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

Joséphine Ledamoisel, Bruno Buatois, Rémi Mauxion, Christine Andraud,  
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► **To cite this version:**

Joséphine Ledamoisel, Bruno Buatois, Rémi Mauxion, Christine Andraud, Melanie McClure, et al.. Sending mixed signals: convergent iridescence and divergent chemical signals in sympatric sister-species of Amazonian butterflies. 2025. hal-04878610

**HAL Id: hal-04878610**

**<https://hal.science/hal-04878610v1>**

Preprint submitted on 10 Jan 2025

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

4 Joséphine Ledamoisel<sup>1,2,3,4</sup>, Bruno Buatois<sup>5</sup>, Rémi Mauxion<sup>3</sup>, Christine Andraud<sup>4</sup>, Melanie  
5 McClure<sup>6</sup>, Vincent Debat<sup>1,2</sup>, Violaine Llaurens<sup>1,2</sup>

6 <sup>1</sup> Centre Interdisciplinaire de Recherche en Biologie (UMR 7241, Collège de  
7 France/CNRS/INSERM), Collège de France 11 place Marcelin Berthelot 75005 Paris, France

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

11 <sup>3</sup> Smithsonian Tropical Research Institute (STRI), Apartado, Panamá 0843-03092, Republic of  
12 Panama

13 <sup>4</sup> Centre de Recherche sur la Conservation (CRC), CNRS, MNHN, Paris, France

14 <sup>5</sup> CEFE, Univ Montpellier, CNRS, EPHE, IRD, Montpellier, France.

15 <sup>6</sup> Laboratoire Écologie, Évolution, Interactions des Systèmes Amazoniens (LEEISA), Université  
16 de Guyane, CNRS, IFREMER, Cayenne, France

17 Corresponding author: Joséphine Ledamoisel

18 **Email:** [josephine.ledamoisel@outlook.fr](mailto:josephine.ledamoisel@outlook.fr)

19 **Author Contributions:** J.L, V.D and V.L designed the research; J.L. and R. M. performed the  
20 behavioral experiments; J.L. acquired the iridescence data; J.L, B.B, M.M, C.A contributed to  
21 analytic tools; J.L and B.B acquired and analyzed the chemical data; J.L. performed all statistical  
22 analyses; J.L, B.B, R.M, M.M, C.A, V.D and V.L wrote the paper; V. D., V.L. supervision.

23 **Competing Interest Statement:** No competing interest.

24 **Classification:** Biological Science, Evolution

25 **Keywords:** Selection, Trait diversification, Mimicry

26 **This PDF file includes:**

27 Main Text  
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36

## 37 **Abstract**

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

## 57 **Significance Statement**

58 By changing the visual aspect of animals in movement, iridescence likely impacts multiple visual  
59 interactions within and among species, and might therefore shape ecological interactions and  
60 species diversification in sympatry. Here we study the relative importance of predation-driven  
61 mimicry and sexual selection on the evolution of iridescence on the wings of sister-species of  
62 *Morpho* butterflies. We finely quantify this conspicuous wing phenotype and used behavioral  
63 experiments to test whether the sexual preference for this trait can vary between allopatric and  
64 sympatric populations of sister-species. We find that convergent iridescence in sympatric *Morpho*  
65 species, putatively driven by predators and supporting the hypothesis of evasive mimicry, impairs  
66 species recognition and could promote the divergence in alternative chemical cues involved in mate  
67 discrimination.

68

## 69 **Main Text**

70

## 71 **Introduction**

72

73 Identifying the evolutionary factors promoting diversification and co-existence of closely-related  
74 species in sympatry is a major challenge. Since the evolution of traits within species in sympatry  
75 can affect ecological interactions between individuals, it either fuels or impairs the speciation  
76 process. Closely-related species often share similar suites of traits because of their shared  
77 evolutionary history. Furthermore, in sympatry, habitat filtering excludes species with suites of traits  
78 mal-adapted to local conditions, therefore enhancing trait similarity (1). The shared selective  
79 pressures encountered by individuals living in sympatry also often promote the evolution of locally  
80 adapted traits within species and even trait convergence among species (2). That said, closely-  
81 related sympatric species often display divergent suites of traits because they are usually

82 partitioned in divergent ecological niches (3). The co-existence in sympatry may also promote  
83 divergent evolution of traits in incipient species, because of competitive interactions between  
84 individuals or as a result of reinforcement (4). The evolution of traits in incipient species living in  
85 sympatry thus depends on the balance between convergence and divergence, resulting either in  
86 (i) evolutionary trade-offs of traits jointly influenced by antagonistic natural and sexual selective  
87 pressures (5, 6) or (ii) convergence in some traits and divergence in others (7).

88  
89 Theoretical and empirical studies have shown that sexual selection may favor the evolution of  
90 preferences for locally-adapted traits within species (8–10). For instance, the predator-deterrent  
91 coloration of poison frogs is also detected and used as mating cues by females (11). Similarly,  
92 habitat-dependent coloration of sympatric cichlid fish is also used as a visual cue for mate  
93 recognition (12). Yet, sexual interactions are likely to occur between individuals from closely-related  
94 species when they live in sympatry, and similar preferences for adaptive traits may thus result in  
95 substantial reproductive interference (13). For example, because sympatric amphibians can  
96 sometimes share the same mating cues and preferences, they often engage in misdirected mating  
97 attempts towards heterospecifics, a potentially deleterious behavior (14). In addition, hybrids, when  
98 produced, can be unfit, thus favoring the evolution of sexual preferences for species-specific cues,  
99 rather than locally-adapted traits (15). Comparing variations in adaptive traits in sympatric vs.  
100 allopatric populations of recently-diverged species, and testing the sexual preference for those  
101 traits can shed light on the selective processes targeting traits modulating reproductive isolation  
102 and co-existence in sympatry.

103  
104 In butterflies, the evolution of wing color patterns can be influenced by both natural and sexual  
105 selection. The visual discrimination of wing color pattern has been shown to enable intraspecific  
106 recognition during courtship in many species (16, 17). However, the evolution of wing color patterns  
107 is also strongly influenced by the visual effect perceived by predators (18, 19). Whether these  
108 selective pressures promote trait convergence or divergence in sympatric species might depend  
109 on their relatedness: for instance, a recent study in *Papilionidae* showed multiple color pattern  
110 convergences between distantly-related species living in sympatry, while divergent colorations are  
111 found in closely-related species (20). Positive selection acting on divergent traits involved in  
112 species recognition could be increased because of higher reproductive interference in closely  
113 related species than in distantly related taxa (21). In sympatric species with chemical-defenses,  
114 such as *Heliconinii* butterflies, local predation pressures tend to promote the convergence of similar  
115 conspicuous warning wing patterns (*i.e.* Müllerian mimicry, (22, 23)). But the costs associated with  
116 hybrid production in turn favor the evolution of alternative divergent mating cues in mimetic  
117 butterflies (24), and divergence in male pheromone bouquets and female attraction has been found  
118 among mimetic sister species (25). Similarly, the evolution of specific visual mate recognition  
119 signals, limiting reproductive interference but indistinguishable by predators, can also be promoted  
120 on the wings of mimetic butterflies (26). Thus, ecological interactions among recently-diverged  
121 species living in sympatry can impact the evolution of traits involved in both local adaptation and  
122 mate recognition and may trigger the evolution of alternative traits. Investigating variations in traits  
123 used as mating cues in allopatric vs. sympatric populations of sister-species can help ascertain  
124 how trait evolution might favor speciation and co-existence in sympatry.

