

# Field evidence for colour mimicry overshadowing morphological mimicry

Alberto Corral-Lopez<sup>1,2</sup>  | Javier Edo Varg<sup>3</sup>  | Yiselle P. Cano-Cobos<sup>4</sup> | Rafael Losada<sup>5</sup> | Emilio Realpe<sup>4</sup>  | David Outomuro<sup>3</sup> 

<sup>1</sup>Department of Ethology/Zoology, Stockholm University, Stockholm, Sweden

<sup>2</sup>Department of Zoology and Biodiversity Research Centre, University of British Columbia, Vancouver, Canada

<sup>3</sup>Section for Animal Ecology, Department of Ecology and Genetics, Evolutionary Biology Centre, Uppsala University, Uppsala, Sweden

<sup>4</sup>Laboratorio de Zoología y Ecología Acuática, Departamento de Ciencias Biológicas, Universidad de los Andes, Bogotá, Colombia

<sup>5</sup>Centro de Investigaciones en Microbiología y Parasitología Tropical (CIMPAT), Departamento de Ciencias Biológicas, Universidad de los Andes, Bogotá, Colombia

## Correspondence

David Outomuro  
Email: outomuro.david@gmail.com

## Present address

David Outomuro, Department of Biological Sciences, University of Cincinnati, Rieveschl Hall, Cincinnati, OH 45221, USA

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## Abstract

1. Imperfect mimicry may be maintained when the various components of an aposematic signal have different salience for predators. Experimental laboratory studies provide robust evidence for this phenomenon. Yet, evidence from natural settings remains scarce.
2. We studied how natural bird predators assess multiple features in a multicomponent aposematic signal in the Neotropical 'clear wing complex' mimicry ring, dominated by glasswing butterflies.
3. We evaluated two components of the aposematic signal, wing colouration and wing morphology, in a predation experiment based on artificial replicas of glasswing butterflies (model) and Polythoridae damselflies (mimics) in their natural habitat. We also studied the extent of the colour aposematic signal in the local insect community. Finally, we inspected the nanostructures responsible for this convergent colour signal, expected to highly differ between these phylogenetically distinct species.
4. Our results provide direct evidence for a stronger salience of wing colouration than wing morphology, as well as stronger selection on imperfect than in perfect colour mimics. Additionally, investigations of how birds perceive wing colouration of the local insect community provides further evidence that a UV-reflective white colouration is being selected as the colour aposematic signal of the mimicry ring. Using electron microscopy, we also suggest that damselflies have convergently evolved the warning colouration through a pre-adaptation.
5. These findings provide a solid complement to previous experimental evidence suggesting a key influence of the cognitive assessment of predators driving the evolution of aposematic signals and mimicry rings.

## KEYWORDS

field predation experiment, Ithomiini, mimicry ring, Neotropics, Polythoridae, wing colour, wing morphology

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## 1 | INTRODUCTION

Mimicry of aposematic warning signals (Bates, 1862; Müller, 1879) has provided a central example of the strength of natural selection in promoting adaptation (Kikuchi & Pfennig, 2013; Ruxton et al., 2004; Sherratt, 2002). Yet, mimics with poor resemblance to their models are common across taxa (the so-called 'imperfect mimicry'), posing a major challenge for evolutionary theory (Chittka & Osorio, 2007; Kikuchi & Pfennig, 2013; Sherratt, 2002; Sherratt & Peet-Paré, 2017). Importantly, aposematic signals are now understood as a suite of traits that all contribute, to a certain extent, to educate predators, that is, aposematic signals are multicomponent (e.g. morphology, colour, pattern, locomotion, chemical cues, etc.; Kazemi et al., 2015; Kikuchi et al., 2016; Kitamura & Imafuku, 2015; Malcicka et al., 2015; Outomuro et al., 2016; Penney et al., 2014; Srygley, 2007). Among all the hypotheses suggested to explain the evolution of imperfect mimicry (reviewed in Kikuchi & Pfennig, 2013), the relaxed selection hypothesis has accumulated the most empirical support: selection against imperfect mimicry is weak (Penney et al., 2012; Sherratt, 2002; Sherratt & Peet-Paré, 2017). One of the underlying reasons is related to

predator cognition processes (Chittka & Osorio, 2007; Gamberale-Stille et al., 2012; Kazemi et al., 2015; Kikuchi & Dornhaus, 2018; Sherratt & Peet-Paré, 2017). When predators learn to discriminate between multiple components of the aposematic signal (e.g. colour, pattern, shape), only the most salient cues are preferentially used to categorize aposematic prey as unpalatable, that is, some cues overshadow others, potentially leading to human-perceived imperfect mimicry (Gamberale-Stille et al., 2018; Kazemi et al., 2014, 2015). Previous laboratory experiments provide solid evidence for colour signals overshadowing pattern and shape in aposematic prey predated by birds (e.g. Aronsson & Gamberale-Stille, 2008; Kazemi et al., 2014, 2018; Rönkä et al., 2018). Hence, it should be expected that in natural systems where bird predation plays a central role (e.g. mimetic insects), the evolution of imperfect mimicry may have been driven by colour signals overshadowing other aposematic traits.

