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Ambient light and mimicry as drivers of wing transparency in Lepidoptera

Mónica Arias[1,2], Jérôme Barbut[3], Rodolphe Rougerie[2], Manon Dutry[1], Mireia Kohler[1], Baptiste Laulan[1], Caroline Paillard[1], Serge Berthier[4], Christine Andraud[5], Marianne Elias[2], Doris Gomez[1,4]

Affiliations

[1]CEFE, Univ Montpellier, CNRS, EPHE, IRD, Univ Paul Valéry Montpellier 3, Montpellier, France

[2]Institut de Systématique, Evolution, Biodiversité, CNRS, MNHN, Sorbonne Université, EPHE, Université des Antilles, 45 rue Buffon CP50, Paris, France

[3]MNHN, Direction des collections, 45 rue Buffon CP50, Paris, France

[4]INSP, Sorbonne Université, CNRS, Paris, France

[5]CRC, CNRS, MNHN, Paris, France

Corresponding author email: moarias@gmail.com

Abstract

Transparency reduces prey detectability by predators. While the proportion of transmitted light in aquatic species is higher as light availability increases, less is known about such variation in terrestrial species. Transparency has evolved several times in the typically opaque winged Lepidoptera order (moths and butterflies), displaying a large diversity of degrees. Using two complementary approaches, we explore how the evolution of the differences in light transmittance relates to habitat openness, daytime activity and mimicry syndrome (bee/wasp versus dead-leaf mimic). First, by exposing artificial moth-like prey to wild avian predators in open and closed habitats, we show that survival increases at higher proportions of transmitted light in open habitats. Second, by analysing the evolution of wing

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features and ecological traits in 107 clearwing species, we confirm that diurnal open-habitat species show higher light transmittances than diurnal closed-habitat species. Additionally, bee/wasp mimics are more often diurnal and have higher and less variable light transmittances than dead-leaf mimics, which are more often nocturnal. Bright conditions, such as open habitats during the day, and mimicry of insects with transparent wings seem to promote high light transmittance. Habitat openness, daytime activity and species interactions play a crucial role in determining transparency design.

Keywords: transparency, irradiance, mimicry, nocturnality, Lepidoptera, Batesian mimicry, habitat openness

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Introduction

Transparency is common in aquatic environments where it reduces detectability by predators, especially in the pelagic environment where there is nowhere to hide (Johnsen 2014). However, the amount of light that is transmitted by transparent tissues varies between organisms. Based on the observation of 29 zooplankton species, Johnsen and Widder (1998), found that light transmittance could range from 50 to 90%, with the most transparent species living in lighter, shallower places. In a theoretical approach, they show that efficiency at detecting transparent species depends on visual performance (for instance, minimum contrast that predator can detect, also known as contrast threshold), which varies with ambient light conditions: in shallow places with bright light conditions, high visual performance (low contrast threshold values) selects for high transmittance, and only highly transparent organisms are effectively undetected. Instead, at deeper areas with dimmer light conditions, visual systems are less performing (high contrast threshold values), thus organisms can be poorly transparent and be effectively undetected (Ruxton et al. 2018, Figure S1). On land, studies in primates have also shown higher visual performance at brighter light conditions as the retinal cells increase their neural response more strongly when mean luminance is higher (Purpura et al. 1988; Ghim and Hodos 2006). Similarly, visual performance increases with ambient light luminance in other terrestrial vertebrates such as birds (Hodos et al. 1976; Kassarov 2003; Lind et al. 2013). Given the pervasive property of variation of visual performance with light availability, selection should favour different transmittance levels in different light environments on land.

On land, transparency is present in only a handful of linages, including Centrolenidae frogs, *Gaeotis* semi-slugs, and insects, where transparent wings are

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common. Transparency also reduces detectability of prey on land (Arias et al. 2019, 2020; Mcclure et al. 2019). Recently, Gomez et al (2020) reported that transparency has evolved multiple times independently in Lepidoptera (butterflies and moths), order of mostly opaque winged species, where it is produced by a large diversity of structures and configurations conferring different transmittance levels. Although this study did not investigate the links between transparency and habitat, it suggested in several aspects an important role of visual predators in driving the evolution of transparency (for instance diurnal species were reported to transmit more light than nocturnal species). Hence, we expect diurnal terrestrial transparent species that live in more open habitats - with brighter light conditions - to transmit more light compared to species flying at night and/or living in more closed habitats - with dimmer light conditions-. In addition, at low light availability, different levels of transmittance can confer similar protection against predators. Therefore, nocturnal and/or closed-habitat species might show larger variation in light transmittance, as neither high nor low transmittance levels are strongly selected.

Predation risk can be reduced not only by reducing detectability, but also by mimicking inedible elements such as leaves (masquerade, Skelhorn et al. 2010) or unprofitable prey (batesian mimicry, Bates 1862), and transparency might help achieving such mimicry. Several clearwing Lepidoptera exhibit brownish colorations that combined with transparent surfaces that presumably perceived as “holes” can resemble dead leaves (example of a potential dead-leaf mimic in Fig. 1a). Other small clearwing moths and butterflies exhibit yellow and dark stripes on their bodies and highly transparent wings (example in Fig. 1b), resembling harmful species for predators, such as bees and wasps. Mimicry syndrome could concur in driving transmittance differences to have different optima in bee/wasp and leaf mimics, but

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whichever factor (light availability versus mimicry) is driving its evolution is still unknown. Bees and wasps often live in open habitats (Grundel et al. 2010; Yamaura et al. 2012), and their mimics should do as well. High light transmittance should be selected in bees, wasps and their mimics because the reduction of detectability already conferred by their small body sizes, can be enhanced by higher levels of transparency (Gomez et al. 2020), and because light conditions are brighter in open habitats. By contrast, dead-leaf mimics might show a larger variation in body size, may more often live in closed habitats, where more leaves fall down and should therefore exhibit a broader diversity of light transmittance, as a range of transmittance levels can contribute to mimic leaves in decomposition and confer similar protection against predators. The expected differences in light transmittance should also be associated to variations in morphology and coloration of wing scales, with absent or reduced scales for bee/wasp mimics and classically shaped scales for leaf mimics.