125  
126 Here we focus on the evolution of mating cues in the neotropical butterfly genus *Morpho*, where  
127 multiple closely-related species co-exist in sympatry (27). In some *Morpho* species, striking  
128 iridescent blue coloration is displayed on the dorsal side of the wings, due to nanostructures on

129 wing scales (28). Variations in the reflected wavelength depending on the angle of observation  
130 and/or illumination (*i.e.* iridescence) have been documented in *Morpho* species (29). The light  
131 signal reflected by iridescent surfaces can be very directional, as hue and brightness of iridescent  
132 objects can drastically change depending on the light environment or the observer's position (30).  
133 Directional iridescent signals generated in animals could then enhance recognition by mates while  
134 remaining poorly detected by predators (31). For instance, in some hummingbird species, when  
135 males tilt their head in a specific direction, flashes of bright coloration can be observed in these  
136 otherwise darkly-colored birds (32). The specific directional signal produced by the iridescent  
137 feathers is a cue during courtship, suggesting that the antagonistic sexual and natural selective  
138 pressures finely shape the evolution of iridescence. Similarly, male *Hypolymnas* butterflies display  
139 abrupt shifts in UV brightness on their wings and their courtship behavior enhances the production  
140 of flashes perceived by females (33). How much sexual selection shapes the evolution of  
141 iridescence in sympatric *Morpho* species is currently unknown, but behavioral experiments carried  
142 out in the field in Amazonian Peru highlighted strong visually-based territorial interactions among  
143 males from sympatric species and limited species discrimination based on female coloration in  
144 males (7).

145  
146 This raises questions on the key visual cues involved in mate choice, given that the iridescent blue  
147 coloration shared by closely-related species encountered within the understory is likely selectively  
148 constrained by predators. The iridescent bright blue dorsal coloration of *Morpho* wings contrasts  
149 with the brown and matte ventral side, and generates a peculiar visual effect during flight. The  
150 combination of the blue flashes produced by the alternate exposure of the bright blue vs. brown  
151 sides of the wings during flapping flight, in addition to erratic flight trajectories makes these *Morpho*  
152 very difficult to follow by predators (34), potentially enhancing their evasive capabilities (35, 36).  
153 Experimental trials with evasive prey have shown that predators learn to avoid prey they repeatedly  
154 fail to catch (37). The display of iridescent wings could thus be associated with a higher survival  
155 rate in nature because of both (i) direct effects, through successful escape of predator attacks, and  
156 (ii) indirect effects, by limiting predation attempts by birds recognizing the blue signals and  
157 refraining from attacking (38). This indirect effect could promote the evolution of convergent blue  
158 patterns in sympatric species, similar to the mimicry observed in species with chemical-defenses.  
159 In line with this hypothesis, repeated local convergence in the proportion of iridescent blue vs. black  
160 areas on the dorsal side of the wings have been documented in the sister-species *Morpho helenor*  
161 and *Morpho achilles* living in sympatry throughout the Amazonian basin: indeed, parallel  
162 geographic variations in the width of the dorsal blue band was detected in these sympatric species  
163 (39). Precise quantification of variations in iridescence is now needed to assess the respective  
164 effects of selection by predators and mates, that may drive convergent vs. divergent evolution of  
165 iridescence in sympatric species sharing the same micro-habitats. Investigating this convergent vs.  
166 divergent evolution of visual cues that may act as pre-zygotic barriers to gene flow between these  
167 two species will shed light on the mechanisms allowing their genetic divergence and coexistence  
168 in sympatry.

169  
170 To do so, we quantified variations of iridescence in the two sister species *M. helenor* and *M. achilles*  
171 in different geographic localities to investigate the antagonistic effects of selective pressures  
172 generated by mate recognition and shared predation. We used behavioral experiments to test for  
173 the effect of this color variation in visual signals in mate preferences. Finally, we studied variations  
174 in the volatile compounds produced by wild males and females from both species to explore the

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

177

## 178 Results

179

180 We investigated differences in wing iridescence, color perception and color pattern-based mate  
181 choice of two sister species of *Morpho*, *M. helenor* and *M. achilles*, which diverged ~ 3.6 Mya (40).  
182 We relied on geographic variations of wing color patterns within *M. helenor* (Fig. 1) to compare the  
183 iridescence of three different populations of *M. helenor* (subspecies *M. helenor bristowi*, *M. helenor*  
184 *theodorus* and *M. helenor helenor*) and test the associated visual effect using visual models.

185

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

187 The gray area represents the whole distribution of *M. helenor*, and the dotted area indicates the  
188 localities where *M. helenor* is in sympatry with *M. achilles*. Note that the different subspecies of *M.*  
189 *helenor* are found in different localities and display substantial variations in the proportion of black  
190 vs. blue areas on the dorsal sides of their wings. For example, *M. h. bristowi* and *M. h. theodorus*  
191 are allopatric in Ecuador, with their respective distribution separated by the Andes such that they  
192 are never in contact. While *M. h. bristowi* is found on the Pacific side of the country and has a wide  
193 blue band on the dorsal side of its wings, *M. h. theodorus* is found in Western Amazonia and  
194 displays a narrow blue band on the dorsal side of its wings. However, *M. helenor* is sympatric with  
195 *M. achilles* throughout the Amazonian rainforest. In French Guiana, the subspecies *M. helenor*  
196 *helenor* and *M. achilles achilles* display convergent dorsal color patterns with thin blue bands (39).]

197

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

199

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

205 We found that hue and brightness always vary depending on the angle of incidence of the light, as  
206 confirmed by PERMANOVAs detecting a significant effect of the “Angle” for all methods of  
207 measurements (Fig. 2 and statistical analyzes in Table S1 and S5) confirming that in both species,  
208 both sexes are iridescent. Second, the brightness of *Morpho* wings is highly sexually dimorphic, as  
209 the effect of sex is always significant. The brightness measured with the “tilt” protocol (Fig. 2.F and  
210 2.G) shows that males tend to be brighter than females in both Ecuador and French Guiana.

211

212 **[Fig. 2: Differences of hue and brightness on the proximo-distal plane of *Morpho* wings (A)**

213 Illustration of the two protocols used to assess the differences in *Morpho* wing reflectance. The  
214 “Specular” set-up allows for the quantification of the wing color variations, while the “tilt” set-up can  
215 be used to quantify brightness variation for each *Morpho* wing (see Appendix for extended  
216 methods). Variations of hue (B and C) and brightness (D and E) calculated from the wing  
217 reflectance measured with the “Specular” set-up, and variations of brightness calculated from the  
218 wing reflectance measured from the “tilt” set-up (F and G). Those hue and brightness parameters  
219 were calculated for the sympatric *M. h. helenor* and *M. a. achilles* (first column in green and blue)  
220 and for the allopatric *M. h. theodorus* and *M. h. bristowi* (second column in orange and purple) on  
221 the proximal-plane plane of their wings (I= illumination on the internal side of the wings, E=



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

224

225 To finely appraise the complete spectrum of variation in iridescence within and among *Morpho*  
226 species, we then used a multivariate approach allowing to analyze the full reflectance spectra  
227 obtained from both proximo-distal and anteroposterior measurements. At the intraspecific level,  
228 significant divergence of iridescence between populations was detected (Fig. 3.B). In contrast,  
229 iridescence did not differ between species living in sympatry (PERMANOVA,  $p$ -value = 0.084),  
230 suggesting that iridescence converges in sympatry (Fig. 3.A). In both cases, the interaction term  
231 taxon\*sex was non-significant, suggesting that the sexual dimorphism of iridescence was similar  
232 for all taxa.

233

234 **[Fig. 3: Characterization and perception of the iridescent coloration of *Morpho* butterflies.**  
235 (A) and (B): PCAs showing the variation in iridescence for both sexes, (A) in the two sympatric  
236 species from French Guiana (*M. h. helenor* vs. *M. a. achilles*) and (B) in the two allopatric  
237 Ecuadorian subspecies of *M. helenor* (*M. h. theodorus* vs. *M. h. bristowi*). Each point represents  
238 the global signal of iridescence of each individual, corresponding to the 21 complete reflectance  
239 spectra obtained from the 21 tested angles of illumination. The results of the PERMANOVA are  
240 shown on the top left corner of each graph. (C) The chromatic distances (*i.e.* the visual  
241 discrimination rate by a visual model) of the wing reflectance measured with the “Specular” set-up  
242 on the proximo-distal plane. Visual modeling was used to calculate the chromatic contrast of blue  
243 coloration between allopatric *M. helenor* subspecies (red) and between the two sympatric sister-  
244 species *M. helenor* and *M. achilles* (green), as perceived by a *Morpho* visual system for every angle  
245 of illumination measured on the proximo-distal plane. The chromatic contrast likely perceived by  
246 UV-sensitive birds is shown in gray. Chromatic contrast of the female wings (top) and male wings  
247 (bottom). The threshold of discrimination is shown by the dotted line and set to 1 *Just Noticeable*  
248 *Difference (JND)*.]

