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

Joséphine Ledamoisel[1,2,3,4], Bruno Buatois[5], Rémi Mauxion[3], Christine Andraud[4], Melanie McClure[6], Vincent Debat[1,2], Violaine Llaurens[1,2]

[1] Centre Interdisciplinaire de Recherche en Biologie (UMR 7241, Collège de France/CNRS/INSERM), Collège de France 11 place Marcelin Berthelot 75005 Paris, France

[2] Institut de Systématique, Evolution et Biodiversité (ISYEB UMR 7205 CNRS/MNHN/SU/EPHE/UA), Muséum National d'Histoire Naturelle - CP50, 45 rue Buffon, 75005 PARIS, FRANCE

[3] Smithsonian Tropical Research Institute (STRI), Apartado, Panamá 0843-03092, Republic of Panama

[4] Centre de Recherche sur la Conservation (CRC), CNRS, MNHN, Paris, France

[5] CEFE, Univ Montpellier, CNRS, EPHE, IRD, Montpellier, France.

[6] Laboratoire Écologie, Évolution, Interactions des Systèmes Amazoniens (LEEISA), Université de Guyane, CNRS, IFREMER, Cayenne, France

*Corresponding author

Email: josephine.ledamoisel@outlook.fr (JL)

Abstract

The evolution of traits in closely related species living in sympatry strongly depends on both shared selective pressures and reproductive interference. 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 mate recognition and courtship. We used spectrophotometry, behavioral experiments, visual modeling and chemical analyses to characterize the evolution of visual and chemical traits in two closely-related species, *Morpho helenor* and *Morpho achilles*. We specifically compared trait variation between samples from allopatric and sympatric populations of *M. helenor* to test the effect of ecological interactions with *M. achilles* on trait evolution. We quantified the differences in wing iridescence and tested for variations in the sexual preference for this trait. We found a strong similarity in iridescence between *M. helenor* and *M. achilles* in sympatry while the iridescence of *M. helenor* diverged in allopatry, suggesting that predation favors local resemblance. Although intraspecific behavioral experiments showed that iridescent signals could be used as visual cues during intraspecific mate choice, the strong resemblance of the iridescent signals between species may impair these species visual recognition. In contrast, the divergent chemical bouquets detected between species suggest that the visual similarity of sympatric *Morpho* species may have favored the divergence of alternative traits involved in species recognition, such as chemical cues.

Introduction

Numerous ecological interactions can impact trait evolution in closely-related species in sympatry, leading to trait convergence or divergence (terHorst et al., 2018). Closely-related species often display similar suites of traits because of their shared evolutionary history (Blomberg et al., 2003). Local selection can also act as a filter and prevent trait divergence (Keddy, 1992), therefore enhancing trait similarity between closely-related species when they occur in sympatry (Chazot et al., 2014). Trait similarity among sympatric species within a given ecological niche can thus stem from retention of locally adapted ancestral traits or from evolutionary convergence (Muschick et al., 2012). Selective pressures promoting the retention of locally adapted traits within species and/or trait convergence among sympatric species can also be due to local ecological interactions: for instance, shared predation pressure may promote the convergence of predator-deterrent traits in sympatry, but allow the trait to differentiate in allopatry (Mallet, 1999).

However, when closely-related sympatric species share a given trait, either as a result of ancestry and/or convergence, they often diverge in other traits because (1) they may be partitioned in different ecological niches (Berlocher & Feder, 2002), or (2) as a result of character displacement due to reproductive interference (Grether et al., 2020) or reinforcement due to poor hybrid fitness (Butlin & Smadja, 2018). As a result, traits involved in sexual competition or mate choice tend to diverge significantly more often between species in sympatry compared to allopatry (Haavie et al., 2004; Marko, 2005).

In this study, we investigate how ecological interactions in sympatry can constrain natural and sexual selection shaping trait evolution. We specifically focus on traits submitted to both natural and sexual selection, and compare differences in these traits in allopatric vs. sympatric ranges.

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; Soni et al., 2024). 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). To determine to what extent ecological interactions shape trait evolution, it is thus necessary to compare patterns of trait evolution in sympatry and allopatry: allopatric populations indeed allow to estimate background levels of divergence or similarity that arise in the absence of direct ecological interactions (Pfennig & Pfennig, 2009). 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 can 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 risk of detection and/or recognition by predators (Finkbeiner et al., 2014; Oliver et al., 2009). Whether these opposite selective pressures ultimately promote trait convergence or divergence in sympatric species might depend on their relatedness: for instance, a recent study in *Papilionidae* showed multiple color pattern convergences between distantly-related species living in sympatry, while divergent

colorations are found in closely-related species (Puissant et al., 2023). Divergence in traits involved in species recognition could be favored because of higher reproductive interference in closely related species than in distantly related taxa (Pfennig & Pfennig, 2009). In sympatric species with chemical-defenses, such as *Heliconinii* butterflies, local predation pressures tend to promote the convergence of similar conspicuous warning wing patterns compared to allopatric species (*i.e.* Müllerian mimicry, Joron et al., 1999; Merrill et al., 2014). But the costs associated with hybrid production in turn favor the evolution of alternative divergent mating cues in mimetic butterflies (Estrada & Jiggins, 2008), and divergence in male pheromone bouquets and female attraction has been found among mimetic sister species (González-Rojas et al., 2020). Similarly, the evolution of specific visual mate recognition signals, limiting reproductive interference but indistinguishable by predators, can also be promoted on the wings of mimetic butterflies (Llaurens et al., 2014).

Here we focus on the evolution of mating cues in the neotropical butterfly genus *Morpho,* where multiple closely-related species co-exist in sympatry (Blandin & Purser, 2013). In the *Morpho* species observed in the understory, striking iridescent blue coloration is displayed on the dorsal side of the wings, due to specific wing scale structure (Giraldo et al., 2016; Siddique et al., 2013). 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). While the iridescent blue color is probably ancestral to the diversification of the understory clade (Chazot et al., 2021), the precise reflectance spectra at different angle likely differs among *Morpho* species. Directional iridescent signals generated in animals can likely enhance recognition by mates while remaining poorly detected by predators (Endler, 1992). In birds (Simpson & McGraw, 2019) and butterflies (White et al., 2015), the specific directional signal produced by the iridescent trait can be used as a cue during courtship, suggesting that the antagonistic sexual and natural selective pressures may finely tune the evolution

115 of iridescent effects. How much sexual selection shapes the evolution of iridescent properties in
116 sympatric *Morpho* species is currently unknown, but behavioral experiments carried out in the field
117 in Amazonian Peru highlighted strong visually-based territorial interactions among males from
118 sympatric species and limited species discrimination based on female coloration in males (Le Roy,
119 Roux, et al., 2021).
120
121 This raises questions on the key visual cues involved in mate choice, given that the iridescent blue
122 coloration shared by closely-related species encountered within the understory is also likely under
123 selection by predators. The iridescent bright blue dorsal coloration of *Morpho* wings contrasts with
124 the brown and matte ventral side, and generates a peculiar visual effect during flight. The
125 combination of the blue flashes produced by the alternate exposure of the bright blue *vs*. brown
126 sides of the wings during flapping flight, in addition to erratic flight trajectories, makes these
127 *Morpho* very difficult to catch by bird predators (Young, 1971), potentially enhancing their evasive
128 capabilities (Murali & Kodandaramaiah, 2020). Experimental trials with evasive prey have shown
129 that predators learn to avoid prey they repeatedly fail to catch (Páez et al., 2021). The display of
130 iridescent wings could thus be associated with a higher survival rate in nature because of both (i)
131 direct effects, through successful escape of predator attacks, and (ii) indirect effects, by limiting
132 predation attempts by birds recognizing the blue signals and refraining from attacking, as
133 highlighted by butterfly release experiments investigating the hunting behavior of wild
134 insectivorous birds in Brazil (Pinheiro et al., 2016). Mark recapture experiments in the field with
135 manipulated dorsoventral contrasts in wild Morphos have suggested that dynamic flash coloration
136 can reduce predation rate (Vieira-Silva et al., 2024). This indirect effect could promote the
137 evolution of convergent blue patterns in sympatric species, similar to the mimicry observed in
138 species with chemical-defenses (Joron et al., 1999; Merrill et al., 2014). In line with this hypothesis,
139 repeated local convergence in the proportion of iridescent blue *vs*. black areas on the dorsal side of

