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: Joséphine Ledamoisel

Email: josephine.ledamoisel@outlook.fr

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

Competing Interest Statement: No competing interest.

Classification: Biological Science, Evolution

Keywords: Selection, Trait diversification, Mimicry

This PDF file includes:

Main Text
Figures 1 to 5
Tables 1 to 2

Abstract

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

Significance Statement

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

Main Text

Introduction

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

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

Theoretical and empirical studies have shown that sexual selection may favor the evolution of preferences for locally-adapted traits within species (8–10). For instance, the predator-deterrent coloration of poison frogs is also detected and used as mating cues by females (11). Similarly, habitat-dependent coloration of sympatric cichlid fish is also used as a visual cue for mate recognition (12). 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 (13). For example, because sympatric amphibians can sometimes share the same mating cues and preferences, they often engage in misdirected mating attempts towards heterospecifics, a potentially deleterious behavior (14). In addition, hybrids, when produced, can be unfit, thus favoring the evolution of sexual preferences for species-specific cues, rather than locally-adapted traits (15). Comparing variations in adaptive traits in sympatric *vs*. allopatric populations of recently-diverged species, and testing the sexual preference for those traits can shed light on the selective processes targeting traits modulating reproductive isolation and co-existence in sympatry.

In butterflies, the evolution of wing color patterns can be influenced by both natural and sexual selection. The visual discrimination of wing color pattern has been shown to enable intraspecific recognition during courtship in many species (16, 17). However, the evolution of wing color patterns is also strongly influenced by the visual effect perceived by predators (18, 19). Whether these selective pressures 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 (20). Positive selection acting on divergent traits involved in species recognition could be increased because of higher reproductive interference in closely related species than in distantly related taxa (21). In sympatric species with chemical-defenses, such as *Heliconinii* butterflies, local predation pressures tend to promote the convergence of similar conspicuous warning wing patterns (*i.e.* Müllerian mimicry, (22, 23)). But the costs associated with hybrid production in turn favor the evolution of alternative divergent mating cues in mimetic butterflies (24), and divergence in male pheromone bouquets and female attraction has been found among mimetic sister species (25). 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 (26). Thus, ecological interactions among recently-diverged species living in sympatry can impact the evolution of traits involved in both local adaptation and mate recognition and may trigger the evolution of alternative traits. Investigating variations in traits used as mating cues in allopatric *vs*. sympatric populations of sister-species can help ascertain how trait evolution might favor speciation and co-existence in sympatry.

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

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

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

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

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

Results

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

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

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 Appendix for extended methods). We then extracted for each spectrum three commonly-used colorimetric variables: Hue, Brightness and Chroma (analyzed in S4)

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 Table S1 and S5) confirming that in both species, both sexes are iridescent. Second, the brightness of *Morpho* wings is highly sexually dimorphic, as the effect of sex is always significant. The brightness measured with the “tilt” protocol (Fig. 2.F and 2.G) shows that males tend to be brighter than females in both Ecuador and French Guiana.

[Fig. 2: Differences of hue and brightness on the proximo-distal plane of *Morpho* wings (A) Illustration of the two protocols used to assess the differences in *Morpho* wing reflectance. The “Specular” set-up allows for the quantification of the wing color variations, while the “tilt” set-up can be used to quantify brightness variation for each *Morpho* wing (see Appendix for extended methods). Variations of hue (B and C) and brightness (D and E) calculated from the wing reflectance measured with the “Specular” set-up, and variations of brightness calculated from the wing reflectance measured from the “tilt” set-up (F and G). Those hue and brightness parameters were calculated for the sympatric *M. h. helenor* and *M. a. achilles* (first column in green and blue) and for the allopatric *M. h. theodorus* and *M. h. bristowi* (second column in orange and purple) 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 Table S1 for the PERMANOVA analyses describing those graphs.]

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, significant divergence of iridescence between populations was detected (Fig. 3.B). In contrast, iridescence did not differ between species living in sympatry (PERMANOVA, *p-value* = 0.084), suggesting that iridescence converges in sympatry (Fig. 3.A). In both cases, the interaction term taxon*sex was non-significant, suggesting that the sexual dimorphism of iridescence was similar for all taxa.

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

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

Evidence of mate preferences based on visual cues within species

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

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

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

[Table 1: 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.]

[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.]

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

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

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

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

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

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

In a final experiment (experiment 4), we tested whether males from the two sympatric species from French Guiana (*M. achilles* and *M. helenor*) 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 (from either *M. helenor* or *M. achilles* species) touched the female models, they did approach them in the cages. The approach rate and the number of approaches did not significantly differ between *M. helenor* males (rate = 61%; *mean* = 4.21, *sd* = 5.34) and *M. achilles* males (rate = 66%; *mean* = 7.18, *sd* = 8.29; *binomial GLM: p-value = 0.626*), revealing similar levels of male courtship motivation. The independent analysis of the mean preference per species shows that neither *M. helenor* (*Wilcoxon test, p-value = 0.503*) nor *M. achilles* (*Wilcoxon test, p-value = 0.406*) males approached significantly more con-specific females as compared to the hetero-specific females in the cage (Fig. 4.C), suggesting that the difference among the convergent color patterns and blue iridescence of female *M. helenor* and *M. achilles* is not perceived by males.