249

250 We then modeled the perception of color contrast in the visual systems of *M. helenor* and UV-  
251 sensitive birds respectively, and tested whether the differences of reflectance observed between  
252 sexes and species at different angles of illumination could potentially be perceived by a *Morpho*  
253 observer or a putative predator. As sexual dimorphism on wing reflectance was detected, we  
254 measured the chromatic distances for males and females separately (Fig. 3.C). As the results were  
255 similar along the two directions of measurements (antero-posterior and proximo-distal), only  
256 proximo-distal results are shown below (antero-posterior results in S6). *Morpho* butterflies and  
257 predators can visually perceive the difference in the blue coloration between the allopatric  
258 populations of *M. helenor*: for both *Morpho* and bird visual systems the mean chromatic contrasts  
259 are always higher than 1 JND, for both sexes and for all angles. In contrast, the mean chromatic  
260 contrasts measured between sympatric species were below 1 JND: the minute differences  
261 observed between sympatric species are thus unlikely to be discriminated either by *Morpho* or bird  
262 predators.

263

264 *Evidence of mate preferences based on visual cues within species*

265

266 We then performed a series of mate choice experiments to identify the visual cues used during  
267 mate choice within *M. helenor*. We specifically tested for preferences based on the differences in

268 color measured between the two previously studied populations of *M. helenor* (*M. h. bristowi* and  
269 *M. h. theodorus*), which significantly differs in both wing pattern and iridescence.

270

271 We first conducted a tetrad experiment using the allopatric subspecies *M. h. bristowi* and *M. h.*  
272 *theodorus*. The highest number of mating events occurred between the two assortative pairs (see  
273 Table 1). More than half of the matings occurred between a female and a male of the *M. h. bristowi*  
274 population. Indeed, a Chi-square test performed on the contingency table showed a significant  
275 departure from random mating ( $X\text{-squared} = 4.98$ ,  $p\text{-value} = 0.04$ ), confirmed by a Fisher exact test  
276 ( $p\text{-value} = 0.04$ ). The time until an assortative mating ( $mean = 48$  min,  $sd = 38$  min) or until a  
277 disassortative mating ( $mean = 48$  min,  $sd = 38$  min) however did not significantly differ ( $T\text{-test; stat} =$   
278  $-0.27$ ,  $p\text{-value} = 0.79$ ).

279

280 **[Table 1: Number of mating events involving the different possible pairs of males and**  
281 **females from the two subspecies of *M. helenor* (*bristowi* vs. *theodorus*) in 30 tetrad**  
282 **experiments.]**

283

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

285 Probabilities of (A) approaching and (B) touching a con-subspecific female for *M. h. bristowi* males  
286 (purple) and *M. h. theodorus* males (orange) from Ecuador, as well as the probabilities of (C)  
287 approaching a con-specific female for *M. h. helenor* males (blue) and *M. a. achilles* males (green)  
288 from French Guiana, measured during *Experiment 1*. The dotted line indicates the expected  
289 probability of approaching/touching a model if no preference is present. The  $p\text{-values}$  of Wilcoxon  
290 tests testing for the significant departure from the 0.5 probability are shown next to the  
291 corresponding graphs.]

292

293 Different pairs of female dummies were presented to the *M. helenor* males to investigate assortative  
294 preference between (sub)species (experiment 1), discrimination of the wing pattern (experiment 2)  
295 and discrimination of the iridescent coloration (experiment 3) (see the experimental details in the  
296 M&M section).

297

298 *Experiment 1* tested the male assortative visual preference using two dummies each harboring the  
299 wings of a female of a different *M. helenor* subspecies. *M. h. theodorus* males did not show any  
300 preference for any dummy female, as shown by the probability of approaches and touches that  
301 were not different from the random 0.5 expectation (approaches: Wilcoxon test,  $p\text{-value} = 0.764$ , Fig.  
302 4.A; touches: Wilcoxon test,  $p\text{-value} = 0.287$ , Fig. 4.B). *Morpho helenor bristowi* males, in contrast,  
303 approached and touched a con-subspecific *M. h. bristowi* dummy female significantly more often  
304 than expected by chance alone (approach: Wilcoxon test,  $p\text{-value} = 0.0448$ , Fig. 4.A; touch:  
305 Wilcoxon test,  $p\text{-value} < 0.001$ , Fig. 4.B), suggesting a preference toward the *M. h. bristowi* females  
306 based on the recognition of visual cues alone.

307

308 *Experiment 2* tested male pattern-based discrimination, using two dummies harboring the wings of  
309 *M. h. bristowi* females (i.e. same iridescence), but one of which was modified so as to present the  
310 thin blue bands found in *M. h. theodorus* (i.e. a different pattern). *M. h. bristowi* males tended to  
311 approach and touch significantly more often the wild-type *M. h. bristowi* female model as compared  
312 to the modified *M. h. bristowi* female model exhibiting a *theodorus* pattern (Wilcoxon tests on  
313 approaches;  $p\text{-value} = 0.0188$ , on touches;  $p\text{-value} < 0.001$ , see S7). As both female dummies  
314 shared the same blue iridescence, the choice of *M. h. bristowi* males was likely influenced by the



315 difference in the pattern displayed by the dummy females, suggesting that the color pattern is used  
316 by males during mate choice.

317

318 *Experiment 3* tested male color-based mate discrimination using two dummies harboring the wings  
319 of females from the two subspecies (i.e. different iridescence), but those of *M. h. bristowi* were  
320 modified so as to resemble *M. h. theodorus* (i.e. same pattern). *Morpho helenor theodorus* males  
321 tended to approach and touch significantly more often the *M. h. theodorus* female model compared  
322 to the artificially modified, theodorus-like *M. h. bristowi* female dummy (Wilcoxon test on  
323 approaches;  $p$ -value = 0.0201, on touches;  $p$ -value = 0.0343, see S8). As both dummy females  
324 shared the same color pattern, the preference expressed by *M. h. theodorus* males likely stems  
325 from the differences in the iridescent blue color suggesting color-based discrimination in males.  
326 Nevertheless, in experiment 2 and 3, modified dummies were strongly rejected by males. We  
327 cannot entirely rule out that the artificial modifications performed on our dummies could generate  
328 a specific visual rejection by the males.

329

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

332

333 In a final experiment (experiment 4), we tested whether males from the two sympatric species from  
334 French Guiana (*M. achilles* and *M. helenor*) have a visual preference toward their conspecific  
335 females: two female dummies harboring the wings of females from the two species were presented  
336 to each male. While none of the males (from either *M. helenor* or *M. achilles* species) touched the  
337 female models, they did approach them in the cages. The approach rate and the number of  
338 approaches did not significantly differ between *M. helenor* males (rate = 61%; mean = 4.21, sd =  
339 5.34) and *M. achilles* males (rate = 66%; mean = 7.18, sd = 8.29; binomial GLM:  $p$ -value = 0.626),  
340 revealing similar levels of male courtship motivation. The independent analysis of the mean  
341 preference per species shows that neither *M. helenor* (Wilcoxon test,  $p$ -value = 0.503) nor *M.*  
342 *achilles* (Wilcoxon test,  $p$ -value = 0.406) males approached significantly more con-specific females  
343 as compared to the hetero-specific females in the cage (Fig. 4.C), suggesting that the difference  
344 among the convergent color patterns and blue iridescence of female *M. helenor* and *M. achilles* is  
345 not perceived by males.

346

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

349

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

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

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

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

369

370 **[Fig. 5: Divergent chemical compounds found on the genitalia of sympatric *Morpho* males.**  
371 nMDS representation of the differences in the (A) C8 to C16 chemical compounds and (B) C16 to  
372 C30 chemical compounds found in the genitalia of *M. h. helenor* and *M. a. achilles* males and  
373 females, calculated using Bray-Curtis distances. *M. h. helenor* are shown in blue (males are in dark  
374 blue and females in light blue), and *M. a. achilles* are shown in green (males are dark green and  
375 females are light green). The result of the PERMANOVA (999 permutations) testing the effect of  
376 sex and species on the chemical composition of *M. h. helenor* and *M. a. achilles* are shown on  
377 each figure.]