140 the wings has been documented in the sister-species *Morpho helenor* and *Morpho achilles* living
141 in sympatry throughout the Amazonian basin (Llaurens et al., 2021). Precise quantification of
142 variations in iridescence is now needed to assess the respective effects of selection by predators
143 and mates, that may drive convergent *vs*. divergent evolution of iridescence in sympatric and
144 allopatric ranges.
145
146 First, we quantified iridescence in allopatric vs. sympatric populations of *M. helenor* subspecies.
147 Since the coloration is expected to be more similar within than among species under neutral
148 evolution, we used allopatric populations of *M. helenor* as a baseline to assess convergence of
149 iridescence between two sympatric species (*M. helenor* and *M. achilles*). We then conducted
150 behavioral experiments to test the effect of variation in iridescence on mate recognition, using two
151 subspecies of *M. helenor* displaying different iridescent phenotypes. This intraspecific comparison
152 allows identifying the visual cues used in mate choice in *M. helenor,* teasing apart the effects of
153 iridescence and/or wing pattern. We then tested whether those visual cues are used in species
154 recognition between sympatric *M. helenor* and *M. achilles*. Finally, we studied variations in the
155 volatile compounds produced by wild males and females from sympatric populations of the two
156 species to explore the evolution of potentially alternative traits, such as chemical cues, possibly
157 acting as a reproductive barrier.
158

159 Results

160
161 We investigated differences in wing iridescence, color perception and color pattern-based mate
162 choice of two sister species of *Morpho*, *M. helenor* and *M. achilles*, which diverged ~ 3.6 Mya
163 (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 perception of these patterns by predators and mates 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 dimorphic, as the effect of sex was always significant. The brightness measured with the "tilt" protocol (Fig 2.F and 2.G) showed that males tend to be brighter than females in all populations.

[Fig 2: Differences of hue and brightness on the proximo-distal plane of *Morpho*]

To finely appraise the complete spectrum of variation in iridescence within and among *Morpho* species, we then used a multivariate approach allowing to analyze the full reflectance spectra obtained from both proximo-distal and anteroposterior measurements. At the intraspecific level,

189 significant divergence of iridescence was detected between allopatric subspecies of *M. helenor* (Fig
190 3.A). In contrast, iridescence did not differ between species living in sympatry (PERMANOVA,
191 F=2.09, df=1, *p-value* = 0.084), suggesting that iridescence converges in sympatry (Fig 3.B). In
192 both cases, the interaction term taxon*sex was non-significant, suggesting that the sexual
193 dimorphism of iridescence was similar for all taxa.
194
195 **[Fig 3: Characterization and perception of the iridescent coloration of *Morpho* butterflies.**]
196
197 We then modeled the perception of color contrast in the visual systems of *M. helenor* and UV-
198 sensitive birds respectively, and tested whether the differences of reflectance observed between
199 sexes and species at different angles of illumination could potentially be perceived by a *Morpho*
200 observer or a putative predator. As sexual dimorphism of wing reflectance was detected, we
201 measured the achromatic distances and chromatic distances for males and females separately.
202 Achromatic distances results (S6 Fig) suggest that neither birds nor *Morpho* butterflies could
203 theoretically differentiate the brightness of the wings of sympatric or allopatric individuals, based
204 on visual modelling. Concerning chromatic distances, as the results were similar along the two
205 directions of measurements (antero-posterior and proximo-distal), only proximo-distal results are
206 shown in Fig 3.C (antero-posterior results in S7 Fig). Results suggest that *Morpho* butterflies and
207 predators can theoretically visually perceive the difference in the blue coloration between the
208 different subspecies of *M. helenor:* mean chromatic contrasts were always higher than 1 JND, for
209 both sexes and for all angles, using both bird and *Morpho* visual models. However, this effect was
210 stronger for females. In males, confidence intervals were only strictly significant for the 0°

illumination using the *Morpho* visual model, suggesting a weak discrimination at most angles of observation.

In contrast, the mean chromatic contrasts measured between sympatric species were always 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

Experimental evidence has shown that *M. helenor* males are attracted to blue coloration when patrolling in the wild (Le Roy, Roux, et al., 2021). We thus performed a series of mate choice experiments to determine the nature of the visual cues used in mate choice in *M. helenor*. We focused on the two previously studied allopatric subspecies of *M. helenor* (*M. h. bristowi* and *M. h. theodorus*), which significantly differ in both wing pattern and iridescence.

We first tested whether mating tended to be preferentially assortative by conducting a tetrad experiment involving one male and one female of both subspecies. The highest number of mating events occurred between the two assortative pairs (see Table 1). Indeed, a Fisher's exact test performed on the contingency table showed a significant departure from random mating (*p-value = 0.04*). The time until an assortative mating (*mean* = 48 min, *sd* = 38 min) or until a disassortative mating (*mean* = 48 min, *sd* = 38 min) however did not significantly differ (*T-test; stat= -0.27, p-*

231 *value = 0.79*). Note that the effects were clearly driven by the bolder behavior of *M. h. bristowi*
232 individuals, as half of the mating events occurred between a female and a male of this subspecies.
233
234 **[Table 1: Number of mating events involving the different possible pairs of males and females**
235 **from the two subspecies of *M. helenor* (*bristowi vs. theodorus*) in 30 tetrad experiments**.]
236
237 **[Fig 4: Morpho male preference based on visual cues alone.]**
238
239 To test whether this assortative mating was driven by visual cues and to identify these cues, we
240 performed a series of experiments using dummy butterflies treated with hexane to remove any
241 olfactory cue. We specifically focused on studying allopatric *M. h. theodorus* and *M. h. bristowi*
242 because they both differ in their iridescent signal and in their iridescent pattern. Different pairs of
243 female dummies were presented to *M. helenor* males to investigate assortative preference between
244 (sub)species (experiment 1), discrimination of the wing pattern (experiment 2) and discrimination
245 of the iridescent coloration (experiment 3) (see the experimental details in the M&M section).
246
247 *Experiment 1* tested male assortative visual preference using two dummies each harboring the
248 wings of a female of a different *M. helenor* subspecies. *M. h. theodorus* males did not show any
249 preference for any female dummy, as shown by the probability of approaches and touches that were
250 not different from the random 0.5 expectation (Binomial GLMM approaches: Estimate=-
251 0.121±0.091 SE, z=-1.332, *p-value=0.183*, Fig 4.A; touches: Estimate=1.600±1.094 SE, z=1.462,
252 *p-value=0.144*, Fig 4.B). *M. h. bristowi* males, in contrast, approached and touched a con-
253 subspecific *M. h. bristowi* dummy female significantly more often than expected by chance alone
254 (Binomial GLMM approaches: Estimate=0.430±0.105 SE, z=4.11, *p-value* < 0.0001, Fig 4.A;

255 touches: Estimate=1.646±0.273 SE, z=6.029, *p-value* < 0.0001, Fig 4.B), suggesting a preference
256 toward the *M. h. bristowi* females based on the recognition of visual cues alone.
257
258 *Experiment 2* tested male pattern-based discrimination, using two dummies harboring the wings of
259 *M. h. bristowi* females (i.e. same iridescence), but one of which was modified so as to present the
260 thin blue bands found in *M. h. theodorus* (i.e. a different pattern). *M. h. bristowi* males tended to
261 approach and touch significantly more often the wild-type *M. h. bristowi* female model as compared
262 to the modified *M. h. bristowi* female model exhibiting a *theodorus* pattern (Binomial GLMMs
263 approaches; Estimate=0.599±0.089 SE, z=6.751, *p-value* < 0.0001, touches;
264 Estimate=2.805±0.944 SE, z=2.972, *p-value* = 0.003, see S8 Fig). As both female dummies shared
265 the same blue iridescence, the choice of *M. h. bristowi* males was likely influenced by the difference
266 in the pattern displayed by the dummy females, suggesting that the color pattern is used by males
267 during mate choice.
268
269 *Experiment 3* tested male color-based mate discrimination using two dummies harboring the wings
270 of females from the two subspecies (i.e. different iridescence), but those of *M. h. bristowi* were
271 modified so as to resemble *M. h. theodorus* (i.e. same pattern). *M. h. theodorus* males tended to
272 approach and touch significantly more often the *M. h. theodorus* female wild-type model compared
273 to the artificially modified, theodorus-like *M. h. bristowi* female dummy (Binomial GLMMs on
274 approaches; Estimate=0.348±0.121 SE, z=2.867, *p-value* = 0.004, on touches;
275 Estimate=1.172±0.465 SE, z=2.521, *p-value* = 0.012, see S9 Fig). As both dummy females shared
276 the same color pattern, the preference expressed by *M. h. theodorus* males likely stemmed from the
277 differences in the iridescent blue color, suggesting color-based discrimination in males.
278 In experiment 2 and 3, modified dummies were each time strongly rejected by males. Although all
279 dummies contained traces of black marker to control for manipulation and treatment, we cannot

entirely rule out that the artificial modifications performed on dummies could generate a specific visual rejection by males, because we cannot be sure whether butterflies perceive these modifications as equivalent to natural coloration. These experiments nevertheless suggest that visual cues – color pattern and iridescence – jointly contribute to mate choice in *M. helenor*.