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

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

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

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

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

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

Discussion

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

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

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

results in a reduction in attacks from predators that have experienced and learned to avoid the escape capabilities of these prey, consistent with the hypothesis of escape mimicry (44, 45).
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 (7). 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 (46). 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, consistent with a similar effect of divergent micro-habitats of males and females in both species (47, 48).
Altogether, our results show that natural selection exerted by predators may have favored the evolution of mimetic iridescent color patterns, as well as sexual dimorphism in sympatric *Morpho* species.

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

Our tetrad and male mate choice experiments in *M. helenor* butterflies clearly show assortative mating based on wing coloration. This result is consistent with the high attraction generated by blue coloration in 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 (7). 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 (49), birds (50), mammals (51)) but patterns can also inform mate choice (52, 53). In jumping spiders, both pattern and coloration are important cues used during mate choice (54). 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 (18). 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*.

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 strong decrease in responses to our visual dummies. The joint convergence of the 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. This is further supported by our male choice experiment showing that although males are attracted by blue iridescent colors and approach each female, they do not preferentially interact with their conspecific female dummies. Selection on visual preference might be relaxed in Amazonian areas where the two sister species are found in sympatry: the shared predator communities may strongly favor convergent evolution of dorsal iridescent color pattern, making visual signals poor species recognition cue.

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

Conclusions

Overall, our study shows that local ecological interactions could result in iridescent color patterns and mate preferences evolving separately in closely-related sister species. We find evidence of converging iridescent patterns in sympatry suggesting that predation could play a major role in the evolution of iridescence, by favoring the emergence of evasive mimicry in a similar manner as the Müllerian mimicry of chemically-defended species (38). There are nevertheless striking differences between the two types of mimicry: while a wide diversity of conspicuous color patterns are involved in Müllerian mimicry (60), the variation of iridescent patterns involved in evasive mimicry might be more tightly constrained, because iridescence itself directly participates in survival via visual confusion (35). Moreover, switches in color pattern is generally considered as a magic trait facilitating speciation in chemically defended species (61), and Müllerian mimicry frequently occurs among distantly-related species (20). Here we observe the co-existence of *Morpho* sister-species displaying convergent color patterns, suggesting that speciation can be promoted by the evolution of traits other than visual such as olfactory chemical cues, or by the specialization in other ecological temporal niches (7).
Altogether, this study shows how convergent trait evolution might lead to mutualistic interactions enhancing species coexistence (62), and alter how selection acts on other traits. It illustrates how the feedback between trait evolution and ecological interactions can fuel speciation and co-existence in sympatry.

Materials and Methods

I. Study system

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

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

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

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 (63)). 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 S1 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 (64). Since no difference was detected between replicates for any of the tested angles (S12), 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 butterfly and bird observers. All reflectance spectra were analyzed using the R package *pavo2* 2.9.0 (65).

a. Extraction and analysis of colorimetric variables

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

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

b. Global iridescence analysis

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

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

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

IV. Mate choice experiments

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

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

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

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

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

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

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

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

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 and *M. a. achilles* individuals (from 9 up to 12 per sex per species) were dissected and
691 extracted in 20µL of dichloromethane, containing 5µL 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 µm, Restek, Bellefonte USA) and
699 helium was used as carrier gas at 1 mL min^{-1} constant flow rate. 1 µL 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^{-}
702 ^{1} rate, finally increased to 340 °C at a 10 °C min^{-1}, 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^{-1} rate and finally
704 increased to 340 °C at a 6 °C min^{-1} 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.

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 (74).

Acknowledgments

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

References

1. N. Chazot, *et al.*, Mutualistic Mimicry and Filtering by Altitude Shape the Structure of Andean Butterfly Communities. *Am. Nat.* **183**, 26–39 (2014).

2. M. Muschick, A. Indermaur, W. Salzburger, Convergent Evolution within an Adaptive Radiation of Cichlid Fishes. *Curr. Biol.* **22**, 2362–2368 (2012).

3. S. H. Berlocher, J. L. Feder, Sympatric Speciation in Phytophagous Insects: Moving Beyond Controversy? *Annu. Rev. Entomol.* **47**, 773–815 (2002).

4. R. K. Butlin, C. M. Smadja, Coupling, Reinforcement, and Speciation. *Am. Nat.* **191**, 155–172 (2018).

5. J. Ellers, C. L. Boggs, THE EVOLUTION OF WING COLOR: MALE MATE CHOICE OPPOSES ADAPTIVE WING COLOR DIVERGENCE IN COLIAS BUTTERFLIES. *Evolution* **57**, 1100–1106 (2003).

6. M. E. Maan, O. Seehausen, Ecology, sexual selection and speciation. *Ecol. Lett.* **14**, 591–602 (2011).

7. C. Le Roy, *et al.*, Convergent morphology and divergent phenology promote the coexistence of Morpho butterfly species. *Nat. Commun.* **12**, 7248 (2021).

8. M. R. Servedio, THE EVOLUTION OF PREMATING ISOLATION: LOCAL ADAPTATION AND NATURAL AND SEXUAL SELECTION AGAINST HYBRIDS. *Evolution* **58**, 913–924 (2004).

9. M. R. Servedio, J. W. Boughman, The Role of Sexual Selection in Local Adaptation and Speciation. *Annu. Rev. Ecol. Evol. Syst.* **48**, 85–109 (2017).

10. G. S. van Doorn, P. Edelaar, F. J. Weissing, On the Origin of Species by Natural and Sexual Selection. *Science* **326**, 1704–1707 (2009).

11. R. G. Reynolds, B. M. Fitzpatrick, ASSORTATIVE MATING IN POISON-DART FROGS BASED ON AN ECOLOGICALLY IMPORTANT TRAIT. *Evolution* **61**, 2253–2259 (2007).