The Neotropical 'clear wing complex' is a mimicry ring dominated by unpalatable glasswing butterflies (tribe Ithomiini; Beccaloni, 1997). This mimicry ring also includes other butterfly species, a number of species of diurnal moths, and is parasitized by some species of Polythoridae damselflies which act as imperfect mimics (Beccaloni, 1997; Outomuro et al., 2016; Figure 1). This insect



**FIGURE 1** The butterfly *Greta andromica* (a) and the damselfly *Euthore fasciata* (b) used respectively as the model and the mimic in the mimicry ring studied. (c–f) Artificial replicas of a control butterfly (c) and a control damselfly (d), a UV-reflective white patch in a butterfly replica (e), and a non-UV-reflective white patch in a damselfly replica (f). Beak marks on the plasticine body can be appreciated in (c) (arrow), and to a very large extent in (f) (arrow). In (d), the small markings (arrow) were done by a mandibulate insect

mimicry ring is mainly predated by birds (e.g. Langham, 2006). The aposematic colour signal of this mimicry ring is an UV-reflective white wing patch, on mostly transparent wings (Beccaloni, 1997; Outomuro et al., 2016; Figure 1). This colour can be considered a relatively rare aposematic colour signal. Indeed, while variation in aposematic colour signals is wide both within and across taxa (Briolat et al., 2019), long-wave rich colours (e.g. red, orange, yellow) are more often present in mimic species given their high contrast against multiple backgrounds, which in turn may facilitate learning processes by predators (Stevens & Ruxton, 2012). The presence of UV reflection in wings of moths and butterflies has been shown to increase predation rates by birds (Lyytinen et al., 2004). Thus, the conspicuousness of the UV-reflective white wing patches to the bird visual system can be under strong predation selection in this mimicry ring. On the other hand, the non-UV-reflective white wing patches of other insects in the local insect community should be under weaker predator selection.

Despite the increasing laboratory evidence supporting the role of predator cognition on promoting different selection regimes on the various components of aposematic signals, we have limited evidence from natural populations (but see Finkbeiner et al., 2014, 2018). A previous study in two mimetic species of Polythoridae damselflies of the 'clear wing complex' established that these species showed advergence of the wing colour patch, but also of wing morphology (Outomuro et al., 2016). Hence, this system offers a remarkable opportunity to characterize the salience of multicomponent aposematic signals in a more natural context, a suggestion that was brought to attention by previous studies (e.g. Kikuchi et al., 2015; Taylor et al., 2017). In parallel, recent experiments with artificial paper replicas of butterflies in natural habitats have provided a valuable tool to study multiple aspects of bird predation-driven mimicry (Finkbeiner et al., 2014, 2017, 2018), with direct application to our study question.

Insect mimicry rings have proven fundamental to answer central questions on evolutionary patterns in relation to warning signals (Rossato et al., 2018). In this study, we aimed to provide further insight on the assessment of a rare colour warning signal by natural predators in a Neotropical insect mimicry ring of butterflies, moths and damselflies. Thanks to prior knowledge on the multicomponent aposematic signal present in butterflies and damselflies within the 'clear wing complex', we aimed to characterize the role that the relative salience of traits can play on the evolution of imperfect mimicry, using experimental evidence from field investigations based on artificial replicas. We also evaluated to what extent this aposematic colour signal was present in the local community of insects using spectrophotometry and physiological models to estimate bird visual perception of the signal. Furthermore, given the convergence of a rare warning signal in largely phylogenetically distinct species, we aimed to further investigate the microscopic organization of the UV-reflective colour signals in the butterflies and damselflies studied. For this, we used high resolution imaging (electron microscopy) and quantified the dimensions of nanostructures present in their wings. Overall, our study is among the firsts to investigate predator cognitive biases in natural populations. Because we also investigated the representation of the colour aposematic signal in the local insect community, and inspected the

proximate causes producing this signal, this study broadens our current understanding of the evolution of mimicry.

## 2 | MATERIALS AND METHODS

### 2.1 | Relative salience of traits in a multicomponent aposematic signal

#### 2.1.1 | Artificial replicas

To study the salience of colouration and morphology in the mimicry ring, we produced artificial replicas of butterflies and damselflies that form part of the 'clear wing complex'. We adapted the experimental procedure in Finkbeiner et al. (2012) to create butterfly and damselfly artificial replicas, based respectively on the glasswing butterfly *Greta andromica* (Hewitson, 1854) and the damselfly *Euthore fasciata* (Hagen in Sélys, 1853; Figure 1a,b). These two species are part of the 'clear wing complex' and they co-occur spatially and temporally in our field location (Outomuro et al., 2016). The glasswing butterfly was chosen as the model and the damselfly as the mimic (presumably Batesian, see Outomuro et al., 2016). We produced three types of butterfly and damselfly replicas: (a) replicas that included an UV-reflective white patch on the wing; (b) replicas that included a non-UV-reflective white patch on the wing; (c) replicas that did not include any white colouration on the wing (Figure 1c–f). Our models thus consisted of two species, that is, butterfly-like and damselfly-like, and three colour treatments, that is, UV-reflective white, non-UV-reflective white and control (no white patch). The butterfly-like replicas represented the models, and the damselfly-like replicas represented the mimics. Moreover, the UV-reflective white patch represents the 'perfect' aposematic colour signal, while the non-UV-reflective white patch represents the 'imperfect' aposematic colour signal.

The left and right wings of butterfly and damselfly replicas were printed on transparency film using a Ricoh Aficio MP C5000 printer with a black MP C5000 print cartridge. Real butterflies show brown and orange colouration in certain areas of the ventral side of the wings, and the damselflies show metallic bluish colouration (Figure 1a,b). Since these colours presumably do not form part of the aposematic signal, and to avoid confounding effects, we kept them black in the artificial replicas (Figure 1c–f). The UV-reflective white wing patch was painted by hand on a third part of the replicas using white Fish Vision UV Reflective Lure & Jig Paint (Reel Wings Decoy Company Inc.; Figure 1e). We also painted by hand a non-UV-reflective white wing patch in another third of the replicas using Titanium white paint (Panduro Hobby AB; Figure 1f). The remaining third of the replicas were not painted (control; Figure 1c,d). Then, left and right wings were glued together and dipped in wax to waterproof the ink and the paint. Finally, the wings were affixed to 0.05 Ø metal wire, for attachment in nature, and to a black plasticine body (Newplast modelling clay and Panduro dough modelling clay; Figure 1c–f). Such artificial replicas resemble the natural resting position of these species when perching on the vegetation.

The plasticine body and the wax on the wings allow detecting marks of attempts of predation.