Visual cues are not pre-zygotic reproductive barriers between the mimetic species M. h. helenor *and* M. a. achilles

We then tested whether these visual cues allowed males to express preferences toward their own species in the sympatric range of *Morpho achilles* and *M. helenor*. In a final experiment (experiment 4), we thus investigated whether males from the two species *M. achilles* and *M. helenor,* sampled in their sympatric range (French Guiana), have a visual preference toward their conspecific females: two female dummies harboring the wings of females from the two species were presented to each male. While none of the males (neither from *M. helenor* nor from *M. achilles*) 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* (rate = 61%; *mean* = 4.21, *sd* = 5.34) and *M. achilles* males (rate = 66%; *mean* = 7.18, *sd* = 8.29; *binomial GLMM: p-value = 0.626*), revealing similar levels of male courtship motivation. The independent analysis of the preference per species (Fig 4.C) shows that *M. helenor* males did not significantly approach their conspecific female more often than the heterospecific one (Binomial GLMM, Estimate=-0.161±0.198 SE, z=-0.813, *p-value* = 0.416), while *M. achilles* males did (Binomial GLMM, Estimate=0.196±0.092 SE, z=2.14, *p-value* = 0.032). However, note that the probability for *M. achilles* to approach one of its conspecifics is only 0.54, which is very low compared to the probability of *M. h. bristowi* to approach (0.61) or touch (0.84) its consubspecific in experiment 1. Visual discrimination thus appears to be lower among species, between the sympatric *M. helenor* and *M. achilles*, than within species, between the allopatric *M. helenor* subspecies, probably due to

306 the remarkable similarity in color pattern and iridescence between the two species in their sympatric
307 range.
308

309 *Divergent chemical profiles observed between males of the sympatric species* M. helenor
310 *and* M. achilles
311

312 We finally investigated the volatile chemical compounds extracted from the genitalia of males and
313 females from the mimetic sympatric species *M. helenor* and *M. achilles*. The nMDS analyses
314 performed (Fig 5) suggested a strong divergence in the chemical profiles between males of the two
315 species, especially for C16 to C30 compounds. This result contrasts with the strong similarity of
316 chemical profiles found between the females of these two species in these longer-chain compounds
317 (S10 Fig).
318 The PERMANOVA of the chemical spectra of *M. h. helenor* and *M. a. achilles* genitalia revealed
319 a significant effect of sex, species (*p-value* < 0.001 on both the C8 to C16 and the C16 to C30
320 datasets, see Fig 5) and a significant interaction between the two variables. When analyzing the
321 results for each sex separately (post-hoc), we found a significant effect of the species variable
322 (*PERMANOVA, df=1, F=10.013, p-value=0.001* for males and *df=1, F=1.928, p-value* =0.049 for
323 females in the C8 to C16 compounds, *PERMANOVA, df=1, F=26.031, p-value=0.001* for males
324 and *df=1, F=2.776, ,p-value = 0.048* for females in the C16 to C30 compounds), suggesting a
325 substantial divergence between species in chemical bouquets, especially marked in males.
326 Next, we performed an Indicator Value Analysis to identify the different compounds significantly
327 associated with each species, separating males and females (Table S2). Among all the annotated
328 compounds, we found traces of beta-ocimene, previously identified as a butterfly pheromone.

329 Interestingly, different proportions of this compound were found on the genitalia of *M. helenor* and
330 *M. achilles* males (Table S2).
331
332 **[Fig 5: Divergent chemical compounds found on the genitalia of sympatric *Morpho* males**.]
333

334 Discussion

335

336 *Convergent iridescence between sympatric* Morpho *butterflies suggests a prominent role*
337 *of predation on the evolution of iridescence*
338

339 Considering divergence time, wing iridescence is expected to be more different between the species
340 *M. achilles* and *M. helenor* than among populations of *M. helenor.* Our results on the variation of
341 iridescent properties of the blue patches of *M. helenor* and *M. achilles* show the opposite trend,
342 with more divergence between populations within *M. helenor* than between *M. helenor* and *M.*
343 *achilles* in their overlapping range. Together with the convergent evolution of the blue band width
344 previously detected in multiple sympatric locations in these two species (Llaurens et al., 2021), this
345 observation is consistent with a primary role of natural selection on the evolution of wing
346 coloration. Sympatric species are indeed submitted to the same abiotic conditions, favoring the
347 evolution of similar adaptations (Chazot et al., 2014). For instance, wing coloration is involved in
348 thermoregulation in butterflies, and similar environmental conditions may constrain the evolution
349 of this trait. Despite this, thermal absorbance in iridescent species appears independent of
350 environmental temperature, as experimentally measured in a large sample of Lepidopteran species
351 (Bosi et al., 2008); similarly, recent experiments have shown that iridescent *Morpho* species are
352 not better at thermoregulating than non-iridescent ones (Bouinier et al., 2025), suggesting that the
353 evolution of structural colors is not driven by selection on thermoregulation. Instead, the results of

354 the visual models showing that these convergent iridescent signals are likely undistinguishable by
355 birds supports an effect of selection by predators (Chouteau et al., 2016; Stuckert et al., 2014). The
356 lack of directionality for the iridescent signal of the wings further supports a limited effect of sexual
357 selection driving divergence in visual signal. Directionality in iridescent signals is indeed expected
358 when sexually selected traits incur a predation cost due to their conspicuousness: directionality
359 might enhance signaling to mates while remaining poorly detected by predators (Endler, 1992).
360 Our study shows that *M. helenor* and *M. achilles* wings display a gradual variation of hue and
361 brightness when changing the illumination/observation angles contrary to other iridescent butterfly
362 species with more directional changes (e.g. *Hypolimnas bolina*; White et al., 2015). Recent
363 predation experiments showed that the gradual change of hue and brightness of iridescence can be
364 involved in crypsis and camouflage: for instance, iridescent beetles are less detected by predators
365 when placed against a leafy background (Kjernsmo et al., 2020; Thomas et al., 2023). However,
366 like most Satyrinae, *Morpho* butterflies tend to rest on leaves with their wings closed, thus hiding
367 the iridescent patterns on the dorsal side of their wings at rest. Furthermore, iridescent species have
368 a very erratic flapping flight, creating very conspicuous flashes (Le Roy, Amadori, et al., 2021;
369 Young, 1971). Gradual variations of hue and brightness during wing movements are thus consistent
370 with the emission of a confusing visual signal that increases the chances of escaping predators. The
371 convergence of visual signals could have been favored because of the indirect advantage that results
372 in a reduction in attacks from bird predators that have experienced and learned to avoid the escape
373 capabilities of these prey (Pinheiro & Freitas, 2014; Ruxton et al., 2004). Coupled with results from
374 predation experiments supporting an effect of dynamic flash coloration as a protective mechanism
375 in *Morpho helenor* (Vieira-Silva et al., 2024), convergence of color patterns in those sympatric
376 butterflies is consistent with the hypothesis of escape mimicry, although this warrants further
377 research.

Interestingly, our reflectance measurements showed that males are generally brighter than females in both species, implying that the flashes generated by males during flight could be more conspicuous. Field experiments have highlighted the territorial behavior of males found patrolling along rivers and displaying competitive interactions with other males (Le Roy, Roux, et al., 2021). This flight behavior strongly differs from female behavior, which are much less frequently observed in the wild and typically spend more time hidden in the understory (Young, 1973). Considering that these ecological niche differences between males and females could result in different predation pressures in contrasted light conditions (open river banks *vs*. cluttered understory), iridescent signals could have evolved differently in the two sexes, and similarly so in both species (Allen et al., 2011; Reimchen & Nosil, 2004).