Here, we investigate whether differences in transmittance found in Lepidoptera clearwing species are related to light availability and/or mimicry using two complementary approaches. First, we tested whether the efficiency of different transmittance levels at reducing detectability changes according to light availability in terrestrial environments, by carrying out a fieldwork experiment with artificial moth-like prey. Second, using actual specimens of butterflies and moths, we tested whether differences in the proportion of transmitted light could be explained by species daytime rhythm, habitat and/or mimicry, by conducting comparative analyses at broad interspecific level. We took advantage of the large dataset of Lepidoptera species analysed by Gomez et al (2020) to add species ecological traits (habitat, daytime rhythm and mimicry, known for 107 species) and relate them to

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clearwing optical and structural traits. Together, these analyses help understanding the evolution of interspecific differences in the proportion of transmitted light on land.

Material and Methods

1. Field experiment

1.a. Artificial butterfly Elaboration

To test for the efficiency of light transmittance at reducing detectability in habitats with different light availability, we elaborated plain grey artificial butterflies with paper wings and a malleable edible body. Following the general methodology described in Arias *et al* (2020), butterflies did not replicate any real local butterfly, but mimicked a general grey moth with closed wings (i. e., a triangular shape). The rather cryptic butterfly colour was chosen based on 120 reflectance measures of green oak *Quercus ilex* trunks. Reflectance measurements were taken using a spectrophotometer (Starline Avaspec-2048 L, Avantes) and a deuterium halogen lamp (Avalight DHS, Avantes) emitting in the 300-700 nm range, including UV, to which some predators of butterfly and moths, such as birds, are sensitive (Chen and Goldsmith 1986). Measurements were done relative to a white reference (lights turned on with no sample) and a dark reference (light turned off with no sample). For reflectance measurements, we used an optic probe (FC-UV200-2-1.5 x 100, Avantes) merging illumination and collection angles. Grey wings (R=155, G=155, B=155) were printed on sketch paper Canson using a HP Officejet Pro 6230 printer. Paper wings were laminated with a Polyester Opale Mat 75µm poach. Four forms of artificial butterflies were used, here listed in order of increasing proportion of light transmittance: completely opaque butterfly “C”; poorly transparent butterfly with 6

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layers of transparent film "T6"; highly transparent butterfly with a single layer of transparent film "T1"; and fully transparent butterfly with no film in the transparent zones "T0". For the butterflies that included transparent elements, triangular windows of 234mm^2 were cut down from the grey triangle and the remaining part was put on top of one or six layers of transparency film 3M for inkjet printing. This transparent film was chosen as it is highly transparent, even in the UV range of the spectrum, and going from 1 to 6 transparent layers permitted a reduction of 50% of the transmittance between treatments (Fig. S2). Such transparent layer was coated with a transparent mate varnish to reduce its shininess. For transparency measurements, we measured specular transmittance from 300 to 1100nm, using a deuterium-halogen lamp (Avalight DHS, Avantes), optical fibres (FC-UV200-2-1.5 x 100, Avantes) and a spectrometer (Starline Avaspec-2048 L, Avantes). Fibres were separated, aligned and 5mm apart and the wing sample was placed perpendicular between them at equal distance (light spot of 1mm diameter). Spectra were taken relative to a dark (light off) and to a white reference (no sample between the fibres) measurement. On top of butterfly wings, we added an artificial grey body. Butterfly bodies were prepared with flour (428 g), lard (250 g) and water (36 g), following Carrol & Sherratt (2013). Yellow, red and blue food edible colourings were used to dye the pastry in grey, imitating artificial wing colour. Such malleable mix permits to register and distinguish marks made by bird beaks from insect jaws. Both paper wings and artificial bodies were measured in spectrometry, similarly to how trunk reflectance was measured (Fig. S2). Bodies could attract predator attention, but it has been previously shown that wingless bodies had the same attractiveness as that of butterflies with large transparent elements (Arias et al. 2020). Artificial butterflies and bodies were pinned to green oak *Quercus ilex* trunks. To avoid ant attacks,

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Vaseline and a piece of highly sticky double-faced transparent tape were stuck in between the wings and the trunk.

1.b. Experimental set up

Predation experiments were performed in May and June 2018 at the zoological park of Montpellier (43.64°, 3.87°) and at La Rouvière forest (close to Montarnaud city 43.65°, 3.64°). This experiment overlapped with the breeding season of local great (*Parus major*) and blue tit (*Cyanistes caeruleus*) populations, when birds are intensively looking for food, which can maximise the number of attacks on our artificial prey. Artificial prey items were pinned every 10 metres on oak trunks of at least 10 cm of diameter and with few or no moss cover. Prey order was randomized by blocks of eight items, two per treatment, before starting the experiment. Initially, prey were evenly disposed in closed and open areas. However, throughout the experiment a stronger predation rate was detected in open areas (see Results section). Therefore, we increased the number of artificial prey released on closed areas to compensate for the differential predation pressure and try to get more similar numbers of attacked prey items in closed and open habitats. We analysed results for both the whole dataset, including larger sample size in closed habitats, and 20 reduced datasets, including each time a different random subset of the closed habitat of the same amount of artificial prey as for the open habitat. Prey were mostly disposed facing north and as perpendicular to the floor as possible, to reduce direct sunlight reflection on them. Prey were checked every 24h. After 96h all prey were removed. When V- or U- marks were detected, or when the body was missing without signals of invertebrate attacks (i.e. no body scraps left on the wings or around the butterfly on the trunk) prey were considered as attacked and wings or

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pins where removed. Instead, if invertebrate attacks were detected, or when prey was missing, it was replaced. Non-attacked prey were considered as censored data.

Canopy cover was used to classify places in open and closed habitat. We took pictures each 20 meters in the trails that were used for the experiment (at forest edges for the open habitat, inside the forest for the closed habitat, examples Fig. S3), using a Canon EOS 550D camera and a fisheye lens. Canopy cover was further quantified using Fiji software (Schindelin et al. 2012). Although canopy cover distribution is unimodal (Hartigans' dip test: D = 0.01, p-value = 0.6), we used a discrete classification, comparable to the one used for real species in the second part of our study. We assumed that places with cover larger than the mean were closed habitats and places with lower covers were open habitats (see Fig. S3 for the distribution of canopy cover in the experimental sites).

To test how different treatments survive along the experiment on both open and closed habitats, Cox proportional hazard regression (Cox 1972) were applied, first for all data points including form, habitat openness and their interaction as explanatory variables, and then, Cox regression were independently applied for each habitat using the different treatments as factors, to further explore the effect of form within each habitat. Overall significance was measured using a Wald test. Statistical analyses were performed in R (R Foundation for Statistical Computing 2014) using the *survival* package (Therneau and Lumley 2009).