We validated the paints used for the white patch by estimating the chromatic and achromatic contrasts in a bird visual system between spectral measurements of the white patch of *G. andromica* and *E. fasciata*, and spectral measurements of the white patch of our replicas after dipping the replicas in wax (see Supporting Information). The chromatic and achromatic contrasts are measured in Just Noticeable Distances (JNDs). In principle, values of JNDs larger than 1 indicate that the observer can discriminate between two reflectance spectra, that is, those spectra are distinctively different (Vorobyev et al., 2001). However, this threshold has been shown to increase depending on the intensity of light, the brightness of the stimuli, and the difference in the spectral composition between stimuli (Olsson et al., 2015). Therefore, we used a threshold of 1 as a very conservative value. We estimated our contrasts for both general types of bird visual systems, namely VS-birds (with the peak sensitivity of the extreme short wavelength above 400 nm) and UVS-birds (peak sensitivity of the extreme short wavelength below 400 nm; Hart, 2001). We found that the chromatic contrasts for the UV-reflective white patch were not larger than 1 for VS-birds (mean  $\pm$  SD = 0.664  $\pm$  0.382; one sample *t*-test:  $t_{239} = -13.632$ ,  $p = 0.999$ ) or for UVS-birds (mean  $\pm$  SD = 1.062  $\pm$  0.650; one sample *t*-test:  $t_{239} = 1.476$ ,  $p = 0.071$ ). In addition, the JND values for the non-UV-reflective white patch were below 1 for VS-birds (mean  $\pm$  SD = 0.687  $\pm$  0.353; one sample *t*-test:  $t_{239} = -13.723$ ,  $p = 0.999$ ) and larger than 1 for UVS-birds (mean  $\pm$  SD = 2.9292  $\pm$  0.822; one sample *t*-test:  $t_{239} = 36.354$ ,  $p < 0.001$ ). We therefore assumed that for the chromatic component, our UV-reflective models were very similar to the natural species, while the non-UV-reflective models were reasonably distinct, especially for UVS-birds.

Regarding the achromatic contrasts, we found that the JND values were much larger than 1 in both visual systems and both for the UV-reflective white patch (VS-birds: mean  $\pm$  SD = 10.445  $\pm$  4.160, one sample *t*-test:  $t_{239} = 35.169$ ,  $p < 0.001$ ; UVS-birds: mean  $\pm$  SD = 10.433  $\pm$  4.140, one sample *t*-test:  $t_{239} = 35.302$ ,  $p < 0.001$ ) and the non-UV reflective white patch (VS-birds: mean  $\pm$  SD = 10.852  $\pm$  3.848, one sample *t*-test:  $t_{239} = 39.661$ ,  $p < 0.001$ ; UVS-birds: mean  $\pm$  SD = 10.839  $\pm$  3.829, one sample *t*-test:  $t_{239} = 39.807$ ,  $p < 0.001$ ). This indicates that our models failed to mimic the achromatic component of wing colouration in the natural species. We decided to keep these levels of luminance in our models because the UV-paint did not have a strong UV component, and we wanted to investigate the role of this component in predator avoidance in our study system. A higher achromatic contrast may improve aposematic signalling to predators (Prudic et al., 2007) and therefore could bias our results in predation rates. However, since the achromatic contrasts were very similar between the UV-reflective and the non-UV-reflective white patches, differences in predation rates between these two treatments should be due to differences in the chromatic component rather than the achromatic component.

The transparency of the wing can reduce predation rates in glasswing butterflies by decreasing detectability (Arias, Elias, et al., 2020; Arias et al., 2019). We measured the light transmitted through the

transparent area of the wing of *G. andromica* and *E. fasciata*, as well as of the plastic replicas (after being dipped in wax). We then estimated the chromatic and achromatic contrasts in a bird visual system between the butterfly wing, the damselfly wing and the plastic replica. The colour of the transparent area was calculated as light reflected from green foliage after passing through the wing or plastic (see Supporting Information). The chromatic and achromatic JND values between the insect wings and the replica were much larger than 1 in both bird visual systems (all cases  $> 6.376$ , see full results in Supporting Information). Therefore, our replicas would be expected to be more conspicuous towards predators than the real insects, potentially increasing the detectability of the artificial replicas. Since the transparency of our replicas is the same across all treatments, any differences in predation rates should be due to differences in the treatments (species or colouration).

## 2.1.2 | Predation experimental procedure

The predation experiment was performed in a piedmont habitat in central Colombia (Santa María de Boyacá, Boyacá, 4°51'52.7"N, 73°15'31.8"W). This habitat is naturally co-inhabited by the two species that our artificial replicas resembled, *E. fasciata* and *G. andromica*. The experiment took place during April 2017, at the end of the dry season, when the abundance of damselflies is higher (Y.P. C.-C., pers. obs.). We placed the artificial butterfly and damselfly replicas in 64 sites along transects in the study area, ranging from 985 to 1,300 m a.s.l. Each replica was attached to a branch, about 1.5–2-m high, using the metal wire attached to the wings and the body, mimicking a resting position of the insect (Figure 1e). The base of the branch was covered with non-toxic anti-insect glue (Supertramp, Inalmet, Colombia), to avoid predation by terrestrial arthropods. To reduce overlap of avian predator territories, sites were separated at least 100 meters between each other (e.g. Finkbeiner et al., 2012). At each site, 30 replicas were placed (five for each species/colouration combination). The replicas were placed with a minimum separation of 4 meters among them. The position of replicas was randomized independently for each site. In total, we used a sample size of 1920 artificial replicas.

We quantified the number of avian attacks by visiting all artificial replicas every 24 hr during 4 consecutive days. A replica was considered attacked if it presented substantial damage in the plasticine abdomen, or if V-shaped pecks were observed in the abdomen or wings of the replica (Figure 1c–f). Replicas attacked by small arthropods (e.g. ants, bees, grasshoppers) presented a characteristic damage with small bites (e.g. Figure 1d) and were not considered as predated in our analyses. Replicas that were predated or damaged were replaced with new replicas of the same treatment for the next experimental days.