378

379

## 380 Discussion

381

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

384

385 Our study revealed a striking similarity in the iridescent coloration of the blue patches in the  
386 sympatric species of *M. helenor* and *M. achilles* in French Guiana, in sharp contrast with the  
387 divergent iridescence observed between allopatric populations of *M. helenor* sampled in different  
388 geographic areas. Together with the convergent evolution of the blue band width previously  
389 detected in these sympatric species (39), this observation is consistent with there being a primary  
390 role of visual predation on the evolution of wing coloration in *Morpho* (41, 42). The results of the  
391 visual models showing that these convergent iridescent signals are likely undistinguishable by  
392 avian predators further supports there being positive selection generated by predators promoting  
393 mimicry in these sympatric species. These results suggest that predation pressure can strongly  
394 impact the evolution of iridescence, and are further supported by the lack of directionality for the  
395 iridescent signal generated by *Morpho* wings. Directionality in iridescent signals is expected when  
396 sexually selected traits incur a predation cost due to their conspicuousness: directionality might  
397 enhance signaling to mates while remaining poorly detected by predators (31). Our study shows  
398 that *M. helenor* and *M. achilles* wings display a gradual variation of hue and brightness when  
399 changing the illumination/observation angles contrary to some other iridescent butterfly species  
400 (e.g. *Hypolimnas bolina*; (33)). The lack of signal directionality observed in these *Morpho* species  
401 thus suggests that the iridescent signal is likely on display for both potential mates and predators  
402 to see. These gradual changes in hue and brightness could also enhance the confusing effect  
403 generated by the alternate exposure of the iridescent and brown side of their wings during erratic  
404 flapping flight (34, 43).

405 Overall, the lack of directionality and the gradually changing aspect of *Morpho* iridescent signals  
406 do not point at any private iridescent signal. Instead, these visual effects are consistent with the  
407 emission of a confusing visual signal that increases the chances of escaping predators. The  
408 convergence of visual signals could have been favored because of the indirect advantage that

409 results in a reduction in attacks from predators that have experienced and learned to avoid the  
410 escape capabilities of these prey, consistent with the hypothesis of escape mimicry (44, 45).  
411 Interestingly, our reflectance measurements showed that males are generally brighter than females  
412 in both species, implying that the flashes generated by males during flight could be more  
413 conspicuous. Field experiments have highlighted the territorial behavior of males found patrolling  
414 along rivers and displaying competitive interactions with other males (7). This flight behavior  
415 strongly differs from female behavior, which are much less frequently observed in the wild and  
416 typically spend more time hidden in the understory (46). Considering that these ecological niche  
417 differences between males and females could result in different predation pressures in contrasted  
418 light conditions (open river banks vs. cluttered understory), iridescent signals could have evolved  
419 differently in the two sexes, consistent with a similar effect of divergent micro-habitats of males and  
420 females in both species (47, 48).  
421 Altogether, our results show that natural selection exerted by predators may have favored the  
422 evolution of mimetic iridescent color patterns, as well as sexual dimorphism in sympatric *Morpho*  
423 species.

424

#### 425 *Evolution of visual and olfactory cues in mimetic sister-species living in sympatry*

426

427 Our tetrad and male mate choice experiments in *M. helenor* butterflies clearly show assortative  
428 mating based on wing coloration. This result is consistent with the high attraction generated by blue  
429 coloration in males, as well as the preference of males for local vs. exotic wing color patterns found  
430 in previous field behavioral experiments carried out in Amazonian Peru (7). Our male mate choice  
431 experiments suggest that both the wing patterning and the iridescent properties of the blue patch  
432 could have an effect on male preference. Furthermore, visual modeling also suggests that the  
433 differences of iridescent coloration between the populations of the same species can be perceived  
434 by mates. Coloration and color perception are key in mate recognition in many taxa (marine  
435 invertebrates (49), birds (50), mammals (51)) but patterns can also inform mate choice (52, 53). In  
436 jumping spiders, both pattern and coloration are important cues used during mate choice (54).  
437 Similarly, in *Heliconius* butterflies, both pigmentary coloration and wing pattern are used in mate  
438 choice, although the preference is hierarchical and coloration is the most important cue (18).  
439 Further experiments are needed to disentangle the relative effects of iridescent coloration and wing  
440 pattern in mate preference in *Morpho* butterflies, but our results show that iridescent color patterns  
441 can be used as mate recognition cues in *M. helenor*.

442

443 Interestingly, intraspecific variations in courtship motivation were detected in our behavioral  
444 experiments within the species *M. helenor*. Individuals from the western areas of the Andes  
445 displayed high courtship motivation while butterflies from the Amazonian area, where *M. helenor*  
446 coexists with *M. achilles*, showed a strong decrease in responses to our visual dummies. The joint  
447 convergence of the color pattern and blue iridescence between *M. helenor* and *M. achilles* in  
448 Amazonia probably makes visual cues poor species recognition signals: visual modeling even  
449 suggests that *M. helenor* cannot perceive the color difference between the two. This is further  
450 supported by our male choice experiment showing that although males are attracted by blue  
451 iridescent colors and approach each female, they do not preferentially interact with their conspecific  
452 female dummies. Selection on visual preference might be relaxed in Amazonian areas where the  
453 two sister species are found in sympatry: the shared predator communities may strongly favor  
454 convergent evolution of dorsal iridescent color pattern, making visual signals poor species  
455 recognition cue.

456

457 The convergent evolution of iridescence in sympatric species might thus promote divergent  
458 evolution of alternative mating cues. By quantifying chemical compounds found on the genitalia of  
459 males and females sampled in Amazonian French Guiana, we did indeed find a strong divergence  
460 in the chemical profiles of sympatric *M. helenor* and *M. achilles*, especially in males. High male  
461 chemical divergence in sympatric species suggests the evolution of female mate choice based on  
462 olfactory discrimination (55). Although all compounds were not identified, some of those that were  
463 have previously been identified as pheromone compounds used during male/female interactions in  
464 butterflies. In particular, beta-ocimene was found on the genitalia of males and is known to  
465 contribute to mate choice in courtship (17) or as an anti-aphrodisiac used by *Heliconius* males to  
466 repel other males after mating with a female (56). Additionally, we found strong divergence between  
467 males for heavier chemical compounds (C16-C30). Because cuticular hydrocarbons can be  
468 involved in mate discrimination during courtship (57), diverging non-volatile compounds could also  
469 be used as a discriminating cue. Although we did not perform mate choices to test for the  
470 discrimination of olfactory cue by females, divergence of odorant cues could be due to reproductive  
471 interference between sympatric species (58, 59). Altogether, our results therefore strongly suggest  
472 that the convergent evolution of iridescent wing pattern between the sympatric *M. helenor* and *M.*  
473 *achilles* in Amazonia, likely promoted by shared predation pressures, have relaxed selection on  
474 visually-based preferences and may have favored the evolution of divergent olfactory cues.

475

#### 476 *Conclusions*

477

478 Overall, our study shows that local ecological interactions could result in iridescent color patterns  
479 and mate preferences evolving separately in closely-related sister species. We find evidence of  
480 converging iridescent patterns in sympatry suggesting that predation could play a major role in the  
481 evolution of iridescence, by favoring the emergence of evasive mimicry in a similar manner as the  
482 Müllerian mimicry of chemically-defended species (38). There are nevertheless striking differences  
483 between the two types of mimicry: while a wide diversity of conspicuous color patterns are involved  
484 in Müllerian mimicry (60), the variation of iridescent patterns involved in evasive mimicry might be  
485 more tightly constrained, because iridescence itself directly participates in survival via visual  
486 confusion (35). Moreover, switches in color pattern is generally considered as a magic trait  
487 facilitating speciation in chemically defended species (61), and Müllerian mimicry frequently occurs  
488 among distantly-related species (20). Here we observe the co-existence of *Morpho* sister-species  
489 displaying convergent color patterns, suggesting that speciation can be promoted by the evolution  
490 of traits other than visual such as olfactory chemical cues, or by the specialization in other  
491 ecological temporal niches (7).

492 Altogether, this study shows how convergent trait evolution might lead to mutualistic interactions  
493 enhancing species coexistence (62), and alter how selection acts on other traits. It illustrates how  
494 the feedback between trait evolution and ecological interactions can fuel speciation and co-  
495 existence in sympatry.

