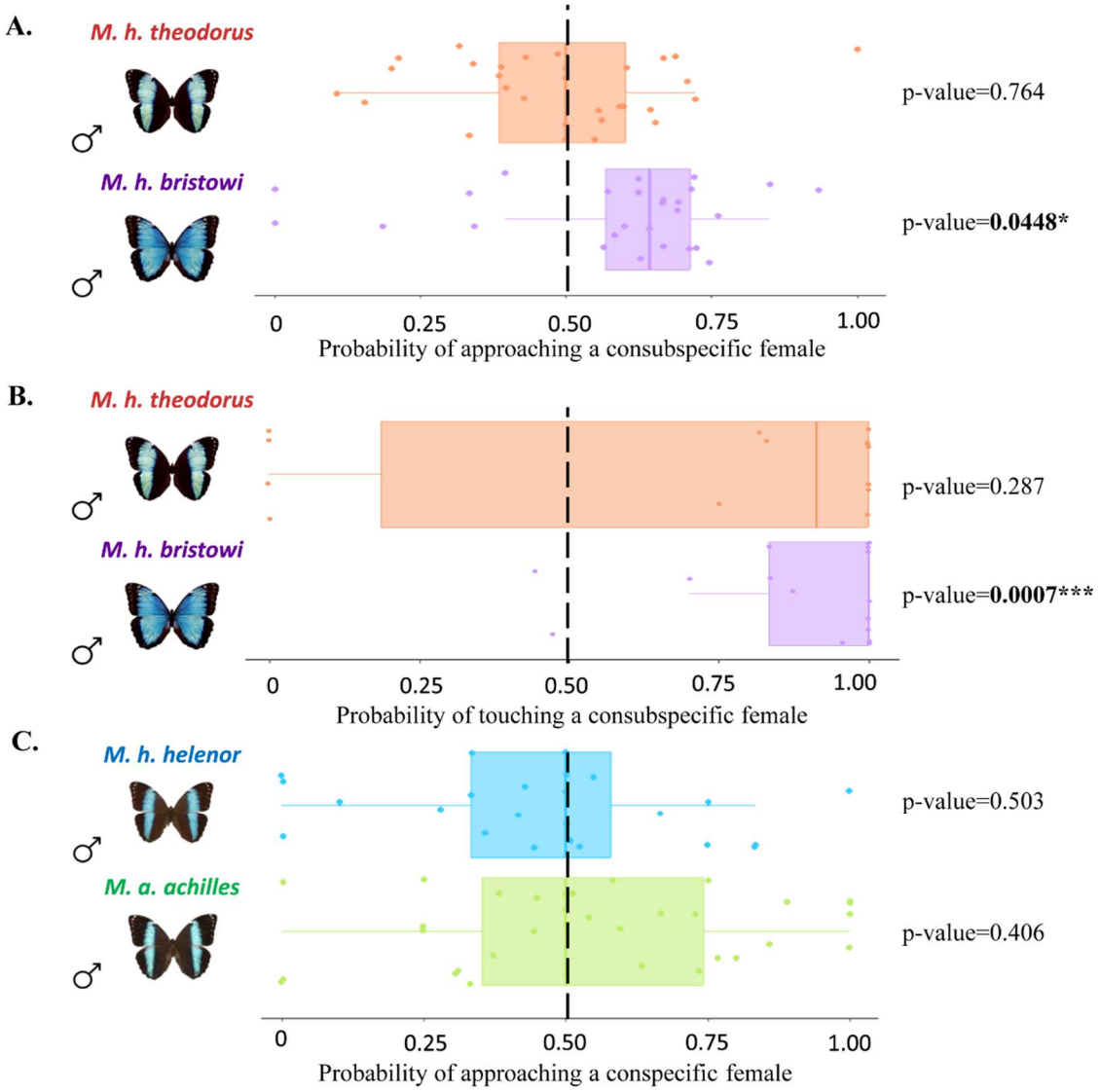
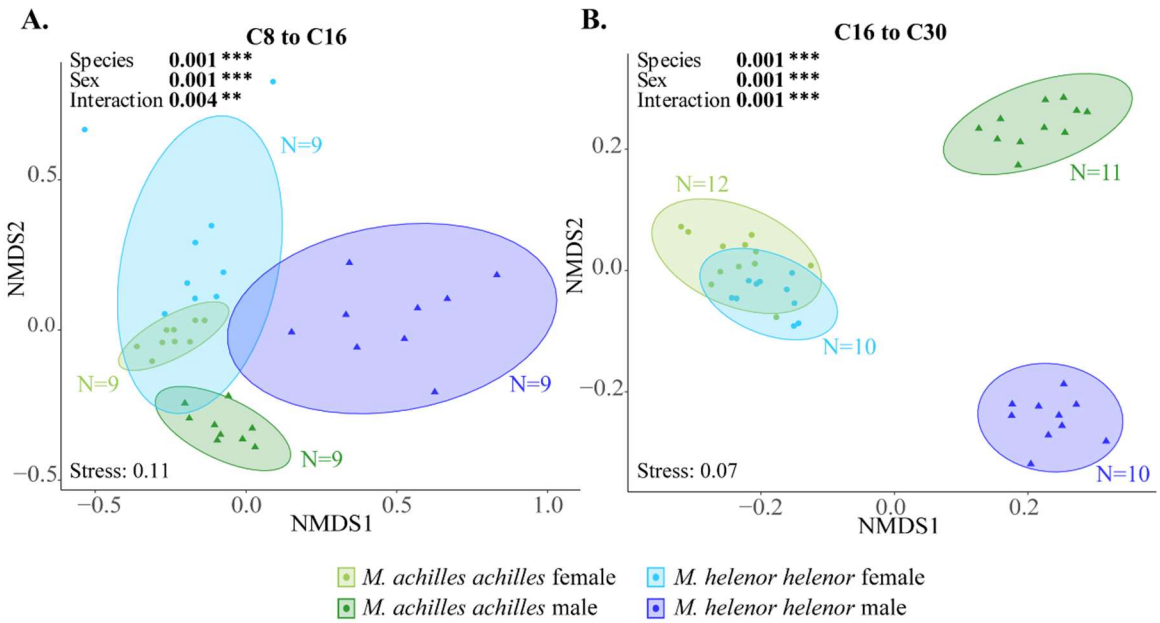