12. O. Seehausen, *et al.*, Speciation through sensory drive in cichlid fish. *Nature* **455**, 620–626 (2008).

13. J. Gröning, A. Hochkirch, Reproductive Interference Between Animal Species. *Q. Rev. Biol.* **83**, 257–282 (2008).

14. S. P. Soni, V. Apte, P. Joshi, V. P. Cyriac, Barking up the wrong frog: global prevalence of misdirected amplexus in anuran amphibians. *Biol. J. Linn. Soc.* blae062 (2024). https://doi.org/10.1093/biolinnean/blae062.

15. L. Maisonneuve, C. Smadi, V. Llaurens, Which cues are sexy? The evolution of mate preference in sympatric species reveals the contrasted effect of adaptation and reproductive interference. *Evol. Lett.* **8**, 283–294 (2024).

16. K. Costanzo, A. Monteiro, The use of chemical and visual cues in female choice in the butterfly Bicyclus anynana. *Proc. R. Soc. B Biol. Sci.* **274**, 845–851 (2007).

17. C. Li, *et al.*, Role of visual and olfactory cues in sex recognition in butterfly Cethosia cyane cyane. *Sci. Rep.* **7**, 5033 (2017).

18. S. D. Finkbeiner, A. D. Briscoe, R. D. Reed, Warning signals are seductive: Relative contributions of color and pattern to predator avoidance and mate attraction in Heliconius butterflies: BUTTERFLY VISUAL SIGNALS. *Evolution* **68**, 3410–3420 (2014).

19. J. C. Oliver, K. A. Robertson, A. Monteiro, Accommodating natural and sexual selection in butterfly wing pattern evolution. *Proc. R. Soc. B Biol. Sci.* **276**, 2369–2375 (2009).

20. A. Puissant, A. Chotard, F. L. Condamine, V. Llaurens, Convergence in sympatric swallowtail butterflies reveals ecological interactions as a key driver of worldwide trait diversification. *Proc. Natl. Acad. Sci.* **120**, e2303060120 (2023).

21. K. S. Pfennig, D. W. Pfennig, Character Displacement: Ecological and Reproductive Responses to a Common Evolutionary Problem. *Q. Rev. Biol.* **84**, 253–276 (2009).

22. M. Joron, I. R. Wynne, G. Lamas, J. Mallet, Variable Selection and the Coexistence of Multiple mimetic forms of the Butterfly Heliconius numata. *Evol. Ecol.* **13**, 721–754 (1999).

23. R. M. Merrill, A. Chia, N. J. Nadeau, Divergent warning patterns contribute to assortative mating between incipient Heliconius species. *Ecol. Evol.* **4**, 911–917 (2014).

24. C. Estrada, C. D. Jiggins, Interspecific sexual attraction because of convergence in warning colouration: is there a conflict between natural and sexual selection in mimetic species? *J. Evol. Biol.* **21**, 749–760 (2008).

25. M. F. González-Rojas, *et al.*, Chemical signals act as the main reproductive barrier between sister and mimetic Heliconius butterflies. *Proc. R. Soc. B Biol. Sci.* **287**, 20200587 (2020).

26. V. Llaurens, M. Joron, M. Théry, Cryptic differences in colour among Müllerian mimics: how can the visual capacities of predators and prey shape the evolution of wing colours? *J. Evol. Biol.* **27**, 531–540 (2014).

27. P. Blandin, B. Purser, Evolution and diversification of Neotropical butterflies: Insights from the biogeography and phylogeny of the genus Morpho Fabricius, 1807 (Nymphalidae: Morphinae), with a review of the geodynamics of South AAmerica. *Trop. Lepidoptera Res.* 62–85 (2013).

28. R. H. Siddique, S. Diewald, J. Leuthold, H. Hölscher, Theoretical and experimental analysis of the structural pattern responsible for the iridescence of Morpho butterflies. *Opt. Express* **21**, 14351 (2013).

29. M. A. Giraldo, S. Yoshioka, C. Liu, D. G. Stavenga, Coloration mechanisms and phylogeny of Morpho butterflies. *J. Exp. Biol.* **219**, 3936–3944 (2016).

30. S. M. Doucet, M. G. Meadows, Iridescence: a functional perspective. *J. R. Soc. Interface* **6** (2009).

31. J. A. Endler, Signals, Signal Conditions, and the Direction of Evolution. *Am. Nat.* **139**, S125–S153 (1992).

32. R. K. Simpson, K. J. McGraw, Interspecific Covariation in Courtship Displays, Iridescent Plumage, Solar Orientation, and Their Interactions in Hummingbirds. *Am. Nat.* **194**, 441–454 (2019).

33. T. E. White, J. Zeil, D. J. Kemp, Signal design and courtship presentation coincide for highly biased delivery of an iridescent butterfly mating signal. *Evolution* **69**, 14–25 (2015).

34. A. M. Young, Wing coloration and reflectance in Morpho butterflies as related to reproductive behavior and escape from avian predators. *Oecologia* **7**, 209–222 (1971).

35. G. Murali, U. Kodandaramaiah, Size and unpredictable movement together affect the effectiveness of dynamic flash coloration. *Anim. Behav.* **162**, 87–93 (2020).

36. A. Vieira-Silva, G. B. Evora, A. V. L. Freitas, P. S. Oliveira, The Relevance of Flash Coloration Against Avian Predation in a Morpho Butterfly: A Field Experiment in a Tropical Rainforest. *Ethology* **130**, e13517 (2024).