## 2.1.3 | Data analyses of predation rates

To evaluate differences in predation between damselfly and butterfly replicas with UV-reflective white, non-UV-reflective white and



no white wing patch (control), we quantified the number of artificial replicas of each treatment attacked by avian predators during the 4 days for each site. Replicas attacked again after replacement were counted as one single attack (Finkbeiner et al., 2014). We analysed predation using two alternative methods: a GLMM with the number of artificial replicas attacked as the response variable; and a Cox proportional-hazards regression model to estimate survival probabilities across the 4-day experiment. The first approach informed of the overall differences of predation rates across the different experimental treatments. The second approach informed of the progression of predation rates during the 4 days that the experiment lasted, and potentially could also inform of changes in the predator response to the different replicas during the course of the experiment. These two analyses, used independently or in combination, are commonplace to evaluate avian predation on artificial butterfly prey in the tropics with similar experimental designs (Dell'Aglio et al., 2016; Finkbeiner et al., 2012, 2014, 2017, 2018; Seymoure & Aiello, 2015).

We assessed differences in predation between treatments using a GLMM with a log-link Poisson distribution. The predated number of replicas of each species and colouration treatment were entered as count numbers per site. The model included the fixed factors species (butterfly and damselfly), and colouration treatment (UV-reflective white, non-UV-reflective white, and control), and the interaction between these two. We included site as a random effect in the model. We did not detect overdispersion in the model by comparing the sum of squared Pearson residuals to the residual degrees of freedom (approximate  $\chi^2$  distribution,  $p = 0.999$ ). We did not detect zero inflation in our dataset, since the number of zeros in our predation data were similar to the number of zeros predicted with a mean equal to the mean count of the data under a Poisson distribution (observed: 255, predicted: 261, ratio: 1.02). We additionally performed diagnostics plots of our Poisson model following guidelines in Harrison et al. (2018). For this, we simulated 10,000 datasets from our model and observed that the proportion of zeros in our real data is comparable to simulated expectation (Figure S1a). Hence, our evaluation of the model again suggested no need to account for zero inflation in the final model used. Similarly, posterior dataset simulations indicated that the model matched the characteristics of the data in relation to the among-site variance observed (Figure S1b). To further inspect differences in predation risk between the three colouration treatments, we conducted pairwise post hoc comparisons, using the EMMEANS package for R (Lenth, 2020).

We studied the survival probabilities of our different artificial replicas quantifying the number of replicas predated each day during the 4-day experiment. We used a Cox proportional-hazards regression model using the function `coxph` in R (Therneau, 2015). The colouration treatment, the species and their interaction term were entered as factors in the model. We further explored the pairwise comparisons of the survival curves using the function `pairwise_survdiff` in the `SURVMINER` R package (Kassambara et al., 2020). We plotted the survival curves using the `survfit` function in the `SURVMINER` R

package (Kassambara et al., 2020). All statistical analyses were performed in R version 4.0.2 (R Core Team, 2020).

## 2.2 | Characterization of the aposematic colour signal

### 2.2.1 | Wing colouration in the butterfly, moth and damselfly community

We hypothesized that the aposematic colour signal of the 'clear wing complex' (a UV-reflective white wing patch on mostly transparent wings) should be restricted to the mimicry ring, and not present in other insects of the same local community that are not part of the mimicry ring (e.g. butterflies and moths with white wing pigmentation but no transparent wings). One day after the completion of the predation experiment, we walked along a linear transect in each of the 64 study sites and collected all butterfly, diurnal moth and damselfly species that presented any type of white/whitish colouration on their wings. This procedure was repeated twice, early in the morning and before dusk. In the following days, more specimens were occasionally collected within the study area, where another study was being developed. For the butterflies and moths, we collected a maximum of two individuals per species and sex, with a total number of species of 20 butterflies ( $N = 46$ ) and two moths ( $N = 3$ ; Table S1). We also collected two species of damselflies in larger numbers ( $N = 17$ ; Table S1), as part of a different study. The collected insects were classified as belonging to the 'clear wing complex' (i.e. with a subapical white patch on a mostly transparent wing), hereafter 'mimicry group' (three species of Ithomiini, two species of damselflies, and one moth species) or not, hereafter 'non-mimicry group' (17 butterfly species and one moth species). Fifteen of the 20 sampled butterfly species had been previously described for the field location (Andrade-C et al., 2017). We note that we were not interested in doing an exhaustive sampling of species of this field location. We believe our species set represents an unbiased sample of butterflies found at that time of the year and along the transects where we performed the predation experiment.

We measured the reflectance of the white wing colouration of the collected specimens using a spectrophotometer (see Supporting Information). We estimated the relative quantum catches using a receptor noise limited model (Vorobyev & Osorio, 1998; Vorobyev et al., 1998) and plotted the data on the avian tetrahedral colour space, which does not account for photoreceptor noise (Endler & Mielke, 2005; Stoddard & Prum, 2008; see details for the modelling in Supporting Information). We did this both for UVS and VS birds. We then estimated the volume occupied in the colour space by the mimicry group and by the non-mimicry group, and the overlap between the volumes. We predicted that the mimicry group would be more spatially concentrated in the avian visual space due to predator convergent selection on the white colour, while the non-mimicry group would be

less spatially concentrated due to weaker predator selection. All spectra processing and modelling was performed in the package PAVO (Maia et al., 2019) for R (R Core team, 2020).

## 2.2.2 | Electron microscopy of wing colouration

The UV-reflective white aposematic signal is convergent between butterflies and damselflies. Since these two groups are phylogenetically distant, it is important to understand how they both can produce such a rare warning signal. Therefore, we studied the nanoarchitecture of the wing colour patches in the model butterfly *G. andromica* and the local mimic damselfly *E. fasciata*. We also included two outgroup local damselfly species that do not have wing colouration (either white or dark), *Cora* sp. Sélys, 1853 and *Hetaerina* sp. Hagen in Sélys, 1853, sampled also in the study site. The genus *Cora* belongs to the damselfly family Polythoridae, similar to *Euthore*. The genus *Hetaerina* belongs to the family Calopterygidae: Calopterygidae and Polythoridae are part of two sister clades under the Superfamily Calopterygoidea (Suvorov et al., 2020).