Fig 4: Morpho male preference based on visual cues alone.

Probabilities of (A) approaching and (B) touching a con-subspecific female for *M. h. bristowi* males (purple) and *M. h. theodorus* males (orange) from Ecuador, as well as the probabilities of (C) approaching a con-specific female for *M. h. helenor* males (blue) and *M. a. achilles* males (green)

984 from French Guiana, measured during *Experiment 1*. The dotted line indicates the expected
985 probability of approaching/touching a model if no preference is present. The *p-values* of Wilcoxon
986 tests testing for the significant departure from the 0.5 probability are shown next to the
987 corresponding graphs.

988



989

990 **Fig 5: Divergent chemical compounds found on the genitalia of sympatric *Morpho* males**.
991 nMDS representation of the differences in the (A) C8 to C16 chemical compounds and (B) C16 to
992 C30 chemical compounds found in the genitalia of *M. h. helenor* and *M. a. achilles* males and
993 females, calculated using Bray-Curtis distances. *M. h. helenor* are shown in blue (males are in dark
994 blue and females in light blue), and *M. a. achilles* are shown in green (males are dark green and
995 females are light green). The result of the PERMANOVA (999 permutations) testing the effect of

sex and species on the chemical composition of *M. h. helenor* and *M. a. achilles* are shown on each figure.

Experiments (Location)	Dummy female 1	Dummy female 2	Live males	Test
1 (STRI)	*M. h. theodorus*	*M. h. bristowi*	*M. h. theodorus* (n = 33) *M. h. bristowi* (n = 28)	Assortative preference within species
2 (STRI)	*M. h. bristowi*	*M. h. bristowi* with thinner blue band	*M. h. bristowi* (n = 28)	Discrimination on the pattern
3 (STRI)	*M. h. theodorus*	*M. h. bristowi* with thinner blue band	*M. h. theodorus* (n = 33)	Discrimination on the iridescent blue
4 (French Guiana)	*M. h. helenor*	*M. a. achilles*	*M. h. helenor* (n = 28) *M. a. achilles* (n=37)	Assortative preference between species

Figure 6: Experimental design of the male choice experiments performed between the Morphos originating from Ecuadorian populations of *M. helenor* (eastern population: *M. h. theodorus* and western population: *M. h. bristowi,* experiments 1 to 3) and from French Guiana (*M. h. helenor* and

1003 *M. a. achilles,* experiment 4), along with the associated preferences and visual cues tested. Note
1004 that the same males were used for experiments 1, 2 and 3.
1005
1006
1007
1008
1009 **Table 1: Tetrad experiment results:** Number of mating events involving the different possible
1010 pairs of males and females from the two subspecies of M. helenor (bristowi vs. theodorus) in 30
1011 tetrad experiments

N=30

	M. h. theodorus female	**M. h. bristowi female**
M. h. theodorus male	6	3
M. h. bristowi male	5	16

1012