37. E. Páez, *et al.*, Hard to catch: experimental evidence supports evasive mimicry. *Proc. R. Soc. B Biol. Sci.* **288**, 20203052 (2021).

38. C. E. G. Pinheiro, A. V. L. Freitas, V. C. Campos, P. J. DeVries, C. M. Penz, Both Palatable and Unpalatable Butterflies Use Bright Colors to Signal Difficulty of Capture to Predators. *Neotrop. Entomol.* **45**, 107–113 (2016).

39. V. Llaurens, Y. Le Poul, A. Puissant, P. Blandin, V. Debat, Convergence in sympatry: Evolution of blue-banded wing pattern in Morpho butterflies. *J. Evol. Biol.* **34**, 284–295 (2021).

40. N. Chazot, P. Blandin, V. Debat, M. Elias, F. L. Condamine, Punctuational ecological changes rather than global factors drive species diversification and the evolution of wing phenotypes in Morpho butterflies. *J. Evol. Biol.* **34**, 1592–1607 (2021).

41. M. Chouteau, M. Arias, M. Joron, Warning signals are under positive frequency-dependent selection in nature. *Proc. Natl. Acad. Sci.* **113**, 2164–2169 (2016).

42. A. M. M. Stuckert, P. J. Venegas, K. Summers, Experimental evidence for predator learning and Müllerian mimicry in Peruvian poison frogs (Ranitomeya, Dendrobatidae). *Evol. Ecol.* **28**, 413–426 (2014).

43. C. Le Roy, *et al.*, Adaptive evolution of flight in Morpho butterflies. *Science* **374**, 1158–1162 (2021).

44. C. E. G. Pinheiro, A. V. L. Freitas, Some Possible Cases of Escape Mimicry in Neotropical Butterflies. *Neotrop. Entomol.* **43**, 393–398 (2014).

45. G. D. Ruxton, M. Speed, T. N. Sherratt, Evasive mimicry: when (if ever) could mimicry based on difficulty of capture evolve? *Proc. R. Soc. Lond. B Biol. Sci.* **271**, 2135–2142 (2004).

46. A. M. Young, Studies on comparative ecology and ethology in adult populations of several species of Morpho butterflies (Lepidoptera: Morphidae). *Stud. Neotropical Fauna* **8**, 17–50 (1973).

47. C. E. Allen, B. J. Zwaan, P. M. Brakefield, Evolution of Sexual Dimorphism in the Lepidoptera. *Annu. Rev. Entomol.* **56**, 445–464 (2011).

48. T. E. Reimchen, P. Nosil, Variable Predation Regimes Predict the Evolution of Sexual Dimorphism in a Population of Threespine Stickleback. *Evolution* **58**, 1274–1281 (2004).

49. J. Baldwin, S. Johnsen, The importance of color in mate choice of the blue crab Callinectes sapidus. *J. Exp. Biol.* **212**, 3762–3768 (2009).

50. S. P. Caro, *et al.*, Mutual mate preferences and assortative mating in relation to a carotenoid-based color trait in blue tits. *Behav. Ecol.* **32**, 1171–1182 (2021).

51. C. Waitt, *et al.*, Evidence from rhesus macaques suggests that male coloration plays a role in female primate mate choice. *Proc. R. Soc. Lond. B Biol. Sci.* **270**, S144–S146 (2003).

52. A. E. Houde, Mate Choice Based Upon Naturally Occurring Color-Pattern Variation in a Guppy Population. *Evolution* **41**, 1–10 (1987).

53. L. Pérez-Rodríguez, R. Jovani, M. Stevens, Shape matters: animal colour patterns as signals of individual quality. *Proc. R. Soc. B Biol. Sci.* **284**, 20162446 (2017).

54. W. Zhou, *et al.*, Sexual selection on jumping spider color pattern: investigation with a new quantitative approach. *Behav. Ecol.* **32**, 695–706 (2021).

55. C. Mérot, B. Frérot, E. Leppik, M. Joron, Beyond magic traits: Multimodal mating cues in Heliconius butterflies. *Evolution* **69**, 2891–2904 (2015).

56. S. Schulz, C. Estrada, S. Yildizhan, M. Boppré, L. E. Gilbert, An Antiaphrodisiac in Heliconius melpomene Butterflies. *J. Chem. Ecol.* **34**, 82–93 (2008).

57. H. Ômura, T. Noguchi, S. Ohta, The male swallowtail butterfly, *Papilio polytes*, uses cuticular hydrocarbons for mate discrimination. *Anim. Behav.* **170**, 133–145 (2020).

58. P. M. B. Bacquet, *et al.*, Selection on male sex pheromone composition contributes to butterfly reproductive isolation. *Proc. R. Soc. B Biol. Sci.* **282**, 20142734 (2015).

59. K. A. Dyer, B. E. White, J. L. Sztepanacz, E. R. Bewick, H. D. Rundle, REPRODUCTIVE CHARACTER DISPLACEMENT OF EPICUTICULAR COMPOUNDS AND THEIR CONTRIBUTION TO MATE CHOICE IN DROSOPHILA SUBQUINARIA AND DROSOPHILA RECENS. *Evolution* **68**, 1163–1175 (2014).

60. J. MALLET, L. E. GILBERT Jr., Why are there so many mimicry rings? Correlations between habitat, behaviour and mimicry in Heliconius butterflies. *Biol. J. Linn. Soc.* **55**, 159–180 (1995).