2. Comparative analyses of museum specimens

2. a. Species selection and phylogeny

To explore the association of light transmittance with different conditions across multiple species we performed phylogenetic comparative analyses, since species are

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not independent. We used the phylogeny of clearwing species by Gomez et al. (2020). In our analyses we included 107 species out of the 123 species included by Gomez et al, for which information about the openness of their habitat was available. These species spanned 31 of the 124 existing Lepidoptera families.

2. b. Ecological data on species habitat, daytime activity and mimicry type

Ecological information was collected from the literature, Lepidoptera experts and our own knowledge for a total of 107 species for activity rhythm (57% of species are diurnal (day-active) species), habitat (56% of species living in open habitats), and mimicry status (14 species were bee/wasp mimics, 12 species were leaf mimics, while the remaining 81 did not fall in either of those categories (hereafter referred to as non-mimics, Table S1 and Fig S4). The selected 107 species do not include species that were reported to live in both open and closed habitats, or to fly at day and night. Visual predators are mostly active during the day and are supposed to be the main selective agents in the evolution of visual signals related to anti-predator defences (Ruxton et al. 2018), thus, nocturnal species are unlikely to face similar selective pressures as diurnal species although both could fly in similarly open habitats. Therefore, daytime rhythm and habitat where coded as a single variable with 4 states (diurnal open habitat, diurnal closed habitat, nocturnal open habitat and nocturnal closed habitat). Bee and wasp mimics were grouped together in the same mimicry category, as both exhibit the striped pattern with black and yellow that advertise their unpalatability (Plowright and Owen 1980) and they exhibit similar body sizes. Butterflies and moths mimicking bees and wasps have forewings longer than their hindwings, and often, strong delimitations of their wing borders and veins by dark coloration, as the hornet moth *Sesia apiformis* (*api* - bee, *formis* -shaped) or

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the broad-bordered bee hawk-moth *Hemaris fuciformis* (Fig 1b). Dead-leaf mimics include brownish moths and butterflies with small or large transparent surfaces and that could touch or not the wing borders (Fig. 1a). *Rotschildia lebeau* has been proposed as a leaf mimic (Janzen 1984) but such mimicry is controversial (Hernández-Chavarría et al. 2004). *Rotschildia ericina* was therefore not considered as a leaf mimic for the analyses.

2. c. Wing structural and optical measurements

Wing structural and optical measurements were taken from Gomez et al, using the following methods. Museum specimens were photographed using a D800E Nikon camera equipped with a 60mm lens, placed on a stand with an annular light. Photos were then analysed using ImageJ (Schneider et al. 2012) to extract descriptors of wing macrostructure: wing length (mm), wing surface (mm^2), and clearwing area (the surface of transparent area in mm^2), for the forewing and hindwing separately. We defined and computed proportion of clearwing area as the ratio clearwing area/wing surface, i.e. the proportion of the total wing area occupied by transparency.

For optical measurements, we followed the same procedure described above for measuring transmittance in artificial prey. For each species and wing, we took five measurements in the transparent zone. We analysed spectral shape using Avicol v6 (Gomez 2006) to extract the mean transmittance over [300-700] nm, which described the level of transparency.

2. d. Physical properties of transparent wings

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Gomez et al (2020) described a large diversity in transparent wing surface characteristics. Using binocular imaging (Zeiss Stereo Discovery V20) and digital microscopic imaging (Keyence VHX-5000), these authors found that transparency could be achieved either with nude membranes or with membranes covered by scales of various types (either classical scales, hair-like scales or both) that could be coloured or transparent, and erected or flat according to their insertion on the wing membrane. Different combinations of structures and configurations are related to different light transmission levels. Therefore, we also explored the evolution of these characteristics and their relationship with mimicry syndrome.

2. e. Analyses of transmittance differences between habitats and mimics

To explore whether differences in the proportion of transmitted light are related to variation in habitat and mimicry we fitted both 1) a linear mixed model including 107 species for which we found data on habitat preference and 2) a Bayesian phylogenetic mixed model, including the phylogeny of the 107 species. Comparisons between the first and the second analysis allowed us to assess the influence, if any, of phylogenetic relationships on the observed trends. Light availability, mimicry and morphological traits were included as explanatory variables in our model. We defined ActHab, a variable combining daytime activity and habitat openness with four levels (diurnal open, diurnal closed, nocturnal open, nocturnal closed) and three contrasts (diurnal vs. nocturnal, open vs. closed habitats, and diurnal open habitat vs. diurnal closed habitat). The variable mimicry has three levels (bee-wasp mimics, leaf mimics and non-mimics) and we tested two contrasts (bee/wasp mimics vs. all other species, and leaf mimics vs. non- mimics). Other explanatory variables included: wing length (as a proxy of butterfly size), proportion of clearwing surface

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and fore-/hindwing, reported by Gomez et al (2020) as correlated to light transmittance. Wing transmittance, wing length and clearwing proportion were obtained from Gomez *et al* (2020). As five measurements per wing (fore- and hindwing), thus ten measurements per species were included in our dataset, wing measures nested in species was considered as a random effect. We fitted different models including different combinations of two- and three-factor interactions and we compared them using the AIC criterion to select the best model. To better understand the effect of each of the included variables, we reported and compared the full model without interactions and the best model including interactions (Table 1). Additionally, we fitted Bayesian phylogenetic mixed models with Markov chain Monte Carlo analyses using the 'mulTree' R package (Guillerme and Healy 2014). Using the best formulated linear mixed model, uninformative priors were chosen, with variance and belief parameter set to 1 for random effects and 0.002 for residual variances (Hadfield 2010). Models were run using two chains of 600,000 iterations, a thinning interval of 300 and a burn-in of 10,000. Fixed effects were considered statistically significant when the probabilities in the 95% credible intervals did not include zero. As Bayesian phylogenetic mixed models consider no interactions between factor levels, several submodels were fitted to test the interactions from the best model obtained according to AIC criterion.