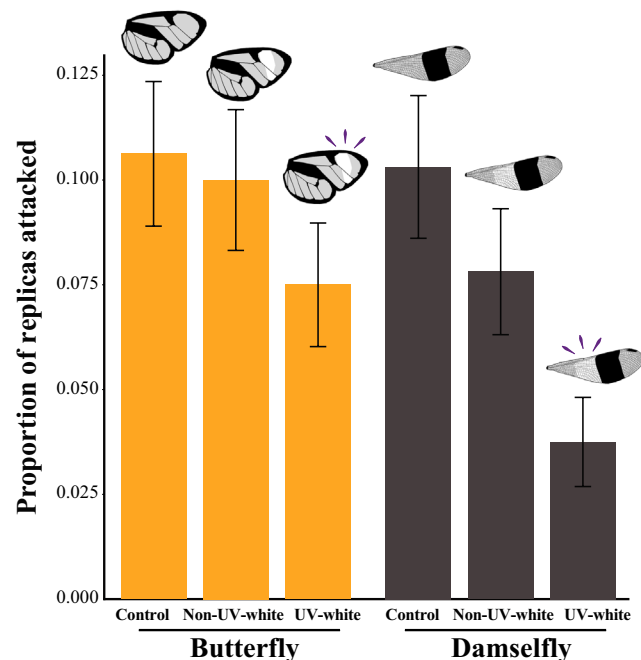
We cut small pieces of the wings using a razor blade. The samples were fixed to a metallic holder using scanning electron microscope conductive double-sided carbon tape followed by a gold coating using a Denton Vacuum Desk IV sputter coater. The samples were observed in a ZEISS EVOHD15 and in a JOEL JSM6490-LV scanning electron microscopes. We took images from white patches and transparent areas of the butterfly *G. andromica*, and the mimic damselfly *E. fasciata*, as well as from a central section in the transparent wing of *Cora* sp. and *Hetaerina* sp. We also took images of the dark wing area of *E. fasciata*. We detected cuticular wax rods on the damselflies wings (see Section 3). We measured the dimensions of those structures (length and width of the rods) in the three wing regions of *E. fasciata*. We also measured the width of the rods in the transparent wings of *Cora* sp. and *Hetaerina* sp. (the quality of the images prevented us from measuring the rod lengths). Measurements were taken using ImageJ 2.1.0 (Rueden et al., 2017). We selected the best images and measured 10 rods per image (N rods measured: length: *E. fasciata* white patch: 10, dark patch: 20, transparent area: 10; N width: *E. fasciata* white patch: 30, dark patch: 20, transparent area: 10, *Cora* sp.: 10, *Hetaerina* sp.: 10). We assessed the differences in length and width between the three regions of the wing of *E. fasciata* using two separate ANOVAs, followed by Tukey tests. We then compared the rods widths of the three regions of *E. fasciata* with *Cora* sp. and *Hetaerina* sp., following a similar analysis.

## 3 | RESULTS

### 3.1 | Salience of traits in the multicomponent aposematic signal

We studied bird predation on butterfly and damselfly replicas placed in nature. Out of the 1920 replicas placed, 160 were

attacked distributed across treatments as follows: butterfly-like: 34 control, 32 non-UV-white and 24 UV-white; damselfly-like: 33 control, 25 non-UV-white and 12 UV-white. Overall, the predation rate was 8.3%, which is fairly similar to previous studies with similar experimental designs (e.g. 6.4% in Finkbeiner et al., 2014; 8.5% in Seymoure & Aiello, 2015; 9.1% in Finkbeiner et al., 2017; 9.5% in Finkbeiner et al., 2018). We found that the colouration treatment of the artificial prey had a significant effect on the number of attacks, but the number of attacks did not depend on the species (GLMM, colouration:  $\chi^2 = 8.474$ ,  $df = 2$ ,  $p = 0.014$ ; species:  $\chi^2 = 2.333$ ,  $df = 1$ ,  $p = 0.127$ ; colouration treatment  $\times$  species:  $\chi^2 = 2.382$ ,  $df = 2$ ,  $p = 0.304$ ; Figure 2). Thus, colour overshadowed morphology in terms of predator attacks of the different artificial replicas. Comparisons between the different types of artificial replicas exposed to natural bird predation showed that predation rates were the highest for the control artificial replicas, intermediate for the replicas with a non-UV-reflective white patch (i.e. the 'imperfect' aposematic signal), and the lowest for the replicas with a UV-reflective white patch (i.e. the 'perfect' aposematic signal; Figure 2). Specifically, post-hoc contrasts of the model for the different colour treatments showed that attacks were significantly lower towards replicas that presented a UV-reflective white patch in relation to control replicas without white wing colouration ( $z = 3.164$ ,  $p = 0.004$ ). In addition, no significant differences in attacks were observed between non-UV and control replicas ( $z = -0.935$ ,  $p = 0.618$ ), or between non-UV and UV replicas ( $z = -2.306$ ,  $p = 0.055$ ). Therefore, the non-UV replicas statistically showed intermediate predation rates between the controls and the UV replicas.



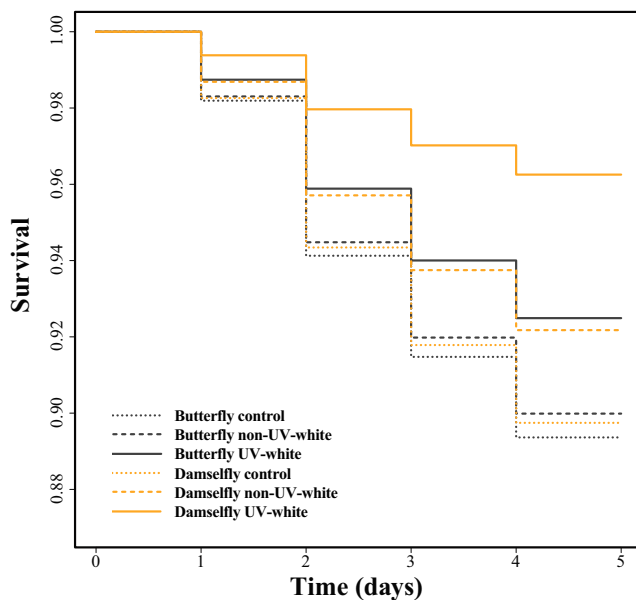
**FIGURE 2** Proportion ( $\pm$ SE) of replicas attacked for each wing morphology (butterfly and damselfly) and wing patch colouration (control: no white wing patch, non-UV-white: non-UV-reflective white wing patch, UV-white: UV-reflective white wing patch)