61. R. M. Merrill, *et al.*, Disruptive ecological selection on a mating cue. *Proc. R. Soc. B Biol. Sci.* **279**, 4907–4913 (2012).

62. G. Boussens-Dumon, V. Llaurens, Sex, competition and mimicry: an eco-evolutionary model reveals unexpected impacts of ecological interactions on the evolution of phenotypes in sympatry. *Oikos* **130**, 2028–2039 (2021).

63. A. Martin, R. D. Reed, wingless and aristaless2 Define a Developmental Ground Plan for Moth and Butterfly Wing Pattern Evolution. *Mol. Biol. Evol.* **27**, 2864–2878 (2010).

64. M. A. Stoffel, S. Nakagawa, H. Schielzeth, rptR: repeatability estimation and variance decomposition by generalized linear mixed-effects models. *Methods Ecol. Evol.* **8**, 1639–1644 (2017).

65. R. Maia, H. Gruson, J. A. Endler, T. E. White, 2: New tools for the spectral and spatial analysis of colour in r. *Methods Ecol. Evol.* **10**, 1097–1107 (2019).

66. J. Oksanen, *et al.*, vegan: Community Ecology Package. https://doi.org/10.32614/CRAN.package.vegan. Deposited 6 September 2001.

67. P. Pirih, M. Ilić, A. Meglič, G. Belušič, Opponent processing in the retinal mosaic of nymphalid butterflies. *Philos. Trans. R. Soc. B Biol. Sci.* **377**, 20210275 (2022).

68. M. Vorobyev, R. Brandt, D. Peitsch, S. B. Laughlin, R. Menzel, Colour thresholds and receptor noise: behaviour and physiology compared. *Vision Res.* **41**, 639–653 (2001).

69. M. E. Brooks, *et al.*, glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. *R J.* **9**, 378–400 (2017).

70. F. Hartig, *DHARMa: Residual Diagnostics for Hierarchical (Multi-Level / Mixed) Regression Models* (2022).

71. M. A. Kneen, H. J. Annegarn, Algorithm for fitting XRF, SEM and PIXE X-ray spectra backgrounds. *Nucl. Instrum. Methods Phys. Res. Sect. B Beam Interact. Mater. At.* **109–110**, 209–213 (1996).

72. T. Pluskal, S. Castillo, A. Villar-Briones, M. Orešič, MZmine 2: Modular framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data. *BMC Bioinformatics* **11**, 395 (2010).

73. O. D. Myers, S. J. Sumner, S. Li, S. Barnes, X. Du, One Step Forward for Reducing False Positive and False Negative Compound Identifications from Mass Spectrometry Metabolomics Data: New Algorithms for Constructing Extracted Ion Chromatograms and Detecting Chromatographic Peaks. *Anal. Chem.* **89**, 8696–8703 (2017).

74. M. Dufrêne, P. Legendre, Species Assemblages and Indicator Species:the Need for a Flexible Asymmetrical Approach. *Ecol. Monogr.* **67**, 345–366 (1997).

Figures and Tables

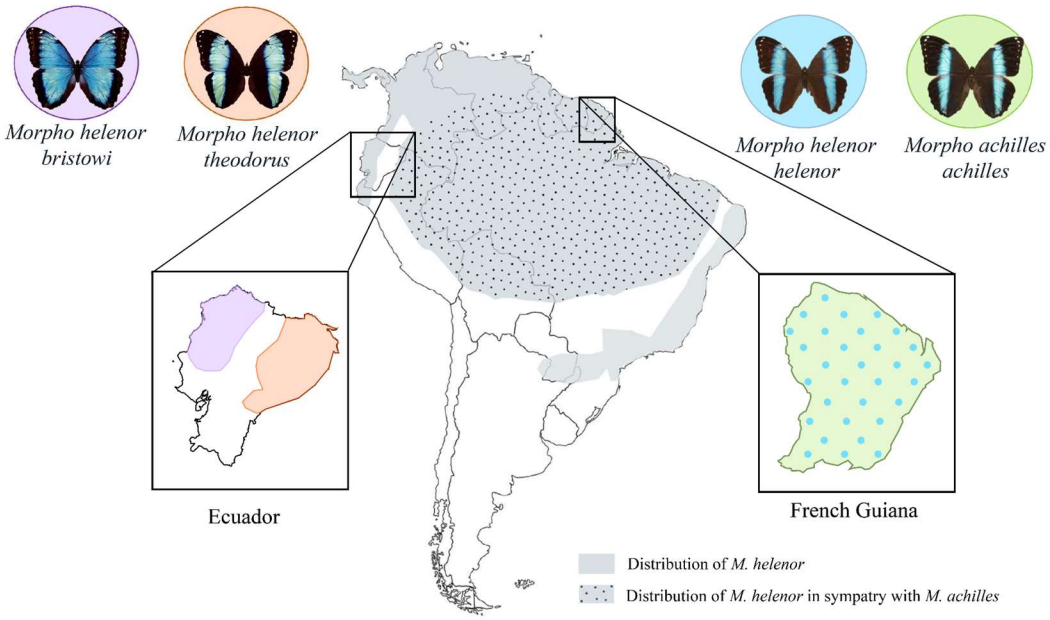

Fig. 1: Distribution of the sister-species *M. helenor* and *M. achilles* across South America. The gray area represents the whole distribution of *M. helenor*, and the dotted area indicates the localities where *M. helenor* is in sympatry with *M. achilles.* Note that the different subspecies of *M.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