Additionally, we performed several phylogenetic generalised least square (PGLS) analyses (1 observation per species) to test for potential correlations between size, proportion of clearwing surface, daytime activity, habitat and mimicry, and between wing ratio (forewing length/hindwing length, usually high in bees and wasps) and mimicry. Using BayesTraits (Pagel and Meade 2013) we additionally explored character coevolution between mimicry and ActHab and between habitat

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and daytime as well as between mimicry and scale characteristics: absence/presence of scales, scale insertion (flat or erected), scale coloration (coloured or transparent) and scale type (hair-like scales and classic scales). When analysing physical wing properties and mimicry evolution, independent analyses were performed for anterior and posterior wings, as differences between wings have been reported by Gomez et al (2020). We calculated the likelihood of the dependent and independent models of evolution of each pair of binary characters, using a Markov-Chain Monte-Carlo approach. We then estimated the likelihood ratio test between them and compared it to a chi-square distribution with 4 degrees of freedom to obtain their statistical significance (Pagel 1994). As this approach can only be applied to binary characters, and mimicry has three factors, models were run including the different possible pairwise combinations to explore: a. evolution of dead-leaf mimicry (including non-mimics and dead-leaf mimics); b. evolution of bee/wasp mimics (including non-mimics and bee/wasp mimics) and c. differences in the evolution of dead-leaf and bee/wasp- mimics (including bee/wasp and dead-leaf mimics). Similarly, for the composed variable ActHab we compared (i) diurnal and nocturnal species flying in open habitats, and (ii) nocturnal species flying in open and in closed habitats. We calculated δ, a new metric inspired in the phylogenetic analog of the Shannon entropy to measure phylogenetic signal in categorical non-ordered data (Borges et al. 2019), for testing for phylogenetic signal in ActHab, daytime activity and mimicry evolution. δ is useful to detect whether the coevolution between traits are homologies (characteristics are shared within a clade) or convergences (characteristics evolved independently but jointly and several times along the phylogeny). For each trait, we compared its evolution in our data with the evolution of a randomly sorted trait (assuming it has no phylogenetic signal), using

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lambda = 0.1, standard deviation of 0.5, 10000 iterations, keeping the 10th iterate and burning-in 100 iterates, parameters from the example script proposed by Borges *et al* (2019). In case of homology, δ should be higher for the real data than for the randomly sorted data.

Finally, we predicted higher variance in light transmittance for those conditions where different levels of transparency might be equally efficient as would be the case for butterflies and moths that are active during the night, that live in closed habitats and/or that are dead-leaf mimics. We applied Fligner-Killeen tests of homogeneity of variance in R to test for difference in variance between (i) diurnal and nocturnal species living in open and in close habitats, and (ii) bee/wasp mimics, leaf mimics and no mimic species. As Gomez et al (2020) reported that transmittance is closely related to wing size, we also tested for differences in the variation of wing length between habitat, daytime activity and mimicry using the test mentioned above.

Results

Fieldwork experiment

In total, 1149 artificial butterflies were used during the experiment and 243 prey were attacked (21.14 %). When data from both open and closed habitats were analysed together, survival was lower in open than in closed areas. In closed areas 651 prey were released, 110 attacked, with an attack rate of 16.89% while in open areas, 498 prey were released, 133 attacked, with an attack rate of 26.71% (Wald test = 34.39, df = 7, $p<0.001$). Results are similar when including the "reduced" datasets (random subsample of the closed habitat set and of the same sample size as the set released in open habitats, Table S2). Additionally, in open habitats the opaque

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form was more attacked than the other forms that had transparency (z= 2.15, p=0.031), but when including the "reduced" dataset, such difference was only detected in 6 out of the 20 random subsets Table S2). When data was analysed independently in each habitat, all prey treatments were similarly attacked in closed areas (Fig. 2a, Wald test = 0.9, df = 3, p = 0.825, and for the reduced dataset Wald test = 0.5, df = 3, p = 0.5), in contrast to open areas (Fig. 2b, Wald test = 9.86, df = 3, p = 0.02). In open areas, opaque artificial butterflies were more attacked than all the other treatments (z = 2.68, p = 0.007, Fig. 2b); artificial butterflies with six layers of transparency were marginally more attacked than those with a single transparency layer (z = 1.839, p = 0.066, Fig. 2b), while artificial butterflies with or without a single transparent layer where similarly attacked and reported the lowest number of attacks (z = -1.61, p = 0.11, Fig. 2b).

Comparative analyses

Relationships between light transmittance, habitat and daytime activity

Habitat and daytime activity partly explain the variations found in light transmittance. Species flying in closed habitats transmit slightly more light than species flying in open habitats (model without interactions t=-1.67, df=101, p = 0.08; model with interactions t=-3.29, df=101, p = 0.001 Table 1). However, diurnal species flying in open habitats transmit more light than diurnal species flying in closed habitats (model with interactions t=2.05, df=101, p =0.043, Table 1, Fig. 3). Transmittance decreases with wing length in diurnal species, but increases with wing length in nocturnal species (interactions between wing length and ActHab: DO>DC t=-3.46, df = 484, p < 0.001; D>N t=-2.33, df = 484, p = 0.02; O>C t = 2.68, df = 484, p = 0.01; Fig. 3). Transmittance increases with the proportion of clearwing

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surface at a similar rate for both wings in most species (Interactions between proportion of clearwing surface and ActHab: DO>DC t= -2.55, df = 484, p = 0.01; D>N t=1.11, df = 484, p = 0.27; O>C t = 3.51, df = 484, p = <0.001). More results were significant when phylogenetic effect was excluded, suggesting some phylogenetic signal in open/close habitat. Results were similar for most Linear and Bayesian mixed models, but differed when testing transmittance in diurnal/nocturnal species (Table 1 for Linear mixed model results and Tables S2 for Bayesian mixed submodels testing interactions included in linear mixed models). Actually, daytime activity has some phylogenetic signal (δ =0.58 for real data vs. δ =0.42 for randomised data), but ActHab not (δ =0.56 for real data vs. δ =0.57 for randomised data). No difference in wing length or the proportion of clearwing surface was detected between diurnal and nocturnal species nor species flying in closed or open habitats (Table S3).

<u>Relationships between light transmittance, transparency structural basis and mimicry</u>

Mimicry explains a large portion of the variation found in light transmitance. Bee/wasp mimics transmit more light than other clearwing species (model without interactions: t =1.77, df = 101, p = 0.08; model with interactions: t = 3.52, df = 101, p<0.001, Table 1, Fig. 4). Light transmittance for bee/wasp mimics is higher at larger proportions of clearwing surfaces (t =2.24, df = 4.84, p = 0.03) and at smaller wing lengths (t =-6.39, df = 484, p < 0.001), in contrast to leaf mimics that exhibit higher trnasmittance at smaller proportions of clearwing surface (t = 3.67, df = 484, p<0.001) and marginally, at larger wing lengths (t = -1.88, df = 484, p = 0.06, Table 1, Fig. 4).