The survival curves supported our results from the GLMM, with significant differences in survival between the colouration treatments, but not between the species (colouration:  $\chi^2 = 10.615$ ,  $df = 2$ ,  $p = 0.005$ ; species:  $\chi^2 = 2.769$ ,  $df = 1$ ,  $p = 0.096$ ; colouration treatment  $\times$  species:  $\chi^2 = 2.547$ ,  $df = 2$ ,  $p = 0.280$ ; Figure 3). Further exploration using pairwise comparisons revealed that the damselfly-like replicas with UV-reflective white patches had significantly higher survival rates than the butterfly-like replicas with non-UV-reflective white patches, and both the control butterfly- and damselfly-like replicas ( $p < 0.009$ ; Figure 3).

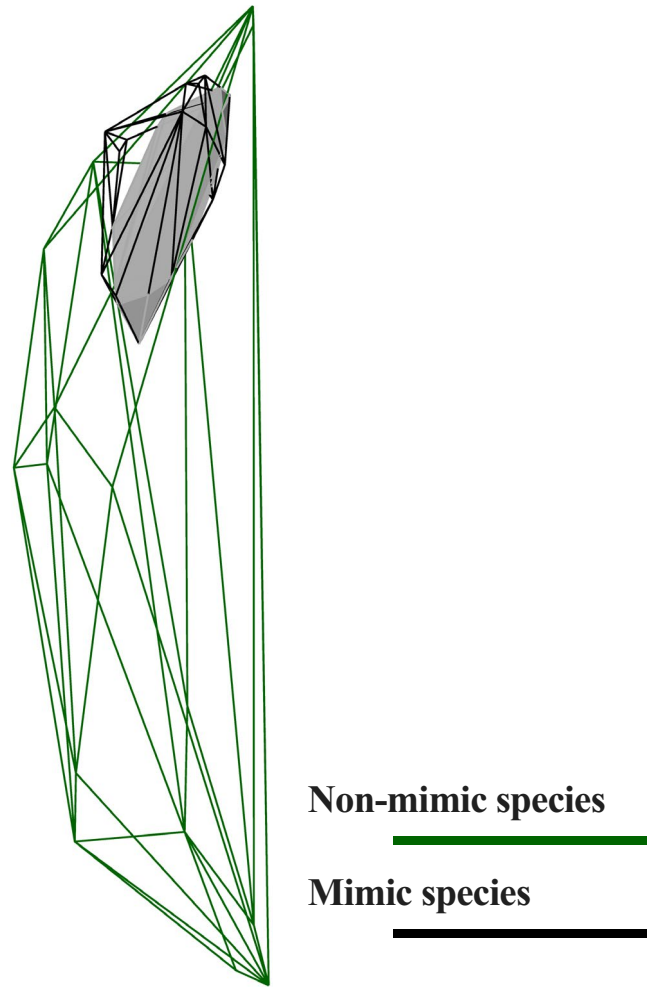
### 3.2 | Characterization of the aposematic colour signal

Using a quantification of the avian visual colour space, we found that non-mimic butterflies (17 species) and moths (one species) occupied a volume 4.5- (VS-birds) and nine-fold (UVS-birds) larger than the butterflies, moths and damselflies of the 'clear wing complex' (6 species), with a 23.9% (VS-birds) and 26.5% (UVS-birds) overlap of the mimic volume on the non-mimic volume (Figure 4).

Evaluation of nanostructures present on the wing surface using scanning electron microscopy showed that the transparent portion of the wing of *G. andromica* was covered by bristle-like scales (Figure 5a), while the white portion had regular-shaped scales (Figure 5b) with ridges not connected by membrane (Figure 5c; see also results for *Greta oto* in Siddique et al., 2015). Therefore, the UV-reflective white is probably produced due to the interaction between light and the nanostructure of those wing scales. For the mimic damselfly *E. fasciata*, the three wing regions (white