496

#### 497 **Materials and Methods**

498

##### 499 I. Study system

500

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

504 *M. helenor* populations of Ecuador: Pupae from the Ecuadorian populations of *M. h. bristowi* and  
505 *M. h. theodorus* were purchased from a breeding farm located in Ecuador (Quinta De Goulaine,  
506 <https://quintadegoulaine.com/es/papillons.php>). These two subspecies of *M. helenor* are found in  
507 western vs. eastern areas of the ecuadorian Andes respectively. These commercially-bought  
508 pupae were then raised in insectaries at STRI in Gamboa, Panama, between January and March  
509 2023.

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

513

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

515

516 A. Measures of wing reflectance

517

518 We investigated the variations in iridescence in *M. h. bristowi*, *M. h. theodorus* (from Ecuador), *M.*  
519 *h. helenor* and *M. a. achilles* (from French Guiana), using 10 females and 10 males of each. We  
520 estimated the iridescence of the blue patches of the dorsal side of the wings by performing a series  
521 of reflectance measurements on wings positioned on a flat surface, following a template to ensure  
522 that the wing orientation remained the same across all measurements. Reflectance spectra  
523 captured at different angles were recorded on the right forewing, at a specific point located in the  
524 wing zone defined by the M3 and Cu1 veins according to the nymphalid ground plan (see (63)).  
525 Wing reflectance was measured using a spectrophotometer (AvaSpec-ULS2048CL-EVO-RS 200-  
526 1100nm, Avantes) coupled with a deuterium halogen light source (AvaLight-DH-S-BAL, Avantes)  
527 for 21 combinations of illumination and observation angles, covering the proximo-distal and  
528 anteroposterior planes of the wing (Fig. 2.A, see S1 for extended methods).

529 These measurements were repeated 3 times for all individuals to estimate the repeatability of the  
530 reflectance measurements at each angle using the R package *rptR* 0.9.22 (64). Since no difference  
531 was detected between replicates for any of the tested angles (S12), the mean of these 3  
532 measurements at each angle for each individual was used in the subsequent analyses.

533

534 B. Statistical analysis of iridescence

535

536 Each illumination/observation position produced a reflectance spectrum whose characteristics (*i.e.*  
537 distribution of reflectance) define the optical properties of the illuminated surface. We focused our  
538 analyses on the wavelength range [300, 700 nm], relevant for butterfly and bird observers. All  
539 reflectance spectra were analyzed using the R package *pavo2* 2.9.0 (65).

540

541 a. Extraction and analysis of colorimetric variables

542

543 To identify the main optical effect produced by the wing at each angle, we extracted three  
544 commonly-used colorimetric variables from each spectrum, using the *summary()* function in *pavo2*:  
545 namely Hue (color in the common meaning of the term - measured as the wavelength at the  
546 maximum reflectance), Brightness (average proportion of reflected light - measured by the mean  
547 reflectance over each spectra) and Chroma (also referred to as saturation, it characterizes the



548 color's purity - measured as the ratio of the reflectance range of each spectra by the brightness).  
549 Because the normality and heteroscedasticity assumptions were not met for the distributions of  
550 values in these three parameters, we relied on permutation-based ANOVAs (*vegan* 2.6.4 package,  
551 (66)) to test the effect of sex, angle of measurement, locality and species on these three colorimetric  
552 variables. To specifically test whether differences in iridescence patterns were observed among  
553 species or localities, we estimated the interaction between the effects of *Morpho* populations and  
554 of the angle of illumination.

555

#### 556 b. Global iridescence analysis

557

558 As iridescence broadly refers to any change in reflectance in relation to illumination and observation  
559 angle (30), we characterized the iridescence of each individual using all complete spectra obtained  
560 at all angular positions (400 wavelengths x 21 angular positions). We applied a Principal  
561 Component Analysis (PCA) to this dataset, where each dot corresponds to the iridescence of an  
562 individual wing and thereby showing the similarities and differences in iridescence among groups.  
563 The samples from Ecuador and French Guiana were analyzed separately to test for more precise  
564 differences in iridescence between allopatric and sympatric *Morpho* butterfly populations. The  
565 coordinates of the 10 first PCs (representing 98% of the explained variance) were then extracted  
566 and a PERMANOVA was performed to test for the effect of sex, locality and species on iridescence  
567 patterns.

568

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

570

571 To investigate whether the variations observed among the different spectra could be perceived by  
572 different observers, we then used the R package *pavo2* 2.9.0 (65) to model both avian and butterfly  
573 visual capabilities. We used the avian UV-sensitive vision model implemented in *pavo* ("avg.uv")  
574 as a proxy for predator vision and specifically built a model of *M. helenor* vision, using the spectral  
575 sensitivity measured in (67). We built an hexachromatic visual model using the *sensmodel* function,  
576 using 345, 446, 495, 508, 570 and 602 nm as the maximum of absorbance in our six simulated  
577 cones respectively (S10). The *vismodel* function was used to compute the quantum catch of each  
578 receptor in the *Morpho* vision model under standard daylight illumination for each spectrum. Finally,  
579 assuming that the discrimination of colors is limited by photoreceptor noise (Receptor-Noise Limited  
580 model, (68)), we estimated the distance between colored stimuli as perceived by Morphos using  
581 the *bootcoldist* function. We used the default options (*weber* = 0.1, *weber.achro* = 0.1) and set the  
582 photoreceptor densities for each cones used in the visual model to 1. The resulting chromatic  
583 distances (*dS*) and confidence interval were then used to test how much the visual capacities of  
584 *Morpho* butterflies could allow the discrimination of wing reflectance depending on sex, localities  
585 and species, at each angle. Similarly, we used the avian UV sensitive visual model implemented  
586 in *pavo* to test the discrimination of *Morpho* wing reflectance by predators. We used  $dS \geq 1$  *Just*  
587 *Noticeable Difference* as the threshold of discrimination.

588

#### 589 IV. Mate choice experiments

590

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

594



595 a. Testing for mate discrimination within *M. helenor*

596

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

607

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

611

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

613

614 To investigate the visual preference of *M. helenor* males for the dorsal color pattern of females, we  
615 performed several choice experiments using dummy butterflies built with actual female wings. To  
616 ensure that preferences were not triggered by olfactory cues, all female wings used to build the  
617 artificial dummies were washed with hexane before any experiment. The details of the experimental  
618 design and specific visual cues tested are described in Table 2.

619

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

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

627 We finally tested the effect of the blue iridescent band within the same black and blue pattern  
628 (Experiment 3) by presenting *M. h. theodorus* males to a *M. h. theodorus* female with a narrow blue  
629 band, and to the modified *M. h. bristowi* female dummy with a narrow band. The wing pattern  
630 modifications were performed using a black permanent marker.

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

634

635 In all experiments, two female dummies were hanging from the ceiling of the cage, 1.5 m apart,  
636 and presented to a live male. The male was released in the experimental cage in front of the two  
637 female dummies and was free to interact with them for 10 minutes. The positions of the female  
638 models were swapped 5 minutes into each trial to avoid potential position-related bias. Two types  
639 of male behaviors were recorded. Each time a male flew within a 40 cm radius from a female model,  
640 an "approach" was recorded. Each time a male touched a female dummy, a "touch" was recorded.  
641 The number of "approaches" and "touches" toward each female dummy per trial was used to

642 determine male preference. Each male performed this experiment 3 times on 3 different days to  
643 account for potential individual variations in mate preference.

644

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

647

648 In order to investigate the importance of color vision in species recognition, we then performed a  
649 male choice experiment with *M. h. helenor* and *M. a. achilles* (experiment 4 in Table 2) in French  
650 Guiana, using natural wings of females *M. h. helenor* and *M. a. achilles* as artificial butterflies  
651 following the exact same protocol.

652

653 **[Table 2:** Experimental design of the male choice experiments performed between the Morphos  
654 originating from Ecuadorian populations of *M. helenor* (eastern population: *M. h. theodorus* and  
655 western population: *M. h. bristowi*, experiments 1 to 3) and from French Guiana (*M. h. helenor* and  
656 *M. a. achilles*, experiment 4), along with the associated preferences and visual cues tested. Note  
657 that the same males were used for experiments 1, 2 and 3.]