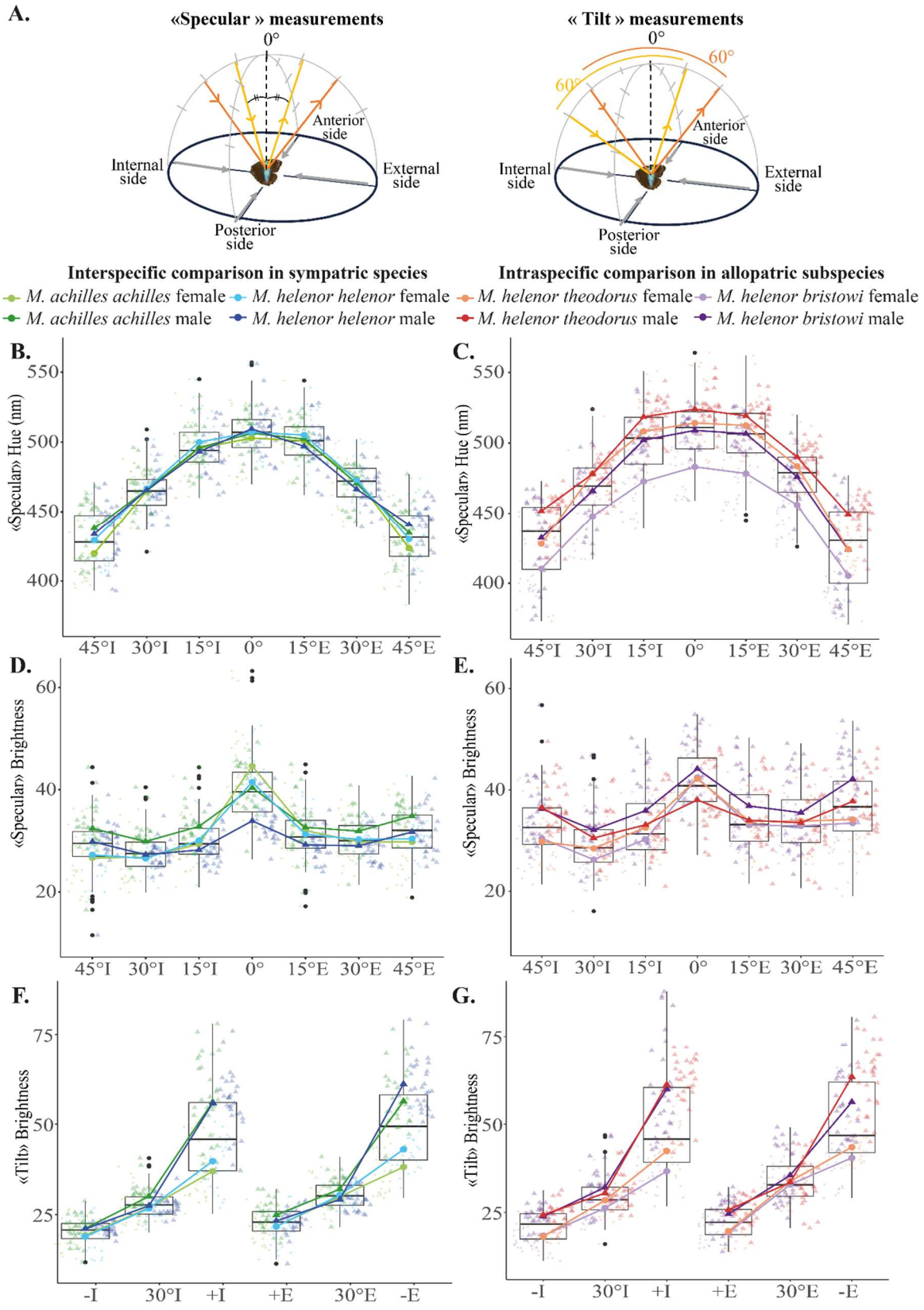
A.
«Specular » measurements
« Tilt » measurements
0°
60°
Anterior side
Internal side
External side
Posterior side
Interspecific comparison in sympatric species
Intraspecific comparison in allopatric subspecies
M. achilles achilles female
M. achilles achilles male
M. helenor helenor female
M. helenor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

Fig. 2: Differences of hue and brightness on the proximo-distal plane of *Morpho* wings (A) Illustration of the two protocols used to assess the differences in *Morpho* wing reflectance. The "Specular" set-up allows for the quantification of the wing color variations, while the "tilt" set-up can be used to quantify brightness variation for each *Morpho* wing (see Appendix for extended methods). Variations of hue (B and C) and brightness (D and E) calculated from the wing reflectance measured with the "Specular" set-up, and variations of brightness calculated from the wing reflectance measured from the "tilt" set-up (F and G). Those hue and brightness parameters were calculated for the sympatric *M. h. helenor* and *M. a. achilles* (first column in green and blue) and for the allopatric *M. h. theodorus* and *M. h. bristowi* (second column in orange and purple) 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 S4 for the PERMANOVA analyses describing those graphs.

.