Although we only compared one sympatric population and one allopatric population, our results on geographic variations in iridescence within *M. helenor* and similarity of iridescent coloration between *M. helenor* and *M. achilles* in sympatry are consistent with an effect of local selection exerted by predators. Further investigation on multiple populations is needed to test whether this trend can be generalized to multiple sympatric locations where other wing patterns can be observed. Additionally, characterizing convergence of iridescence is an indirect method to test for the effect of iridescence in predator deterrence: testing the predation rate of *Morpho* when presented with local or exotic predators would better characterize the direct effect of convergent iridescent in escaping predators in sympatry.

Iridescent color patterns can be used as mate recognition cues in M. helenor

Our tetrad and male mate choice experiments in *M. helenor* butterflies clearly show assortative mating based on wing coloration, mainly driven by the behavior of individuals from the subspecies *M. h. bristowi*. This result is consistent with the high attraction generated by blue coloration on males, as well as the preference of males for local *vs*. exotic wing color patterns found in previous

field behavioral experiments carried out in Amazonian Peru (Le Roy, Roux, et al., 2021). Our male mate choice experiments suggest that both the wing patterning and the iridescent properties of the blue patch could have an effect on male preference. Furthermore, visual modeling also suggests that the differences of iridescent coloration between the populations of the same species can be perceived by mates. Coloration and color perception are key in mate recognition in many taxa (marine invertebrates; Baldwin & Johnsen, 2009, birds; Caro et al., 2021, mammals ; Waitt et al., 2003) but patterns can also inform mate choice (Houde, 1987; Pérez-Rodríguez et al., 2017). In jumping spiders, both pattern and coloration are important cues used during mate choice (Zhou et al., 2021). Similarly, in *Heliconius* butterflies, both pigmentary coloration and wing pattern are used in mate choice, although the preference is hierarchical and coloration is the most important cue (Finkbeiner et al., 2014). Further experiments are needed to disentangle the relative effects of iridescent coloration and wing pattern in mate preference in *Morpho* butterflies, but our results show that iridescent color patterns can be used as mate recognition cues in *M. helenor*.

Evolution of visual and olfactory cues in similar sister-species living in sympatry

Interestingly, intraspecific variations in courtship motivation were detected in our behavioral experiments within the species *M. helenor*. Individuals from the western areas of the Andes displayed high courtship motivation while butterflies from the Amazonian area, where *M. helenor* coexists with *M. achilles,* showed a more limited response to our visual dummies. The joint convergence of both color pattern and blue iridescence between *M. helenor* and *M. achilles* in Amazonia probably makes visual cues poor species recognition signals: visual modeling even suggests that *M. helenor* cannot perceive the color difference between the two, whereas it could make the difference between two *M. helenor* subspecies displaying more divergent coloration. This is further supported by our male choice experiment showing that although *M. helenor* males living

429 in sympatry with *M. achilles* are attracted by blue iridescent colors and approach each female, they
430 do not preferentially interact with their conspecific female dummies. Nevertheless, *M. achilles*
431 males tended to approach significantly more their conspecific females. Previous mate choice
432 experiments performed in the wild suggested that *M. achilles* indeed preferred to fly around *M.*
433 *achilles* females compared to *M. helenor* females (Le Roy, Roux, et al., 2021), suggesting that
434 visual cues are still relevant in mate choice for some *Morpho* species living in sympatry with other
435 *Morpho* butterflies. However, in our experiments, *M. achilles* males approached their conspecifics
436 with a probability of 0.54 only, which is very low compared to what has been observed between
437 intraspecific *M. helenor* subspecies, probably because of the high resemblance between *M. helenor*
438 and *M. achilles* in sympatry.
439
440 The convergent evolution of iridescence in sympatric species might thus promote divergent
441 evolution of alternative mating cues. By quantifying chemical compounds found on the genitalia
442 of males and females sampled in Amazonian French Guiana, we did indeed find a strong divergence
443 in the chemical profiles of sympatric *M. helenor* and *M. achilles,* especially in males. High male
444 chemical divergence in sympatric species suggests the evolution of female mate choice based on
445 olfactory discrimination (Mérot et al., 2015). Although all compounds were not identified, some of
446 those that were have previously been identified as pheromone compounds used during male/female
447 interactions in butterflies. In particular, beta-ocimene was found on the genitalia of males and is
448 known to contribute to mate choice in courtship (Li et al., 2017) or as an anti-aphrodisiac used by
449 *Heliconius* males to repel other males after mating with a female (Schulz et al., 2008). Additionally,
450 we found strong divergence between males for heavier chemical compounds (C16-C30). Because
451 cuticular hydrocarbons can be involved in mate discrimination during courtship (Ômura et al.,
452 2020), diverging non-volatile compounds could also be used as a discriminating cue. Although we
453 did not perform mate choices to test for the discrimination of olfactory cue by females, divergence

of male odorant cues could be due to reproductive interference between sympatric species (Bacquet et al., 2015; Dyer et al., 2014). Altogether, our results therefore 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, may have negatively impact visual discrimination and favored the evolution of divergent olfactory cues. However, determining whether divergence of the chemical profiles of allopatric and visually more divergent *Morpho* butterflies occurs and to what extent would be needed in order to determine whether the divergence observed in sympatric *Morpho* species is truly due to reinforcement.

Conclusions

We found evidence of convergent iridescent patterns in sympatry suggesting that predation could play a major role in the evolution of iridescence. Further work is nevertheless needed to directly test this hypothesis and establish the importance of evasive mimicry in *Morpho* (Pinheiro et al., 2016), similar to the Müllerian mimicry observed in chemically-defended species. There are however 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, a switch 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 should be promoted by the evolution of traits other than visual, such as olfactory chemical cues, or by the specialization into divergent ecological temporal niches (Le Roy, Roux,

et al., 2021). Altogether, this study addresses how convergence in one trait as a result of biotic interactions may alter selection on traits in other sensory modalities, resulting in a complex mosaic of biodiversity.

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), frequently supplying their breeding with wild-caught individuals from Tena (Eastern Ecuador) and Pedro Vicente Madonado (Western ecuador) respectively. No artificial selection was performed. These commercially-bought pupae were then raised in insectaries at STRI in Gamboa, Panama, between January and March 2023, and used for experiments 4 or 5 days after emergence to wait for sexual maturity.

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.

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

A. Measures of wing reflectance

We investigated the variations in iridescence in *M. h. bristowi*, *M. h. theodorus* (from Ecuador), *M. h. helenor* and *M. a. achilles* (from French Guiana), using 10 females and 10 males of each. We estimated the iridescence of the blue patches of the dorsal side of the wings by performing a series of reflectance measurements on wings positioned on a flat surface, following a template to ensure that the wing orientation remained the same across all measurements. Reflectance spectra captured at different angles were recorded on the right forewing, at a specific point located in the wing zone defined by the M3 and Cu1 veins according to the nymphalid ground plan (see Martin & Reed, 2010). Wing reflectance was measured using a spectrophotometer (AvaSpec-ULS2048CL-EVO-RS 200-1100nm, Avantes) coupled with a deuterium halogen light source (AvaLight-DH-S-BAL, Avantes) for 21 combinations of illumination and observation angles, covering the proximo-distal and anteroposterior planes of the wing (Fig 2.A, see Supporting Information Text 1 for extended methods).

These measurements were repeated 3 times for all individuals to estimate the repeatability of the reflectance measurements at each angle using the R package *rptR* 0.9.22 (Stoffel et al., 2017). Since no difference was detected between replicates for any of the tested angles (S12 Fig), the mean of these 3 measurements at each angle for each individual was used in the subsequent analyses.