658

659 a. Statistical analyses

660

661 *Comparing male mating motivation*

662

663 Because we noticed behavioral differences between the species and subspecies of *Morpho* during  
664 the course of the experiments 1-4, we attempted to account for variations in male mating  
665 motivation. The approach and touch rates of males, *i.e.* their willingness to approach and touch  
666 (respectively) any female dummies during each trial, was defined as the proportion of trials where  
667 the males approached and touched (respectively) either female dummies at least once. The  
668 approach rate and touch rate of *M. h. bristowi*, *M. h. theodorus*, *M. h. helenor* and *M. a. achilles*  
669 males were calculated, and a binomial generalized linear model (GLM) was used to test whether  
670 the approach or touch rate was significantly different between males from different populations.

671 All statistical analyses were performed with the R package *glmmTMB* (69). The normality,  
672 homogeneity of variance and overdispersion tests for the GLMs were performed using the R  
673 package *DHARMA* (70). We controlled for potential individual variations by using the male ID and  
674 trial number as a random effect in all the tests.

675

676 *Visually-based male mate preferences*

677

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

684

685 V. Pheromone analysis for sympatric species

686 a. Chemical analyses

687

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

695 The chemical compounds of those samples were analyzed by gas chromatography and time-of-  
696 flight mass spectrophotometry (GC-ToF-MS) using a 8890 GC system (Agilent, Santa Clara, USA)  
697 coupled to a PEGASUS BT mass spectrophotometer (LECO, Saint Joseph, USA). The connected  
698 capillary column was a Rxi-5ms (30 m x 0.25 mm, df = 0.25  $\mu$ m, Restek, Bellefonte USA) and  
699 helium was used as carrier gas at 1 mL min<sup>-1</sup> constant flow rate. 1  $\mu$ L of each sample was injected  
700 in a GC split/splitless injector at 300 °C. Two temperature programs were used: (i) the analysis of  
701 the C8-C16 compounds started at 40 °C maintained for 1 min, then increased at 210°C at 6 °C min<sup>-1</sup>  
702 rate, finally increased to 340 °C at a 10 °C min<sup>-1</sup>, and (ii) the analysis of the C16-C30 compounds  
703 started at 70 °C maintained for 1 min, then increased to 150 °C at 30 °C min<sup>-1</sup> rate and finally  
704 increased to 340 °C at a 6 °C min<sup>-1</sup> rate. Mass spectra were recorded with a 70eV electron impact  
705 energy. Mixtures of alkanes C8 to C19 and C16 to C44 (ASTM® D5442 C16-C44 Qualitative  
706 Retention Time Mix, Supelco, Bellefonte, USA) were injected under the same conditions to be used  
707 as external standards.

708 The chromatograms were converted in netCDF format and operated using *MZmine 2.43* for  
709 compound detection and area integration. To this aim, chromatograms were cropped before 4 min  
710 and after 27 min, and baseline-corrected using the Rolling ball algorithm (71). Mass detection was  
711 performed using the centroid mass detector, and chromatograms of each mass were constructed  
712 using the ADAP algorithm (72, 73). Chromatograms were deconvoluted into individual peaks using  
713 the local minimum search algorithm and aligned on a master peak list with the RANSAC algorithm.  
714 Finally, the Peak Finder gap filling algorithm was used to search for missing peaks, and a matrix  
715 compiling the area under each peak detected for each butterfly sample was extracted. This matrix  
716 was purged from siloxane like compounds as well as compounds present in less than 10% of the  
717 samples. The areas of the blanks were subtracted to the samples, and the final data were used for  
718 statistical analysis. Mass spectra were then visualized using the ChromaTOF® software, compared  
719 to MZmine-selected ones, in order to manually annotate the chemical compounds. Annotation were  
720 based on scan comparison with the NIST2017 database, and linear retention indexes (LRI)  
721 calculated from the alkane mixtures with free database (<https://pubchem.ncbi.nlm.nih.gov/>) and  
722 literature (Adams, 2017).

723

#### 724 b. Statistical analyses of the chemical compounds

725

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

732 We also performed an Indicator Value Analysis to identify the chemical compounds significantly  
733 associated to our different *Morpho* samples with the *mutlipatt* function from the *indicspecies*  
734 package in R using the IndVal index from (74).

735

## 736 Acknowledgments

737

738 The authors would like to thank Aurélie Tournié and Doris Gomez for the advice provided on  
739 iridescence measurements. We are also grateful to Owen McMillan from the Smithsonian  
740 Tropical Research Institute (Panama) and Mathieu Chouteau from LEEISA in French Guiana  
741 (France) for providing facilities to perform the behavioral experiments. We exported the wings of  
742 the butterflies from Panama using exportation permit number PA-01-ARB-028-2023, and declared  
743 to the French authorities the exportation of butterfly wings from French Guiana to the French  
744 metropole. J.L. PhD was funded by an IBEEES grant from Sorbonne Université. This study was  
745 funded by the European Union (ERC-2022-COG - OUTFOTHEBLUE - 101088089). Views and  
746 opinions expressed are however those of the authors only and do not necessarily reflect those of  
747 the European Union or the European Research Council. Neither the European Union nor the  
748 granting authority can be held responsible for them.

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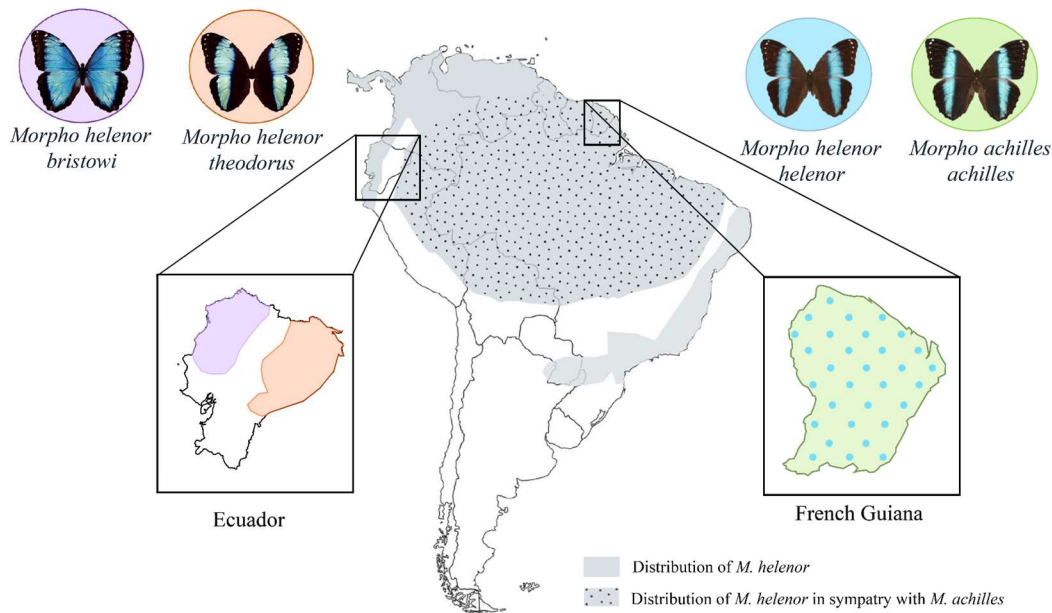
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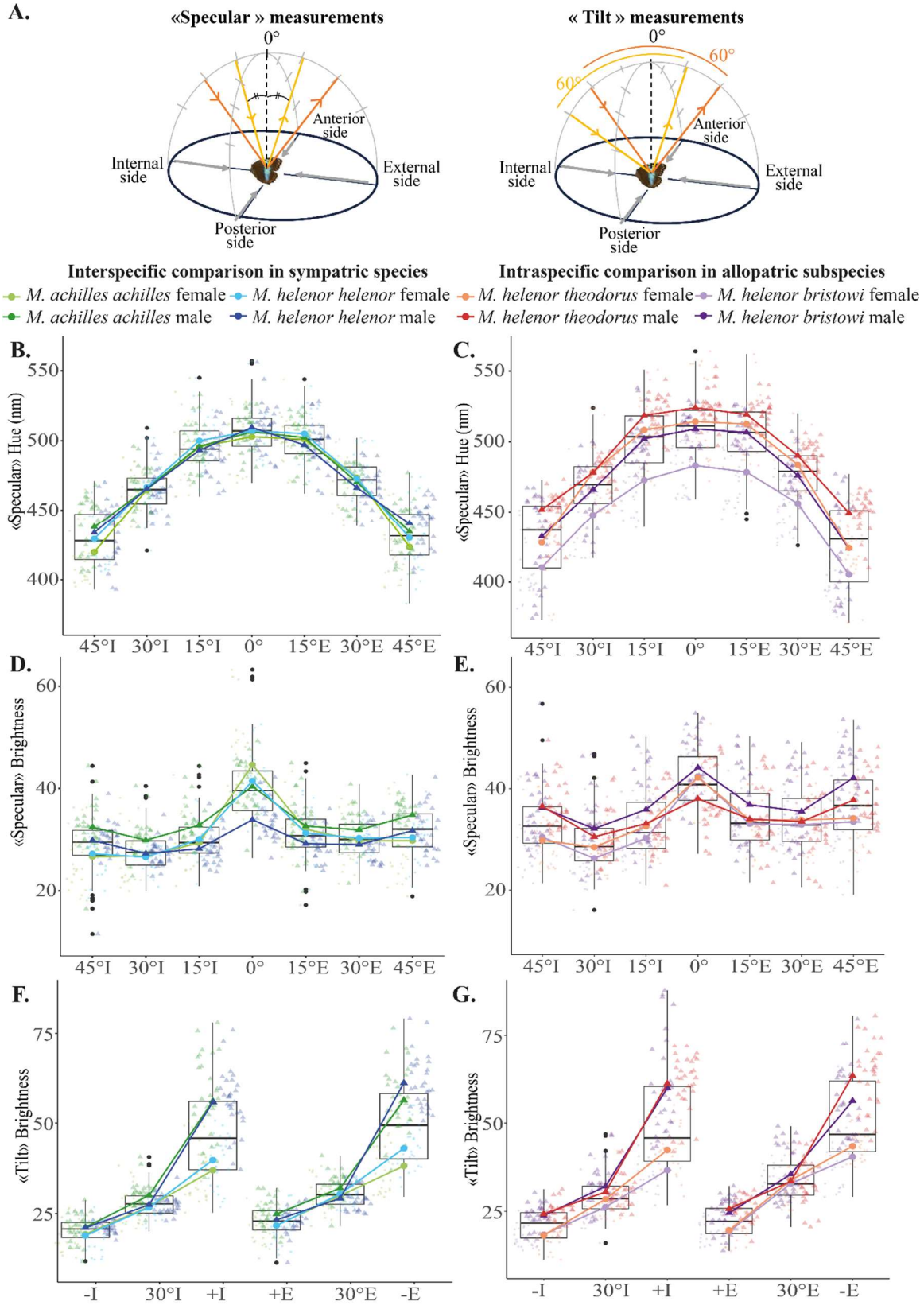
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### Figures and Tables