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Regarding the relationship between mimicry and wing characteristics, leaf mimics have smaller hindwings than non-mimics (PGLS forewing: t=1.66, p = 0.1; hindwing: t=1.99, p = 0.05, Figure 4b), and the length ratio forewing/hindwing is larger for bee/wasp mimics than for any other clearwing species (PGLS t=4.51, p <0.001, Fig. 4e). Additionally, the proportion of clearwing surface is larger for non-mimics and for bee/wasp mimics (PGLS forewing: t=4.005, p < 0.001; hindwing: t=2.86, p=0.005, Fig. 4c). Mimicry syndrome and daytime have likely coevolved: most bee/wasp mimics are diurnal while most leaf mimics are nocturnal (BayesTraits daytime activity and bee/wasp or leaf mimics: LRT=9.5, df = 4, p=0.05, Table 2, Fig S4 & Fig S6).

We found that transmittance varies less in bee/wasp mimics than in leaf-mimics (Fligner-Killeen test for forewing: 15.6, df= 1, p<0.001, for hindwing: 36.14, df = 1, p<0.001), or in other clearwing species (Fligner-Killeen test for forewing: 16.38, df=1, p<0.001; for hindwing: 36.34, df = 1, p<0.001), but there is no difference in the trnasmittance variation in leaf mimics and non-mimic species (Fligneer-Killeen test for forewing: 2.02, df=1, p= 0.155; for hindwing: 0.00005, df = 1, p=0.995). Likewise, wing length varies less in bee/wasp mimics than in leaf mimics (Fligner-Killeen test for forewing: 18.91, df=1, p<0.001; for hindwing: 39.86, df = 1, p<0.001) or in other clearwing species (Fligner-Killeen test for forewing: 39.06, df=1, p<0.001; for hindwing: 59.15, df = 1, p<0.001). Leaf and non-mimics show similar variations in wing length (Fligner-Killeen test for forewing: 0.58, df=1, p=0.447; for hindwing: 2.46, df = 1, p=0.117).

Some scale characteristics are correlated to mimicry evolution. Scales are more often absent and less often coloured when present in hindwings of bee/wasp mimics in comparison to non-mimics wings (BT LRT presence = 24.55, df=4,

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p<0.001, Table S4 & Fig S7; BT LRT colour= 16.14, df=4, p=0.003, Table S4 & Fig S7). Similarly, leaf mimics have less often coloured scales in comparison to non-mimics both in forewing (BT LRT=11.62, df=4, p=0.02) and hindwing (LRT=12.87, df=4, p=0.012, Table S4 & Fig S7). Moreover, leaf mimics only exhibit classic scales in contrast to bee/wasp mimics and non-mimics (LRT=9.33, df=4, p=0.053, Table S4 & Fig S7). These similarities are likely to be evolutionary convergences as mimicry has no phylogenetic signal (δ =0.99 for real data vs. δ =1.002 for randomised data).

Discussion

Fieldwork experiments using artificial prey that differed in their proportion of transmitted light suggest that variation in transparency can be detected only in brighter light conditions, similar to what has been suggested for aquatic environments (Johnsen and Widder 1998). Poorly transparent prey types survived less than highly transparent ones in open environments, while they performed similarly in closed areas. Additionally, less artificial moths were attacked in closed habitats, even after increasing the number of artificial prey in closed habitats to compensate for the higher predation rate often reported for open habitats such as forest edges (Barbaro et al. 2014). By controlling for differences in predation rate between habitats by increasing the number of prey placed in closed habitat our results suggest that selective pressure towards an increase in transmittance is higher in open than in closed habitats.

Our experimental results suggest that if transparency has mostly evolved as a detectability reducer, high transmittance should be more common in open habitats. In comparative analyses, when analysing only diurnal species, clearwing species flying in open habitats transmit indeed more light than species flying in closed

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habitats, in agreement to what would be selected according to the high visual performance of visual predators under bright conditions (Johnsen and Widder 1998). However, overall butterflies and moths living in closed habitats (including both diurnal and nocturnal) transmit similar to slightly more light than species living in open habitats. Gomez et al (2020) reported that poor light transmittance is associated to small proportions of clearwing surface and large wing sizes. Diurnal species flying in closed habitats show higher proportion of clearwing surface than those living in open habitats (Table 1, Fig. 3), probably contributing to the similar/slightly higher values of light transmittance in closed habitats in comparison to the open habitats. Species living in open habitats undergo higher risks of tissue damage by higher exposure to UV light, more wind or higher exposure to direct rainfall, conditions that have been reported as important at shaping moth and butterfly segregation in different habitats (Brown Jr and Hutchings 1997). In case transparency entails costs related to one or several of these factors, selection may promote lower proportions of clearwing surfaces in open habitats, decreasing the vulnerability of clearwing Lepidoptera species to these abiotic effects.

Transparency can decrease prey detectability but it is also involved in mimicry of bees or wasps, and differences in light transmittance seem to be closely associated to mimicry evolution. When mimicry is included in the analysis, we found that bee/wasp mimics have higher transmittance levels, frequently associated to the absence of scales in their wings, and are more often restricted to open habitats, in agreement to our fieldwork results and confirming that high transmittance in open habitats can be a visual optimum in terrestrial environments. As this is not the case for all clearwing species, this highlights the strong effect of biotic and abiotic

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interactions in shaping adaptive peaks, thus the segregation of species under different conditions and the evolution of different features.

We also found that bee/wasp mimics are diurnal (similar to most bees, Somanathan et al. 2009), and have smaller wing sizes and smaller hindwings in comparison to their forewings (as has been reported for wasps and bees, Wootton 1992; Lindauer 2019). Insects that mimic bees or wasps resemble their harmful counterparts in body colour, body shape and flight pattern (for butterflies (Skowron Volponi et al. 2018)) but also in activity patterns (for Diptera (Howarth et al. 2004)). Convergence in high light transmittance along with reduction in variation in wing size and the presence of long and narrow forewings and small hindwings (large fore/hindwing ratio) can contribute to such multicomponent mimicry, that improves prey chances at deceiving a larger set of predators, in case several traits are similarly salient for them (Kazemi et al. 2015) or when different predator species find different cues as salient (Kikuchi et al. 2016). As chitin, primary component of insects exosqueleton, is transparent, clearwings in insects are likely the basal state in absence of selection for colour evolution. However, the evolution of highly transparent wings in diurnal and very active insects, such as pollinators could have been driven by their higher mobility and the openness of the habitat where the majority of nectar sources frequently occurs (van Halder et al. 2011; Baude et al. 2016). This suggest that bees, wasps and their Batesian mimics combine highly transparent wings as they can enhance their crypsis while being highly mobile and also exhibit warning colours that advertise their (potential) harmfulness once they are detected/approached by predators, similar to unpalatable clearwing Ithomiini species (Mcclure et al. 2019). In aquatic environment, transparent forms of *Hippolyte obliquimanus* shrimps are more mobile and exploit a larger diversity of habitats,

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probably because they are cryptic on several backgrounds (Duarte et al. 2016). Whether transparency is also related to higher mobility in insects remains to be properly tested.