B. Statistical analysis of iridescence

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 both butterfly (Briscoe, 2008) and bird observers (Bennett & Théry, 2007). All reflectance spectra were analyzed using the R package

529 *pavo2* 2.9.0 (Maia et al., 2019). The statistical analysis of both colorimetric variables and global
530 iridescence were performed using the iridescence data of 10 males and 10 females from each
531 population (*M. h. bristowi, M. h. theodorus, M. h. helenor* and *M. a. achilles*).
532

533 ## a. Extraction and analysis of colorimetric variables

534

535 To identify the main optical effect produced by the wing at each angle, we extracted three
536 commonly-used colorimetric variables from each spectrum, using the *summary()* function in *pavo2*:
537 namely Hue (color in the common meaning of the term - measured as the wavelength at the
538 maximum reflectance), Brightness (average proportion of reflected light - measured by the mean
539 reflectance over each spectra) and Chroma (also referred to as saturation, it characterizes the color's
540 purity - measured as the ratio of the reflectance range of each spectra by the brightness). For each
541 individual, we thus extracted these 3 variables for each of the 21 spectra obtained for different
542 combinations of illumination/reflectance angles. Because the normality and heteroscedasticity
543 assumptions were not met for the distributions of values in these three parameters, we relied on
544 permutation-based ANOVAs (*vegan 2.6.4* package, Oksanen et al., 2001) to test the effect of sex,
545 angle of measurement, locality and species on these three colorimetric variables. To specifically
546 test whether differences in iridescence patterns were observed among species or localities, we
547 estimated the interaction between the effects of *Morpho* populations and of the angle of
548 illumination.
549

550 ## b. Global iridescence analysis

551

552 As iridescence broadly refers to any change in reflectance in relation to illumination and
553 observation angle (Doucet & Meadows, 2009), we characterized the iridescence of each individual

554 using all complete spectra obtained at all angular positions (400 wavelengths x 21 angular
555 positions). We applied a Principal Component Analysis (PCA) to this dataset, where each dot
556 corresponds to the iridescence of an individual wing and thereby showing the similarities and
557 differences in iridescence among groups. The samples from Ecuador and French Guiana were
558 analyzed separately to test for more precise differences in iridescence between allopatric and
559 sympatric *Morpho* butterfly populations. Additionally, those comparisons correspond to the
560 phenotypes used in the mate choice experiments (see below). The coordinates of the 10 first PCs
561 (representing 98% of the explained variance) were then extracted and a PERMANOVA was
562 performed to test for the effect of sex, locality and species on iridescence patterns.
563

564 III. Modeling how *Morpho* butterflies and avian predators perceive *M.* 565 *helenor*

566

567 To investigate whether the variations observed among the different spectra could be perceived by
568 different observers, we then used the R package *pavo2* 2.9.0 (Maia et al., 2019) to model both avian
569 and butterfly visual capabilities. We used the avian UV-sensitive vision model implemented in
570 *pavo* ("*avg.uv*") as a proxy for predator vision and specifically built a model of *M. helenor* vision,
571 using the spectral sensitivity measured in Pirih et al., 2022. We built an hexachromatic visual model
572 using the *sensmodel* function, using 345, 446, 495, 508, 570 and 602 nm as the maximum of
573 absorbance in our six simulated cones respectively (S11 Fig). The *vismodel* function was used to
574 compute the quantum catch of each receptor in the *Morpho* vision model under standard daylight
575 illumination for each spectrum. Finally, assuming that the discrimination of colors is limited by
576 photoreceptor noise (Receptor-Noise Limited model, Vorobyev et al., 2001), we estimated the
577 distance between colored stimuli as perceived by Morphos using the *bootcoldist* function. We used
578 the default options (*weber* = 0.1, *weber.achro* = 0.1) and set the photoreceptor densities for each
579 cones used in the visual model to 1. The resulting chromatic distances (*dS*), achromatic distances

580 (dL) and confidence intervals were then used to test how much the visual capacities of *Morpho*
581 butterflies could allow the discrimination of wing reflectance (brightness for achromatic distances,
582 hue for chromatic distances) depending on sex, localities and species, at each angle. Similarly, we
583 used the avian UV sensitive visual model implemented in *pavo* to test the discrimination of *Morpho*
584 wing reflectance by predators. We used $dS \geq 1$ *Just Noticeable Difference* as the threshold of
585 discrimination.
586

587 # IV. Mating experiments

588

589 All mate choice experiments took place in cages of similar dimensions (3m x 3m x 2m), in which
590 Morpho butterflies, especially males, were able to fly freely. All *Morpho* butterflies were fed 30
591 minutes before the beginning of the experiments taking place between 8am and 12am.
592

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

594

595 To study the discrimination of mating partners within the species *M. helenor*, tetrad experiments
596 were performed between *M. h. bristowi* and *M. h. theodorus*. For each experiment, one virgin
597 female and one mature male of both *M. h. bristowi* and *M. h. theodorus* were placed within the
598 same cage. Their starting positions in each corner of the cage were randomized. The first mating
599 occurring out of the four possibilities was recorded and the experiment was stopped as soon as the
600 male joined their genitalia. Experiments could also be ended when no mating occurred within 5
601 hours of trial. We carried out N = 33 trials to estimate whether the first mating observed
602 preferentially involved assortative pairs, due to either male and/or female mate preference, of which
603 N=30 trials actually resulted in assortative or disassortative matings. We statistically tested for
604 departure from the random mating expectation using a Fisher's exact test.

605

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

609

610 **b. Identifying specific visual cues used during mate choice by *M.***
611 ***helenor* males**

612

613 As male *Morpho* tend to patrol in the wild to find females, and because reports show that males are
614 attracted to bright blue coloration in the wild (Le Roy, Roux, et al., 2021), we specifically tested
615 for male mate choice using visual cues. Females are also likely to be sensitive to color pattern in
616 mate recognition. However, we did not test for female choice because their behavior was not
617 compatible with experiments involving flying around butterfly dummies: female individuals tended
618 to hide away from the males in our experimental cages. To investigate the visual preference of *M.*
619 *helenor* males for the dorsal color pattern of females, we performed several choice experiments
620 using dummy butterflies built with actual female wings. To ensure that preferences were not
621 triggered by olfactory cues, all female wings used to build the artificial dummies were washed with
622 hexane before any experiment. The details of the experimental design and specific visual cues
623 tested are described in Figure 6.

624

625 First, we tested male preference for the visual appearance of *M. h. bristowi* and *M. h. theodorus*
626 females (Experiment 1). Since *M. h. bristow*i and *M. h. theodorus* females differ both in terms of
627 color patterns (width of blue band) and iridescence, we then conducted experiments to isolate these
628 two components.

We specifically tested the effect of the proportion of blue to black areas (Experiment 2) by presenting males *M. h. bristowi* to female dummies harboring *M. h. bristowi* wings, except one was modified with black ink to resemble the narrow blue band on *M. h. theodorus* female wings.

We finally tested the effect of the blue iridescent band within the same black and blue pattern (Experiment 3) by presenting *M. h. theodorus* males to a *M. h. theodorus* female with a narrow blue band, and to the modified *M. h. bristowi* female dummy with a narrow band. The wing pattern 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). Nevertheless, we cannot entirely discard the specific visual effect of the marker on the dorsal face.

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*

654 In order to investigate the importance of color vision in species recognition, we then performed a
655 male choice experiment with *M. h. helenor* and *M. a. achilles* (experiment 4 in Figure 6) in French
656 Guiana, using natural wings of females *M. h. helenor* and *M. a. achilles* as artificial butterflies
657 following the exact same protocol above.
658
659 [**Figure 6: Experimental design of the male choice experiments**]
660

661 a. Statistical analyses

662
663 *Comparing male mating motivation*
664
665 Because we noticed behavioral differences between the species and subspecies of *Morpho* during
666 the course of the experiments 1-4, we attempted to account for variations in male mating
667 motivation. The approach and touch rates of males, *i.e.* their willingness to approach and touch
668 (respectively) any female dummies during each trial, was defined as the proportion of trials where
669 the males approached and touched (respectively) either female dummies at least once. The
670 approach rate and touch rate of *M. h. bristowi*, *M. h. theodorus, M. h. helenor* and *M. a. achilles*
671 males were calculated, and a binomial generalized linear model (GLMM) was used to test whether
672 the approach or touch rate was significantly different between males from different populations.
673 All statistical analyses were performed with the R package *glmmTMB* (Brooks et al., 2017). The
674 normality, homogeneity of variance and overdispersion tests for the GLMMs were performed using
675 the R package *DHARMa* (Hartig, 2022). We controlled for potential individual variations by using
676 the male ID and trial number as a random effect in all the tests.
677
678 *Visually-based male mate preferences*

679

680 We then tested for male preferential attraction for different dummy females. For each male
681 individual, we recorded the number of approaches and touches directed toward the two female
682 dummies and modeled this as a two-column response variable. Similar to the protocol above, we
683 used a binomial GLMM to test whether individuals preferentially interacted with dummy 1 vs.
684 dummy 2, and added a random effect for individual identity to account for repeated observations
685 from the same individual. A significant signal was detected when the probability of touching a
686 dummy was different from the 0.5 proportion expected under random mating, and interpreted as a
687 signal of preference.