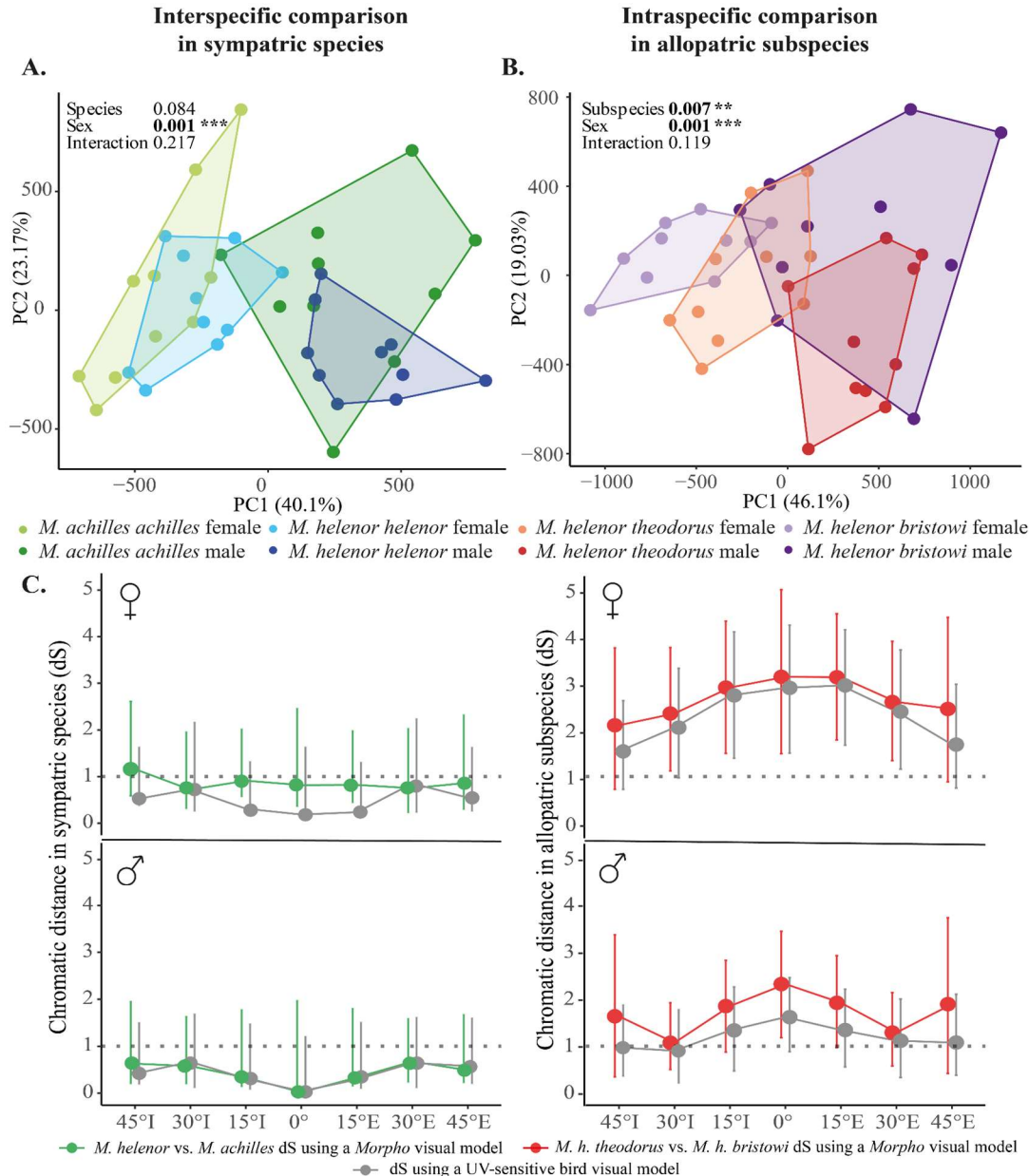
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**Fig. 1: Distribution of the sister-species *M. helenor* and *M. achilles* across South America.** The gray area represents the whole distribution of *M. helenor*, and the dotted area indicates the localities where *M. helenor* is in sympatry with *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 (39).