On the other hand, we found that leaf mimics are larger, are more often found in closed habitats and in contrast to the trends for all other clearwing species, transmit more light at larger wing sizes and lower proportions of clearwing surfaces. Some fully opaque fallen leaf mimic species, such as the comma butterfly (*Polygonia c-album*) exhibit a distinctive pale mark that might decrease their predation by being perceived as 'distractive marks' preventing predators from recognise the prey, thus enhancing background matching (Dimitrova et al. 2009; Olofsson et al. 2013), although it is controversial (Troscianko et al. 2018). Either way, reduced and highly transparent zones as those found in *Orthogonioptilum violascens* might produce a similar appearance to those markings given their high transmittance that might be highly reflective and be perceived as a white mark, conferring thus the potential benefits of the "comma mark" when seen for a given angle, as well as the full crypsis, when no reflections can be perceived. Whether highly transparent windows work as other highly visible elements displayed in cryptic species such as eyespots that deter predators (Stevens et al. 2008, 2009) and that are also important in mate choice (Robertson and Monteiro 2005) remains to be tested.

Other dead-leaf mimics include large transparent surfaces that can expand to the wings edges, as for *Bertholdia* species, or not, as for *Pseudasellodes fenestraria*. In both cases, transparency surfaces are likely disrupting prey shape (as in the first case) and/or prey surface (as in the second example (Costello et al. 2020)), thereby reducing their detectability. Disruptive coloration, especially when touching prey borders, has been broadly studied as it is common in fully opaque cryptic species

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(Cuthill et al. 2005; Schaefer and Stobbe 2006; Stevens and Cuthill 2006; Fraser et al. 2007). Whether transparent surfaces touching wing edges, work as disruptive marks regardless of their transmittance, decreasing even more prey detectability remains to be tested.

Conclusion

Both light availability (daytime activity and habitat openness) and mimicry can concur to fostering variations in light transmittance on land. Our results opens up new questions that need to be investigated in the future, in particular the eventual cost of transparency regarding irradiance, wind and direct rainfall, needed to better understand the evolution and the constraints imposed on transparency on land.

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Figures and tables

a. b.

Figure 1. Examples of a potential dead leaf mimic (a. *Pseudasellodes fenestraria,* Rio Kosnipata, Peru. Photo: © Adrian Hoskins) and a bee/wasp mimic (b. Adult female *Hemaris fuciformis fuciformis*, Catalonia, Spain. Photo: © Tony Pittaway). Forewing length is ~18mm in *Pseudasellodes fenestraria*, ~24mm in *Hemaris fuciformis fuciformis.* Scale in mm.

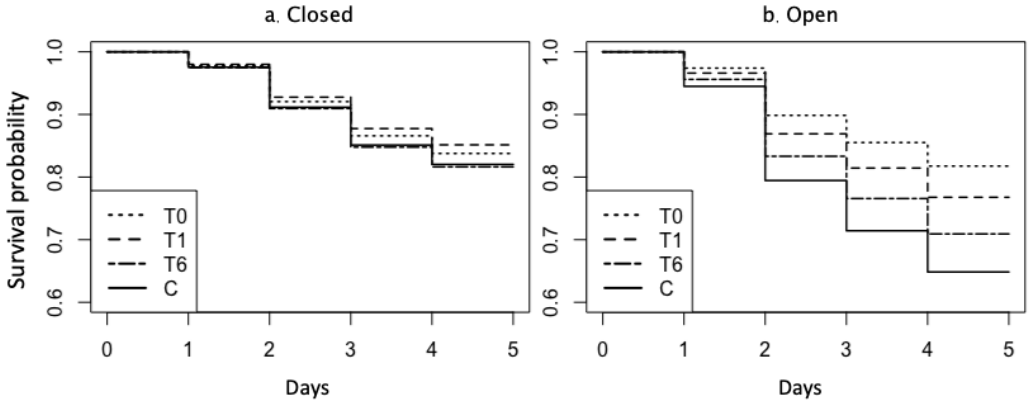

Figure 2. Survival of artificial prey in (a.) closed and (b.) open areas, without any transparent area (C), and with transparent elements covered by 6 (T6), 1 (T1) or non (T0) transparent film layers. Prey placed on tree trunks were monitored for 'survival' every day for 4 days.