688

689 V. Pheromone analysis for sympatric species

690 a. Chemical analyses

691

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

699 The chemical compounds of those samples were analyzed by gas chromatography and time-of-
700 flight mass spectrophotometry (GC-ToF-MS) using a 8890 GC system (Agilent, Santa Clara, USA)
701 coupled to a PEGASUS BT mass spectrophotometer (LECO, Saint Joseph, USA). The connected
702 capillary column was a Rxi-5ms (30 m x 0.25 mm, df = 0.25 µm, Restek, Bellefonte USA) and
703 helium was used as carrier gas at 1 mL min^{-1} constant flow rate. 1 µL of each sample was injected
704 in a GC split/splitless injector at 300 °C. Two temperature programs were used: (i) the analysis of

705 the C8-C16 compounds started at 40 °C maintained for 1 min, then increased at 210°C at 6 °C min^{-1}
706 rate, finally increased to 340 °C at a 10 °C min^{-1}, and (ii) the analysis of the C16-C30 compounds
707 started at 70 °C maintained for 1 min, then increased to 150 °C at 30 °C min^{-1} rate and finally
708 increased to 340 °C at a 6 °C min^{-1} rate. Mass spectra were recorded with a 70eV electron impact
709 energy. Mixtures of alkanes C8 to C19 and C16 to C44 (ASTM® D5442 C16-C44 Qualitative
710 Retention Time Mix, Supelco, Bellefonte, USA) were injected under the same conditions to be
711 used as external standards.
712 The chromatograms were converted in netCDF format and operated using *MZmine 2.43* for
713 compound detection and area integration. To this aim, chromatograms were cropped before 4 min
714 and after 27 min, and baseline-corrected using the Rolling ball algorithm (Kneen & Annegarn,
715 1996). Mass detection was performed using the centroid mass detector, and chromatograms of each
716 mass were constructed using the ADAP algorithm (Myers et al., 2017; Pluskal et al., 2010).
717 Chromatograms were deconvoluted into individual peaks using the local minimum search
718 algorithm and aligned on a master peak list with the RANSAC algorithm. Finally, the Peak Finder
719 gap filling algorithm was used to search for missing peaks, and a matrix compiling the area under
720 each peak (*i.e.* the concentration of each component) detected for each butterfly sample was
721 extracted. This matrix was purged from siloxane like compounds as well as compounds present in
722 less than 10% of the samples. The areas of the blanks were subtracted to the samples, and the final
723 concentration data was used for statistical analysis. Mass spectra were then visualized using the
724 ChromaTOF® software, compared to MZmine-selected ones, in order to manually annotate the
725 chemical compounds. Annotation were based on scan comparison with the NIST2017 database,
726 and linear retention indexes (LRI) calculated from the alkane mixtures with free database
727 (https://pubchem.ncbi.nlm.nih.gov/) and literature (Adams, 2017).
728

b. Statistical analyses of the chemical compounds

In order to quantify variations in chemical compounds detected in the different sample, we performed a nonmetric multidimensional scaling (NMDS) ordination using the Bray-Curtis dissimilarity calculation on the concentrations extracted from the chromatograms. By considering the amount of each compound found in our samples as a variable, we performed a permutation MANOVA with the *adonis2()* function found in the *vegan 2* package on R. We tested whether the sex or the species had a significant effect on the chemical 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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Figures and Tables

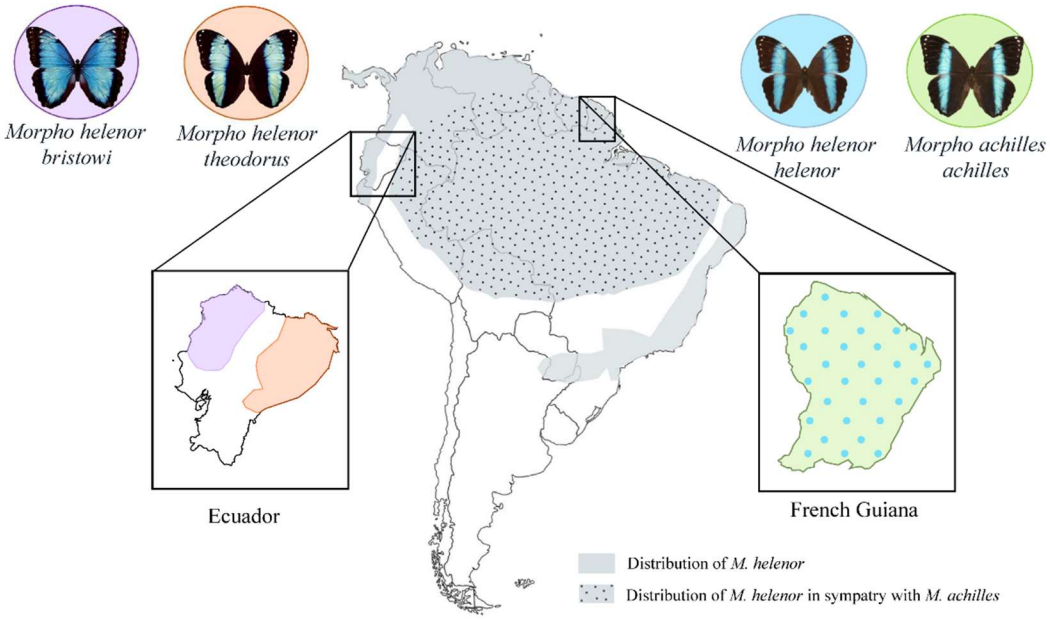
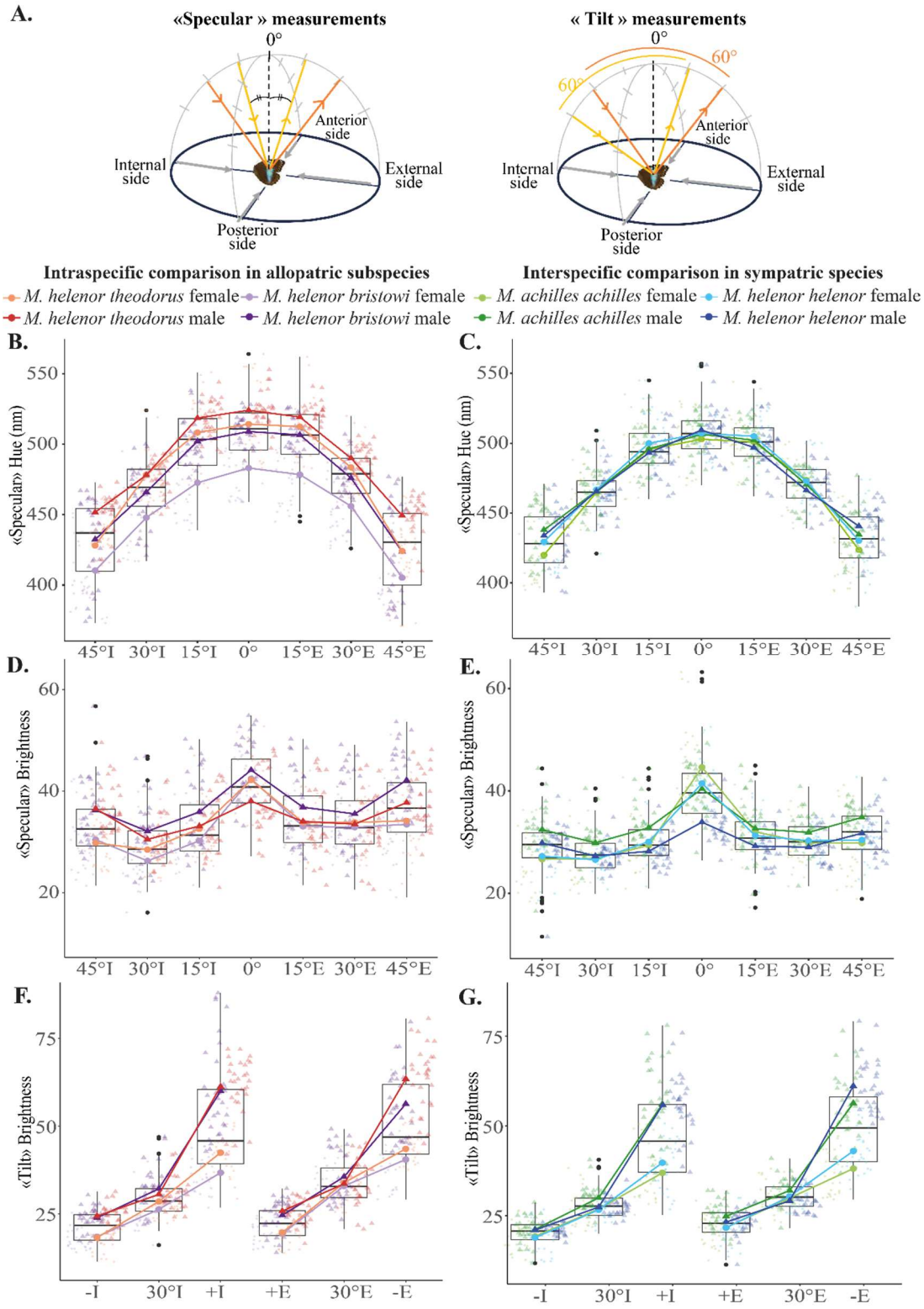