946 **Fig. 2: Differences of hue and brightness on the proximo-distal plane of *Morpho* wings** (A)  
947 Illustration of the two protocols used to assess the differences in *Morpho* wing reflectance. The  
948 “Specular” set-up allows for the quantification of the wing color variations, while the “tilt” set-up can  
949 be used to quantify brightness variation for each *Morpho* wing (see Appendix for extended  
950 methods). Variations of hue (B and C) and brightness (D and E) calculated from the wing  
951 reflectance measured with the “Specular” set-up, and variations of brightness calculated from the  
952 wing reflectance measured from the “tilt” set-up (F and G). Those hue and brightness parameters  
953 were calculated for the sympatric *M. h. helenor* and *M. a. achilles* (first column in green and blue)  
954 and for the allopatric *M. h. theodorus* and *M. h. bristowi* (second column in orange and purple) on  
955 the proximal-plane plane of their wings (I= illumination on the internal side of the wings, E=  
956 illumination on the external side of the wings). See S4 for the PERMANOVA analyses describing  
957 those graphs.  
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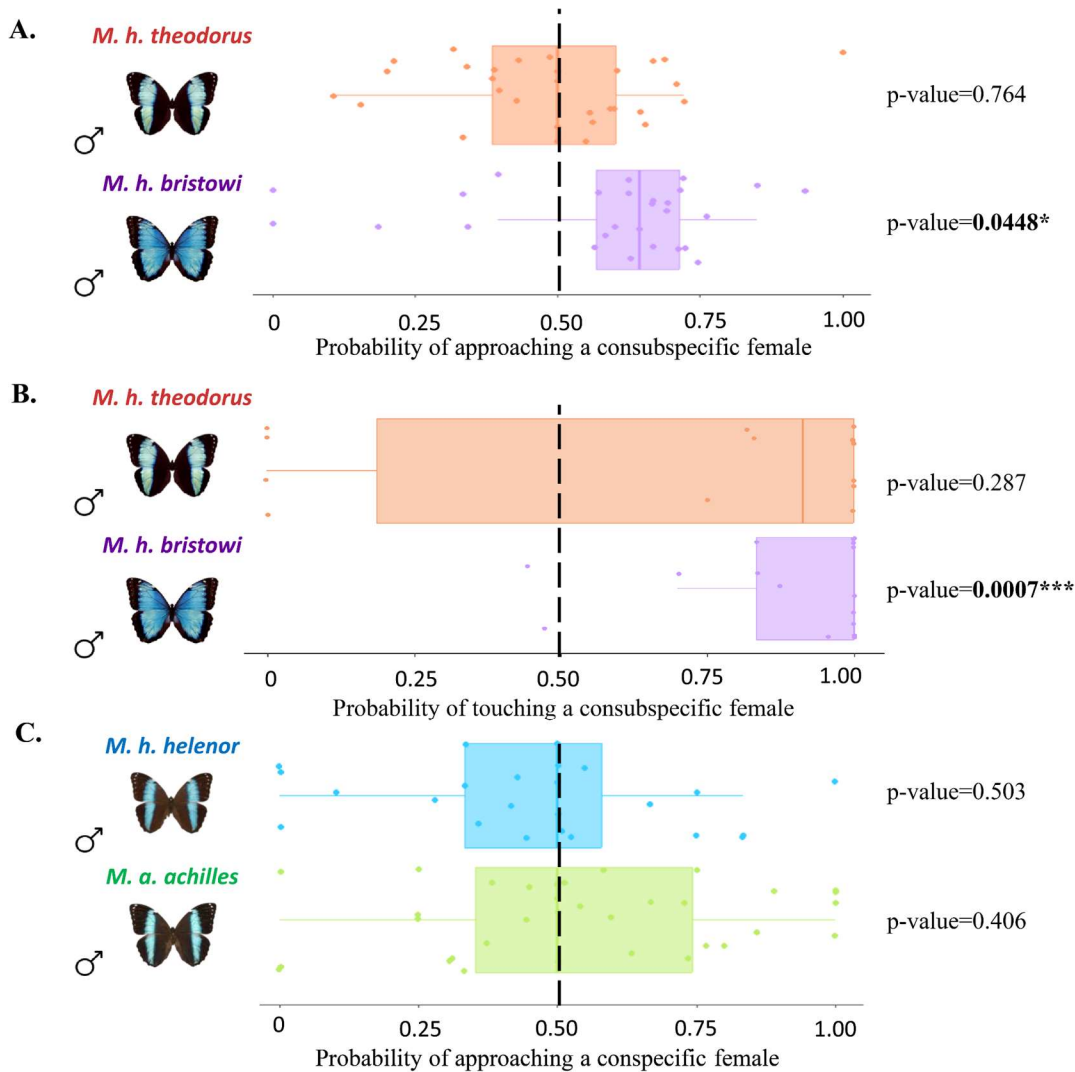


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**Fig. 3: Characterization and perception of the iridescent coloration of *Morpho* butterflies.** (A) and (B) : PCAs showing the variation in iridescence for both sexes, (A) in the two sympatric species from French Guiana (*M. h. helenor* vs. *M. a. achilles*) and (B) in the two allopatric Ecuadorian subspecies of *M. helenor* (*M. h. theodorus* vs. *M. h. bristowi*). Each point represents the global signal of iridescence of each individual, corresponding to the 21 complete reflectance spectra obtained from the 21 tested angles of illumination. The results of the PERMANOVA are shown on the top left corner of each graph. (C) The chromatic distances (*i.e.* the visual discrimination rate by a visual model) of the wing reflectance measured with the “Specular” set-up on the proximo-distal plane. Visual modeling was used to calculate the chromatic contrast of blue coloration between allopatric *M. helenor* subspecies (red) and between the two sympatric sister-species *M. helenor* and *M. achilles* (green), as perceived by a *Morpho* visual system for every angle of illumination measured on the proximo-distal plane. The chromatic contrast likely perceived by UV-sensitive birds is shown in gray. Chromatic contrast of the female wings (top) and male wings



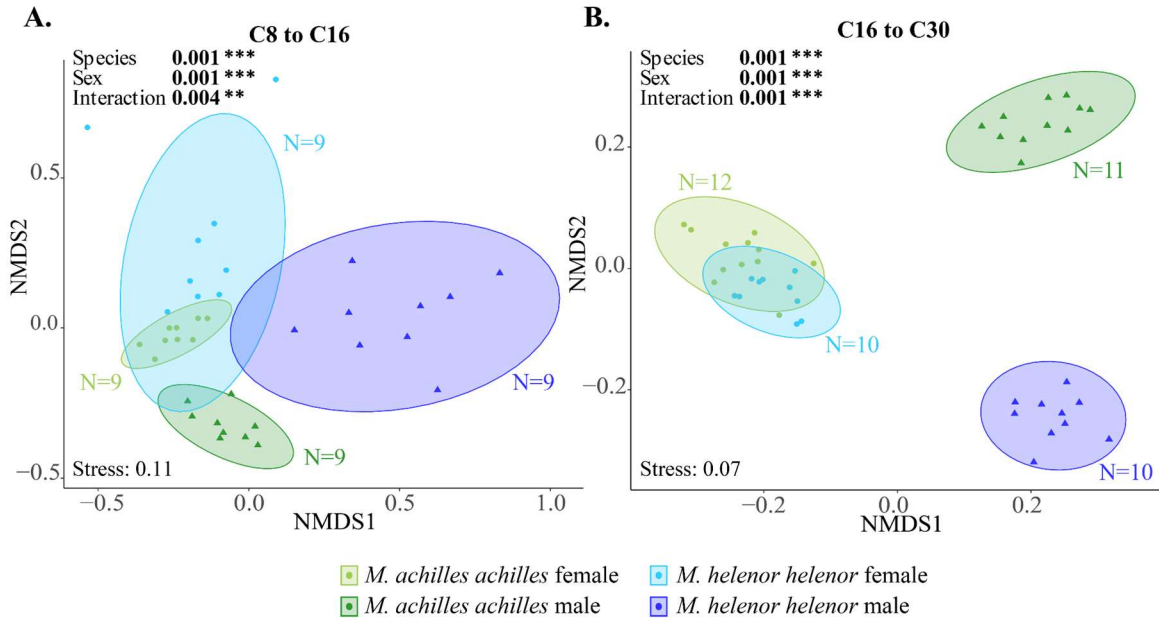
975 (bottom). The threshold of discrimination is shown by the dotted line and set to 1 *Just Noticeable*  
976 *Difference (JND)*.  
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**Fig. 4: Morpho male preference based on visual cues alone.**

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



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









998 **Table 1: Number of mating events involving the different possible pairs of males and**  
999 **females from the two subspecies of *M. helenor* (*bristowi* vs. *theodorus*) in 30 tetrad**  
1000 **experiments**  
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N=30

	♀ <i>M. h. theodorus</i>	♀ <i>M. h. bristowi</i>
♂ <i>M. h. theodorus</i>	6	3
♂ <i>M. h. bristowi</i>	5	16

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1005 **Table 2:** Experimental design of the male choice experiments performed between the  
 1006 Morphos originating from Ecuadorian populations of *M. helenor* (eastern population: *M.*  
 1007 *h. theodorus* and western population: *M. h. bristowi*, experiments 1 to 3) and from French  
 1008 Guiana (*M. h. helenor* and *M. a. achilles*, experiment 4), along with the associated  
 1009 preferences and visual cues tested. Note that the same males were used for experiments 1,  
 1010 2 and 3.

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

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