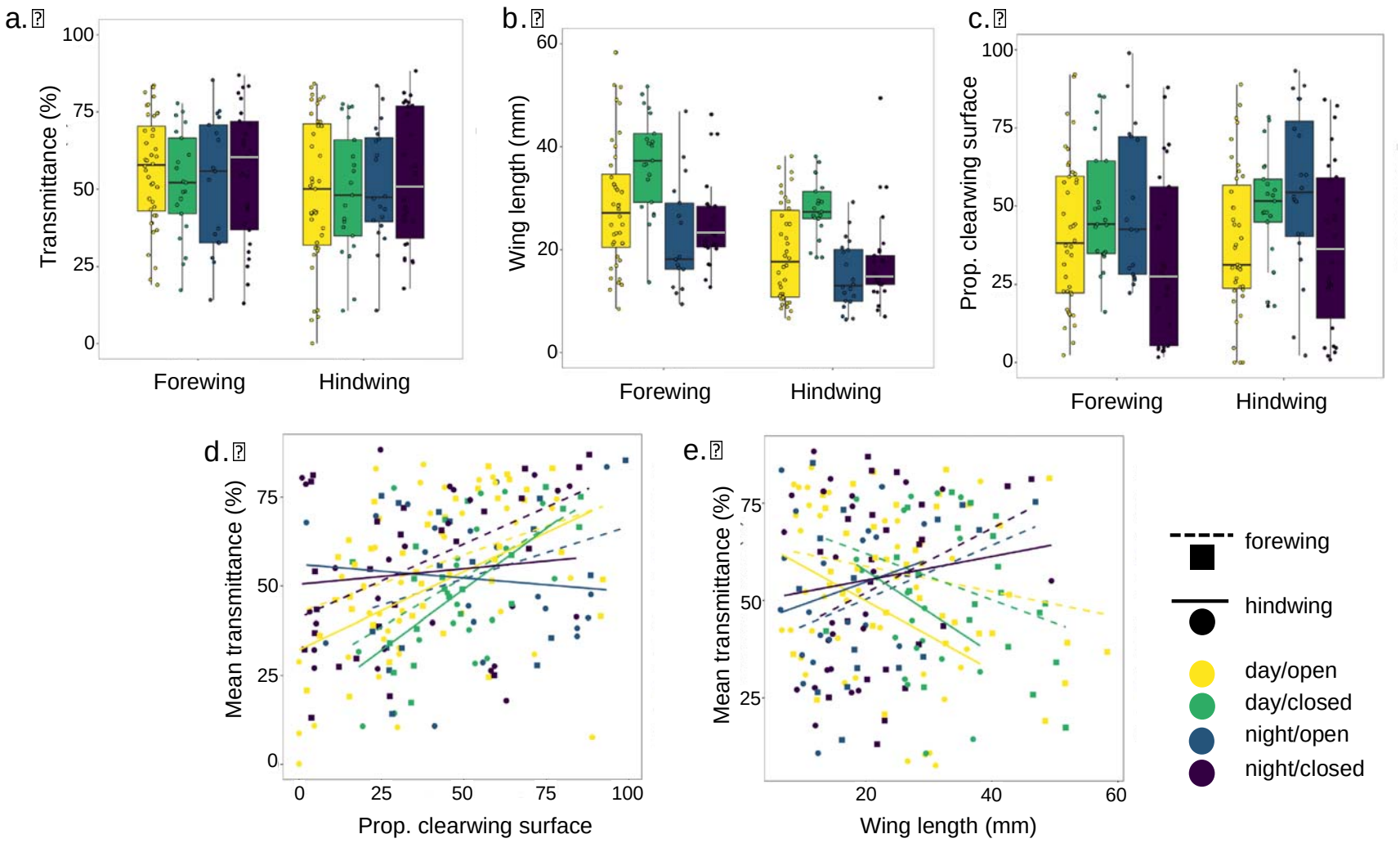

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Figure 3: Variation between diurnal/open (yellow), diurnal/closed (green), nocturnal/open (blue) and nocturnal/closed (purple) in a) mean light transmittance, b) wing length, c) proportion of clearwing surface; and the relationship between transmittance, wing (forewing: square/dashed and hindwing: circle/plain) and d) proportion of clearwing surface or e) wing length. Plotted lines in d. and e. correspond to linear regressions per wing and ActHab level. Values larger than 60 mm were not plotted for clarity reasons but were included in the analyses.

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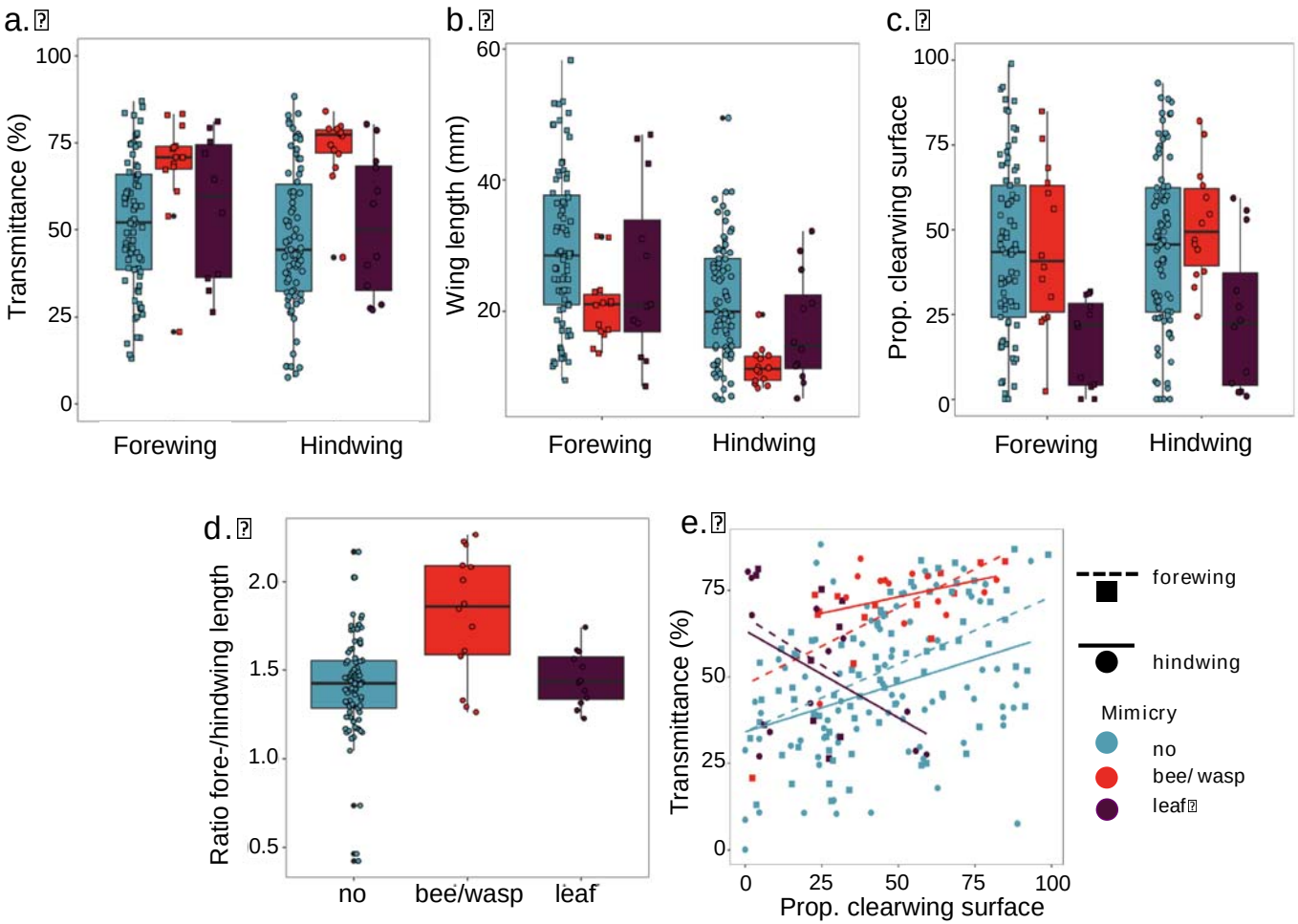

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Figure 4: Variation between bee/wasp mimics (red), leaf mimics (purple) and no mimics (blue) in a) mean light transmittance, b) wing length, c) proportion of clearwing surface, d) Ratio between length of forewing and hindwing per mimicry group and e) in the relationship between wing size and mean light transmittance. Plotted lines in e. correspond to linear regressions per wing and mimicry level.