Fig 1: Distribution of the sister-species *M. helenor* and *M. achilles* across South America. The gray area represents the whole distribution of *M. helenor*, and the dotted area indicates the localities where *M. helenor* is in sympatry with *M. achilles.* Note that the different subspecies of *M. helenor* are found in different localities and display substantial variations in the proportion of black *vs*. blue areas on the dorsal sides of their wings. For example, *M. h. bristowi* and *M. h. theodorus* are allopatric in Ecuador, with their respective distribution separated by the Andes such that they are never in contact. While *M. h. bristowi* is found on the Pacific side of the country and has a wide blue band on the dorsal side of its wings, *M. h. theodorus* is found in Western Amazonia and

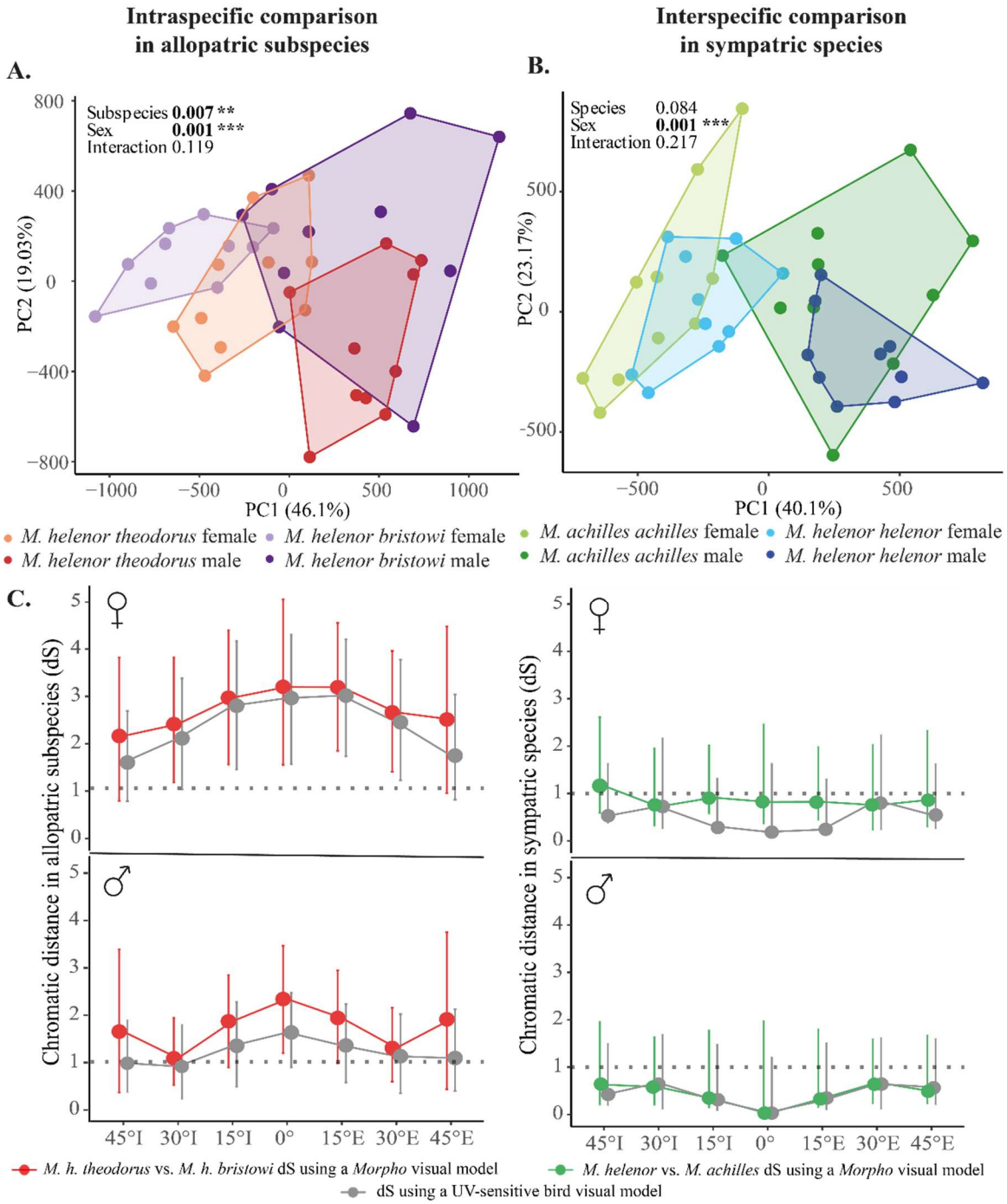
displays a narrow blue band on the dorsal side of its wings. However, *M. helenor* is sympatric with *M. achilles* throughout the Amazonian rainforest. In French Guiana, the subspecies *M. helenor helenor* and *M. achilles achilles* display convergent dorsal color patterns with thin blue bands (Llaurens 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
Intraspecific comparison in allopatric subspecies
M. helenor theodorus female
M. helenor bristowi female
M. helenor theodorus male
M. helenor bristowi male
Interspecific comparison in sympatric species
M. achilles achilles female
M. helenor helenor female
M. achilles achilles male
M. helenor helenor male
B.
«Specular» Hue (nm)
550
500
450
400
45°I 30°I 15°I 0° 15°E 30°E 45°E
C.
«Specular» Hue (nm)
550
500
450
400
45°I 30°I 15°I 0° 15°E 30°E 45°E
D.
«Specular» Brightness
60
40
20
45°I 30°I 15°I 0° 15°E 30°E 45°E
E.
«Specular» Brightness
60
40
20
45°I 30°I 15°I 0° 15°E 30°E 45°E
F.
«Tilt» Brightness
75
50
25
-I 30°I +I +E 30°E -E
G.
«Tilt» Brightness
75
50
25
-I 30°I +I +E 30°E -E

1045

1046 **Fig 2: Differences of hue and brightness on the proximo-distal plane of *Morpho* wings** (A)
1047 Illustration of the two protocols used to assess the differences in *Morpho* wing reflectance. The
1048 "Specular" set-up allows for the quantification of the wing color variations, while the "tilt" set-up
1049 can be used to quantify brightness variation for each *Morpho* wing (see Appendix for extended
1050 methods). Variations of hue (B and C) and brightness (D and E) calculated from the wing
1051 reflectance measured with the "Specular" set-up, and variations of brightness calculated from the
1052 wing reflectance measured from the "tilt" set-up (F and G). Those hue and brightness parameters
1053 were calculated for the allopatric *M. h. theodorus* and *M. h. bristowi* (first column in orange and
1054 purple) and for the sympatric *M. h. helenor* and *M. a. achilles* (second column in green and blue)
1055 on the proximal-plane plane of their wings (I= illumination on the internal side of the wings, E=

illumination on the external side of the wings). See S1 Table for the PERMANOVA analyses describing those graphs.