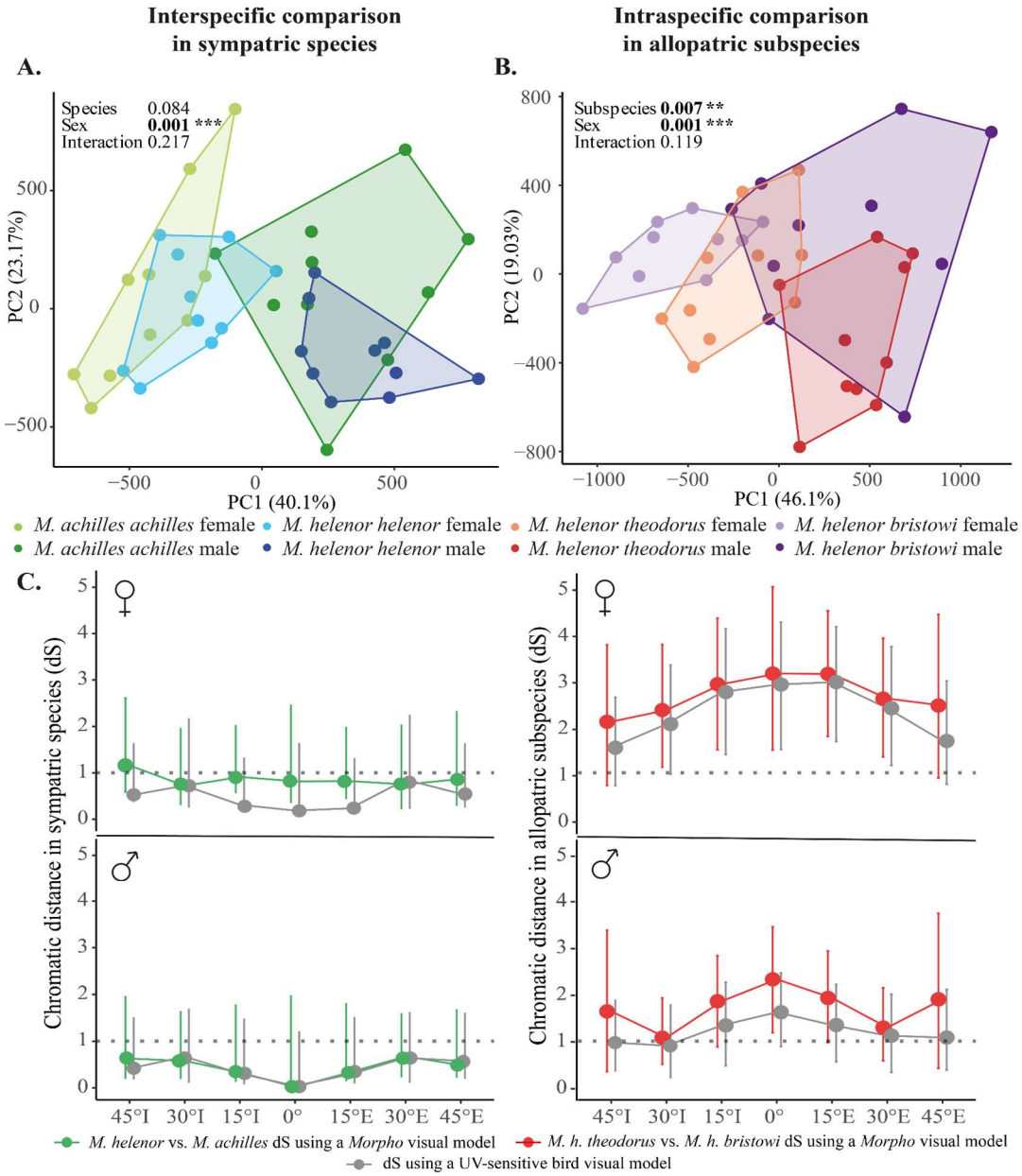

Fig. 3: Characterization and perception of the iridescent coloration of *Morpho* butterflies. (A) and (B) : PCAs showing the variation in iridescence for both sexes, (A) in the two sympatric species from French Guiana (*M. h. helenor* vs. *M. a. achilles*) and (B) in the two allopatric Ecuadorian subspecies 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