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Table 1. Variation of mean proportion of transmitted light according to mimicry, habitat, daytime activity, wing size, wing and proportion of clearwing surface excluding (in the left) or including interactions between them (in the right). For Bayesian estimations see Tables S2 a, b, c and d.

		Estimate ± se	DF	*t*		Estimate ± se	DF	*t*	
Single factors	**(Intercept)**	**48.36±3.03**	**502**	**15.97**	***	**42.77±4.47**	**484**	**9.56**	***
	% clearwing	**0.27±0.02**	**502**	**10.91**	***	**0.27±0.08**	**484**	**3.57**	***
	Mimicry:bee/wasp>leaf+non-mimics	**16.39±5.55**	**101**	**2.95**	**	**31.14±6.7**	**101**	**4.65**	***
	Mimicry:leaf<non-mimics	9.8±6.15	101	1.59		8.09±7.96	101	1.02	
	Wing length	**-0.43±0.07**	**502**	**-6.6**	***	-0.07±0.14	484	-0.5	
	ActHab (DayOpen>DayClosed)	3.18±3.78	101	0.84		**16.18±7.89**	**101**	**2.05**	*
	ActHab (Day>Night)	1.68±1.99	101	0.84		6.63±4.13	101	1.61	
	ActHab (Open>Closed)	-4.62±2.77	101	-1.67	~	**-17.26±5.24**	**101**	**-3.29**	**
	Wing (Forewing>Hindwing)	**3.67±0.39**	**502**	**9.45**	***	1.23±0.66	484	1.87	~
Interactions	Mimicry (bw>l+n.m): % clearwing	-	-	-		0±0.07	484	-0.06	
	Mimicry (leaf<n.m): % clearwing	-	-	-		**-0.37±0.1**	**484**	**-3.72**	***
	Mimicry (bw>l+n.m):W.length	-	-	-		**-0.87±0.15**	**484**	**-5.85**	***
	Mimicry (leaf<n.m):W.length	-	-	-		0.33±0.19	484	1.76	~
	ActHab (DO>DC): % clearwing	-	-	-		**-0.36±0.14**	**484**	**-2.55**	**
	ActHab (D>N): % clearwing	-	-	-		0.08±0.07	484	1.11	
	ActHab (O>C): % clearwing	-	-	-		**0.31±0.09**	**484**	**3.51**	***
	ActHab (DO>DC):Wing length	-	-	-		**-0.89±0.26**	**484**	**-3.46**	***
	ActHab (D>N):Wing length	-	-	-		**-0.32±0.14**	**484**	**-2.33**	*
	ActHab (O>C):Wing length	-	-	-		**0.54±0.2**	**484**	**2.68**	**
	% clearwing surf.:Wing length	-	-	-		0±0	484	-0.5	
	Wing length:Wing (F>H)	-	-	-		0.05±0.02	484	1.91	~
	ActHab (DO>DC):Wing (F>H)	-	-	-		0.55±0.93	484	0.58	
	ActHab (D>N):Wing (F>H)	-	-	-		**2.31±0.48**	**484**	**4.83**	***
	ActHab (O>C):Wing (F>H)	-	-	-		0.02±0.76	484	0.02	
	ActHab (DO>DC): % clearw :W. length	-	-	-		**0.02±0.01**	**484**	**4**	***
	ActHab (D>N): % clearw :W. length	-	-	-		0±0	484	-0.48	
	ActHab (O>C): % clearw :W. length	-	-	-		**-0.01±0**	**484**	**-2.99**	**

Linear mixed model with transmittance as dependent variable and the following explanatory variables: the combination between daytime activity and habitat type (ActHab: nocturnal-open/nocturnal-closed/diurnal-open/diurnal-closed), wing length in mm, proportion of clearwing area (% clearwing), mimicry syndrome (bee/wasp -, leaf - or non-mimics), wing (fore-/hindwing) and the interactions: mimicry syndrome and proportion of clearwing area, mimicry syndrome and wing size, proportion of clearwing area and habitat type, wing size and habitat type, wing size and proportion of clearing area, wing and wing length and proportion of clearwing area, wing length and habitat as explanatory variables. Wing measurements nested in species was considered as random effect. LMM p values below 0.05 are statistically significant. *** stands for p<0.001, ** for p<0.01, * for p<0.05 and ~ for p<0.1.

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Table 2. Results of coevolution tests between habitat, mimicry and daytime activity

Tested characters	Mimicry levels included	L.Dep.	L.Indep.	LR	df	p	
daytime activity/ mimicry	**bee/wasp & leaf mimics**	**-29.13**	**-33.88**	**9.5**	**4**	**0.05**	*
	No- & leaf mimics	**-91.21**	**-98.19**	**13.96**	**4**	**0.01**	***
	No- & bee/wasp mimics	-100.85	-100.86	0.02	4	1	
habitat / mimicry	bee/wasp & leaf mimics	-34.86	-36.78	3.85	4	0.43	
	No- & leaf mimics	-102.27	-102.34	0.13	4	1	
	No- & bee/wasp mimics	-99.73	-101.26	3.05	4	0.55	
habitat/daytime activity	No-, bee/wasp- & leaf mimics	-142.49	-145.1	5.22	4	0.26	
day.open vs night.open /mimicry	bee/wasp & leaf mimics	-17.57	-21.17	7.2	4	0.12	
	No- & leaf mimics	-45.89	-49.05	6.32	4	0.18	
	No- & bee/wasp mimics	-57.48	-57.82	0.68	4	0.95	
night.open vs night.closed /mimicry	bee/wasp & leaf mimics	-19.5	-19.52	0.04	4	1	
	No- & leaf mimics	-50.56	-50.57	0.02	4	1	
	No- & bee/wasp mimics	-36.86	-36.87	0.02	4	1	

Log-likelihood of dependent (L. Dep. Coevolution, 4 parameters estimated) and independent (L. Indep. 8 parameters estimated) evolution models between pairs of binary characters obtained from BayesTraits, log-likelihood ratio (LR), associated degree of freedom (df: difference in number of parameters) and corresponding p-value of the Likelihood Ratio Test. Because BayesTraits only take binary characters, when factors have more than 2 levels (mimicry and the composed character ActHab), we implemented pairwise comparisons of level. Coevolution between mimicry and diurnal species flying either in open or in closed habitats was not tested as no bee/wasp mimic is simultaneously diurnal and closed habitat dweller (Fig S4). A significant p-value means that the dependent model is significantly better than the independent model, indicating a correlated evolution between the traits. ** stands for statistical significance below 0.01 and * for significance values below 0.05.