1061 **Fig 3: Characterization and perception of the iridescent coloration of *Morpho* butterflies.** (A)
1062 and (B): PCAs showing the variation in iridescence for both sexes, (A) in the two sympatric species
1063 from French Guiana (*M. h. helenor* vs. *M. a. achilles*) and (B) in the two allopatric Ecuadorian
1064 subspecies of *M. helenor (M. h. theodorus vs*. *M. h. bristowi*). Each point represents the global
1065 signal of iridescence of each individual, corresponding to the 21 complete reflectance spectra
1066 obtained from the 21 tested angles of illumination. The results of the PERMANOVA are shown on
1067 the top left corner of each graph. (C) The chromatic distances (*i.e.* the visual discrimination rate by
1068 a visual model) of the wing reflectance measured with the "Specular" set-up on the proximo-distal
1069 plane. Visual modeling was used to calculate the chromatic contrast of blue coloration between
1070 allopatric *M. helenor* subspecies (red) and between the two sympatric sister-species *M. helenor* and
1071 *M. achilles* (green), as perceived by a *Morpho* visual system for every angle of illumination
1072 measured on the proximo-distal plane. The chromatic contrast likely perceived by UV-sensitive
1073 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*). Error bars show the confidence intervals calculated during the bootstrap analysis.

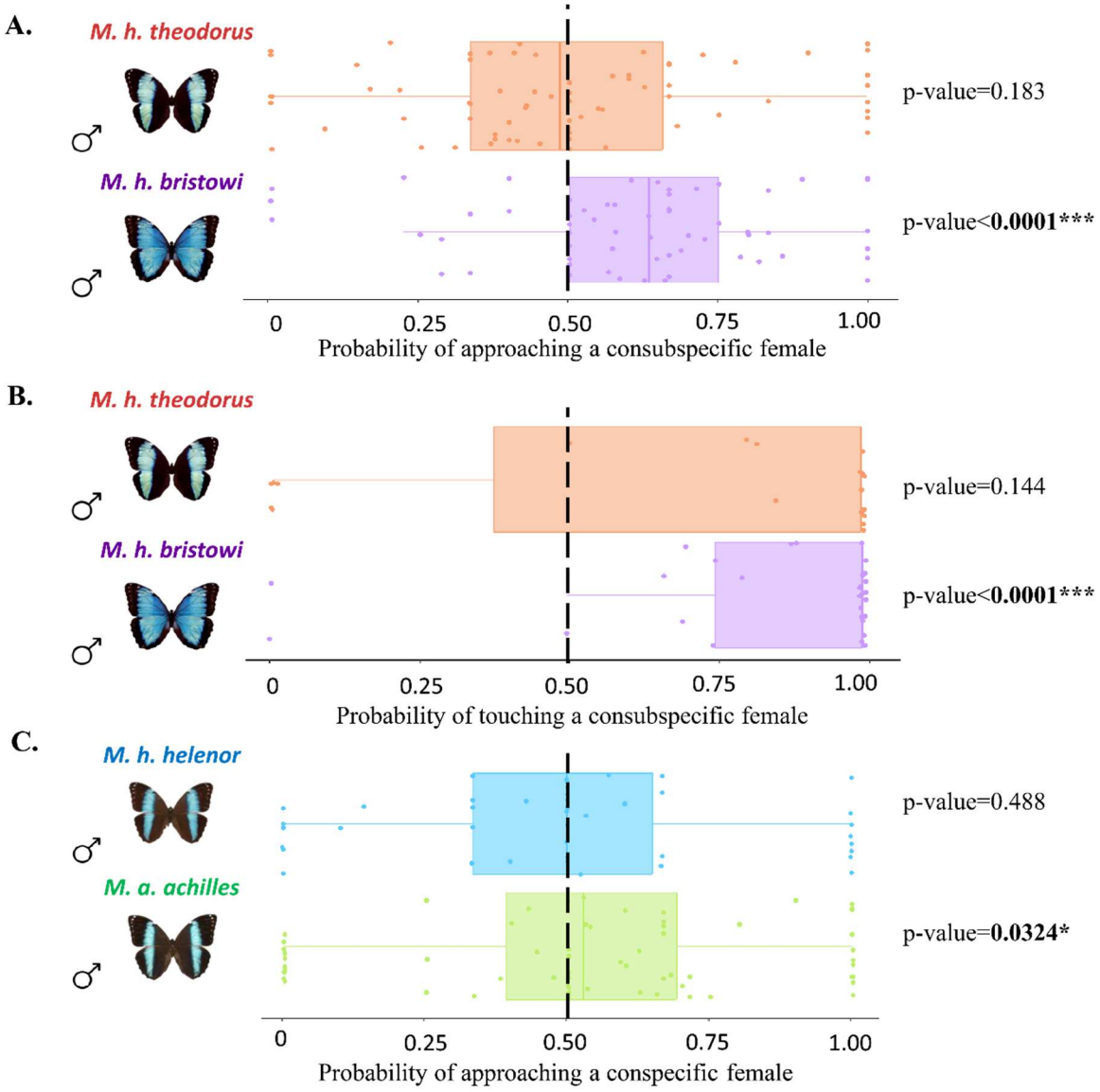
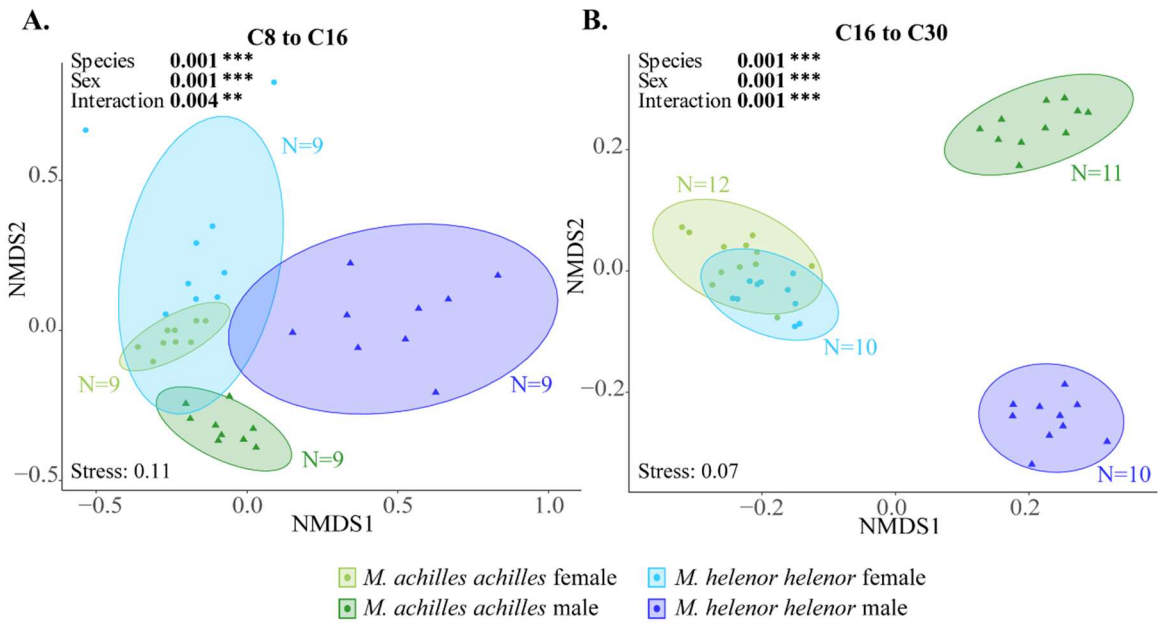


Fig 4: Morpho male preference based on visual cues alone.

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)

1082 approaching a con-specific female for *M. h. helenor* males (blue) and *M. a. achilles* males (green)
1083 from French Guiana, measured during *Experiment 1*. The dotted line indicates the expected
1084 probability of approaching/touching a model if no preference is present. The *p-values* resulting
1085 from the binomial GLMM testing for preferentially directed interactions toward one female dummy
1086 are shown next to the corresponding graphs.
1087



1088

1089 **Fig 5: Divergent chemical compounds found on the genitalia of sympatric *Morpho* males**.
1090 nMDS representation of the differences in the concentration of (A) C8 to C16 chemical compounds
1091 and (B) C16 to C30 chemical compounds found in the genitalia of *M. h. helenor* and *M. a. achilles*
1092 males and females, calculated using Bray-Curtis distances. *M. h. helenor* are shown in blue (males
1093 are in dark blue and females in light blue), and *M. a. achilles* are shown in green (males are dark
1094 green and 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

M. a. achilles, experiment 4), along with the associated preferences and visual cues tested. Note that the same males were used for experiments 1, 2 and 3.

Table 1: Tetrad experiment results: Number of mating events involving the different possible pairs of males and females from the two subspecies of M. helenor (bristowi vs. theodorus) in 30 tetrad experiments

N=30

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