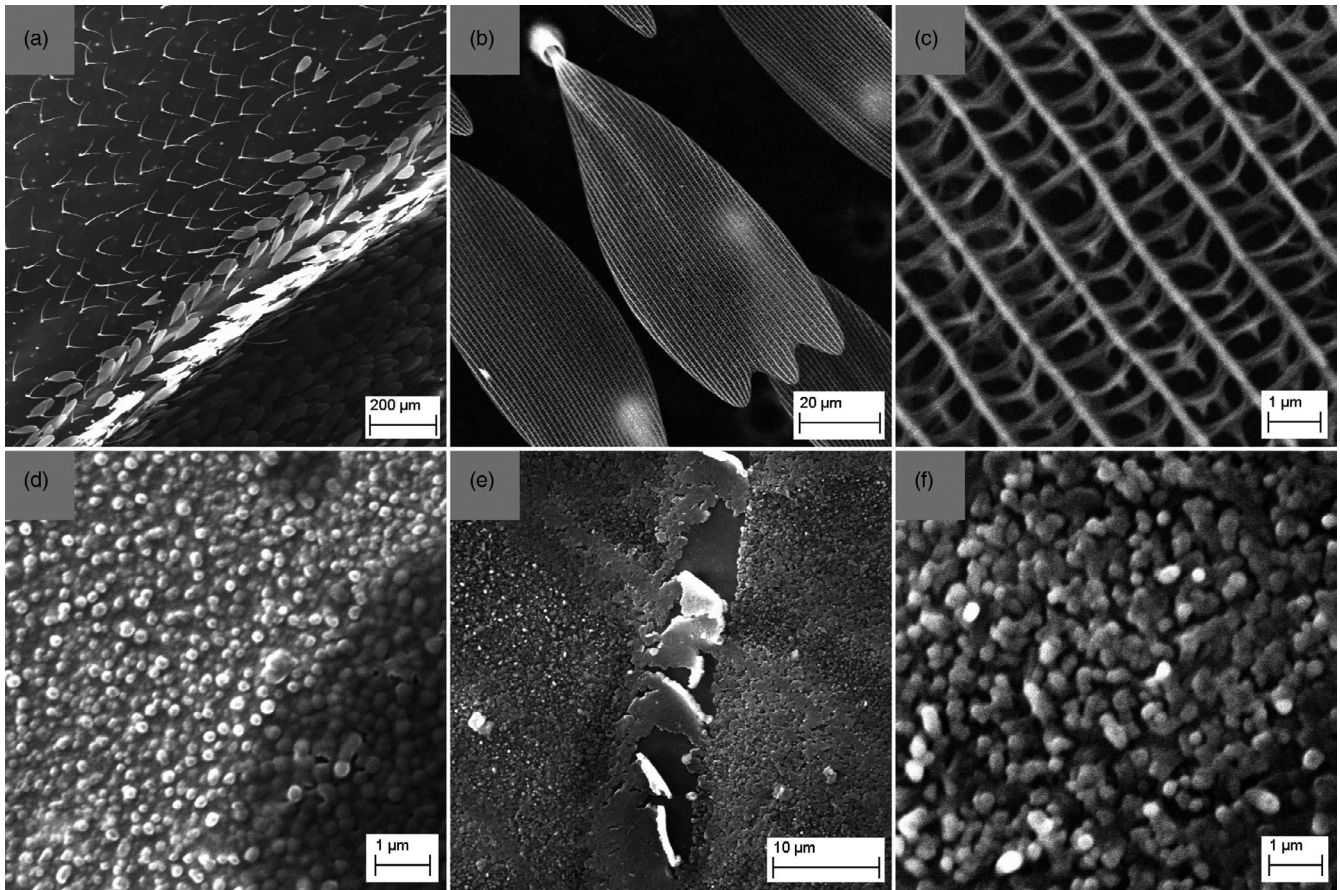
**FIGURE 3** Survival curves of replicas attacked of each wing morphology (butterfly and damselfly) and wing patch colouration (control: no wing patch, non-UV-white: non-UV-reflective white wing patch, UV-white: UV-reflective white wing patch) during the 4-day experiment



**FIGURE 4** Volume occupied in the UVS avian visual space by mimic butterflies, moths and damselflies, and non-mimic species of butterflies and moths. The overlap between both volumes is shown in solid grey

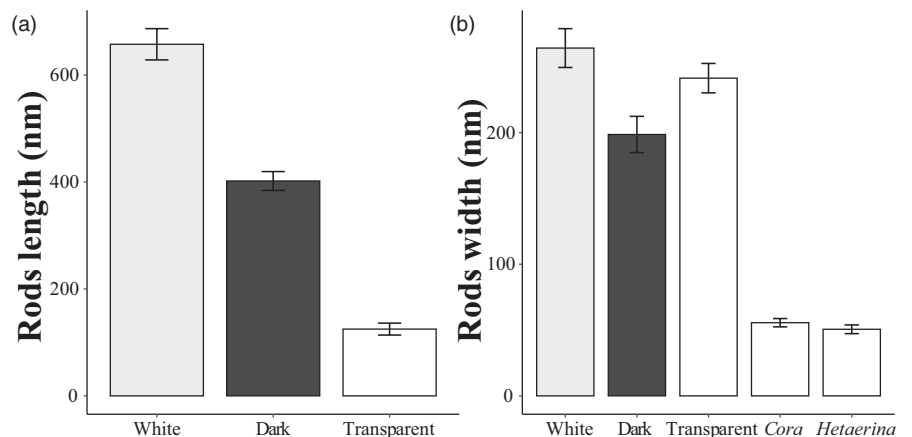
patch, dark area and transparent area) were covered in cuticular wax (Figure 5d-f; see results for the damselfly *Megaloprepus caerulatus* in Schultz & Fincke, 2009). We compared the dimensions of the wax rods between the white, dark and transparent areas of *E. fasciata* wings. The rod lengths differed significantly between wing regions (ANOVA:  $F_{2,57} = 127.400$ ,  $p < 0.001$ ; Figure 6a). The three wing regions differed from each other (Tukey tests, all pairwise comparisons  $p < 0.001$ ), with the longest rods in the white region, followed by the dark and the transparent regions (Figure 6a). The rod widths also significantly differed among wing regions (ANOVA:  $F_{2,57} = 5.418$ ,  $p = 0.007$ ; Figure 6b). The white area showed significantly thicker rods than the dark area, while the transparent area showed values intermediate between the white and the dark areas (Tukey tests,  $p$ -values: white-dark: 0.005; white-transparent: 0.639; transparent-dark: 0.255; Figure 6b). We also evaluated wing samples for two outgroup damselflies without wing colouration, *Cora* sp. and *Hetaerina* sp. We observed that the wax coverage was also present on those two species (Figure S2), but the wax rods were much thinner than in any of the wing areas of *E. fasciata* (ANOVA:





**FIGURE 5** Nanostructures on the wing surface of *Greta andromedica* (a–c) and *Euthore fasciata* (d–f) observed by scanning electron microscopy. (a) Transition between the transparent region of the wing with bristle-like scales (top) to the region with regular-shaped scales (bottom). (b) Close up on the scales of the white patch. (c) Nanoarchitecture of the scales of the white patch, showing ridges without interconnecting membranes. (d) Transparent region of the wing, with small wax rods. (e) Thick disturbed layer of wax in the white region of the wing. (f) White region of the wing, showing large wax rods

**FIGURE 6** Length and width of the cuticular wax rods in damselfly wings. (a) Length of the rods in three regions of the wing of the mimic damselfly *Euthore fasciata*: UV-reflective white patch, dark patch, and transparent region of the wing. (b) Width of the rods in the three regions of the wing of *E. fasciata* (white, dark and transparent), as well as in the transparent wings of the non-mimic outgroup damselflies *Cora* sp. and *Hetaerina* sp



$F_{4,75} = 38.780$ ,  $p < 0.001$ ; Tukey tests: all pairwise comparisons between *Cora* sp. and *E. fasciata*, and *Hetaerina* sp. and *E. fasciata*:  $p < 0.001$ ; Figures 5d–f and 6b; Figure S2). The thickness of the rods did not differ between *Cora* sp. and *Hetaerina* sp. (Tukey test,  $p = 0.999$ ; Figure 6b). Therefore, the UV-reflective white patch in mimic damselflies is probably produced by the interaction between light and enlarged cuticular wax rods.