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

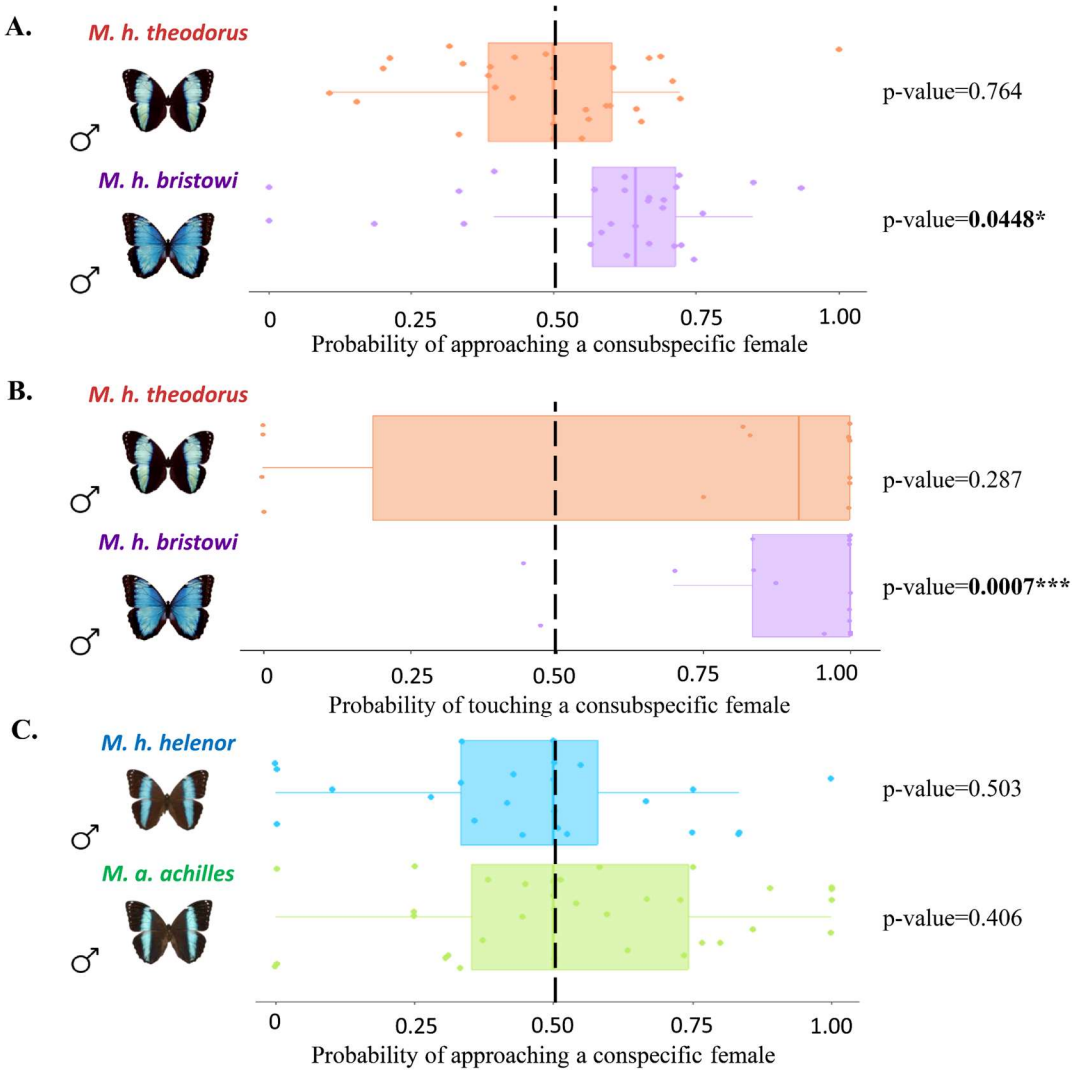

Fig. 4: Morpho male preference based on visual cues alone.
Probabilities of (A) approaching and (B) touching a con-subspecific female for *M. h. bristowi* males (purple) and *M. h. theodorus* males (orange) from Ecuador, as well as the probabilities of (C) approaching a con-specific female for *M. h. helenor* males (blue) and *M. a. achilles* males (green) 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.

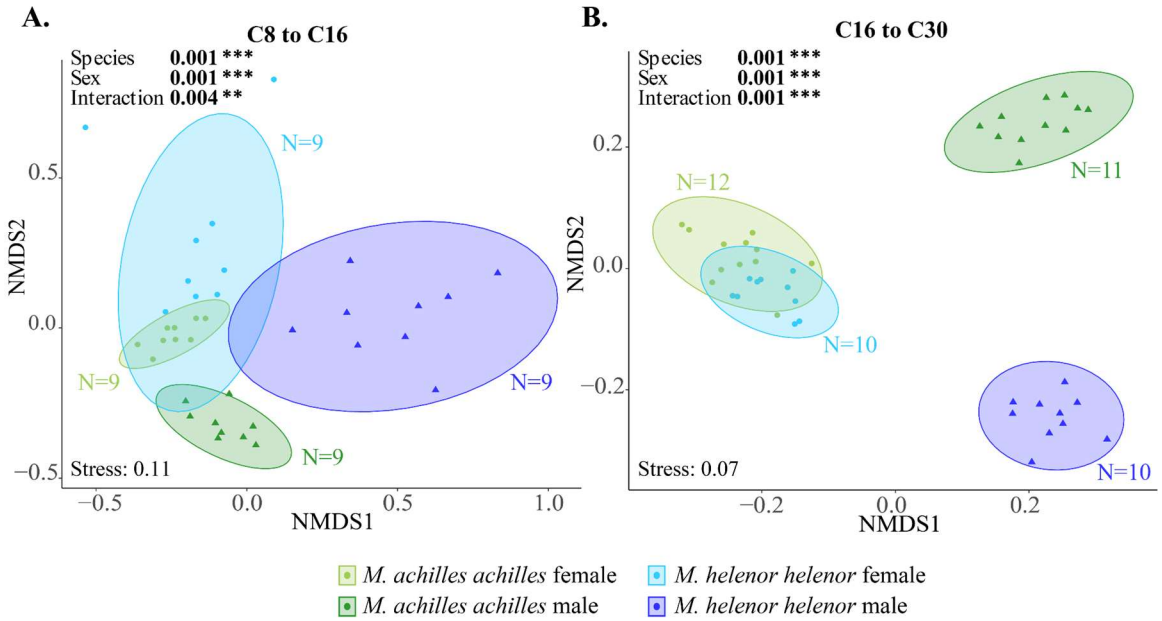

Fig. 5: Divergent chemical compounds found on the genitalia of sympatric *Morpho* males. nMDS representation of the differences in the (A) C8 to C16 chemical compounds and (B) C16 to C30 chemical compounds found in the genitalia of *M. h. helenor* and *M. a. achilles* males and females, calculated using 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**
1001

N=30

	♀ *M. h. theodorus*	♀ *M. h. bristowi*
♂ *M. h. theodorus*	6	3
♂ *M. h. bristowi*	5	16

1002
1003
1004

Table 2: 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.

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