## 4 | DISCUSSION

### 4.1 | Saliency of traits in the multicomponent aposematic signal

Our predation experiment showed that the number of attacks from bird predation differed between the wing colouration treatments but



not between the species treatment. These results provide direct evidence for the higher salience of wing colouration over morphology for avian predators in natural conditions. This also adds support from a natural setting to previous laboratory experiments that showed that avian predators pay primarily attention to colour in relation to other features when evaluating warning signals (Aronsson & Gamberale-Stille, 2008; Kazemi et al., 2014; Rönkä et al., 2018; Terhune, 1977). The relaxed selection hypothesis (Kazemi et al., 2014; Kikuchi & Pfennig, 2013) predicts that selection in multicomponent aposematic signals is more relaxed in certain aposematic cues compared to others, thus leading to different levels of resemblance across cues and imperfect mimicry. Our results add experimental evidence in field conditions that supports this prediction, since morphology showed lower salience than colouration for bird predators. The relaxed selection hypothesis also predicts similar predation risk in perfect and imperfect mimics, which would prevent from further resemblance in the aposematic cue. This is the case for the species treatment, since we did not detect overall differences in predation risk between damselfly- and butterfly-like replicas. However, our results suggest that the colour 'imperfect mimics' (i.e. the replicas with non-UV-reflective white patches) might be under stronger selection than the colour 'perfect mimics' (i.e. the replicas with UV-reflective white patches). This unexpected result opens up new research venues in this field that require further investigation. As our imperfect mimics lack the UV component, a crucial part of the colour aposematic signal, the differences observed may indicate that avian predators are probably fine-tuning colour aposematic signals, and future research should evaluate more subtle differences in this colour cue.

Our characterization of wing colouration in the butterflies, damselflies and diurnal moths present in the local community of a Colombian piedmont habitat adds further evidence to our hypothesis of the colour aposematic signal being probably actively selected by bird predation (see also Outomuro et al., 2016). Specifically, we found that white wing colouration in the species within the mimicry ring occupies a small volume of the avian visual space, probably due to convergence/advergence, while the white wing colouration of non-mimic butterflies and moths occupied a much larger volume of the avian colour space. Interestingly, the reflectance curve of other white-winged butterflies (e.g. other species of Nymphalidae: Stavenga et al., 2014) is different from the glasswing butterflies, and in some cases the wings strongly absorb UV (e.g. Pieridae: Giraldo & Stavenga, 2007). Our findings thus provide novel evidence on how the predator visual system has shaped mimicry evolution in the 'clear wing complex', suggesting that the presence of short wavelength colouration (UV colouration) in white patches on the wing is the foremost feature of the signal, as well as an effective aposematic signal for bird predation in this habitat.

We did not find significant differences in predation rates between the two species. Interestingly, the butterfly-like replicas with UV-reflective white patches were predated twice as much (7.5%) as the damselfly-like replicas (3.75%) with the same colouration treatment. Butterflies are driving the aposematic mimicry ring (Beccaloni, 1997) and are presumably more abundant than damselflies in the study area (damselflies are directly associated with running waters while

butterflies are not). Indeed, the relative abundances of the models and the mimics, as well as the presence of alternative prey, can influence the evolution of mimetic traits (Finkbeiner et al., 2018; Kikuchi & Pfennig, 2013; Lindström et al., 2004). The local predators thus probably have more experience with butterflies than with damselflies, suggesting that damselfly-like replicas might have been perceived as an unfamiliar prey item with the morphology of a damselfly but with the aposematic colour signal of the mimicry ring. The initial reactions of predators to novel mimic prey items (i.e. damselflies) might not be the same as the long-term reactions (i.e. butterflies; Sherratt & Peet-Paré, 2017). We can speculate that the less experienced local predators might be showing a certain level of neophobia to a novel mimic, leading to a higher survival rate of the damselfly-like replicas with UV-reflective white patches. Another possibility is related to the size of the white patch between the damselfly- and the butterfly-like replicas. The wing area that covers the white patch is 33% smaller in butterflies than in damselflies, and so it was in our replicas. A larger colour aposematic cue might result in more aversive predator responses, and this could be driving the higher survival of damselfly- than butterfly-like replicas with UV-reflective white patches. Further field experiments will be necessary to test the effects of differences in size of the colour aposematic cue in predator response.

Our experiment explored the effects of the colour aposematic signal and species in predation. Another important component participating in predator detectability is the transparency of the wings, which characterizes the 'clear wing complex'. Transparency has been shown to confer higher crypsis in glasswing butterflies by reducing predator detectability (Arias, Elias, et al., 2020; Arias et al., 2019). Future research should investigate the combined effects of the relative wing areas with transparency and UV-reflective white colour in bird predation.

## 4.2 | How is the aposematic signal produced?

Our observations of the nanostructure of the wing colouration across species within the mimicry ring and in damselfly outgroups suggest that mimic damselflies have evolved a UV-reflective white patch that resembles that of the butterfly models through a likely pre-adaptation. Dragonflies and damselflies have a crystalline wax coverage over the wings and body, which presumably would decrease wettability (an important adaptation for insects associated with aquatic habitats) and prevent wing contamination (Futahashi et al., 2019; Gorb et al., 2000, 2009; Schultz & Fincke, 2009; Wagner et al., 1996). Moreover, white patches in the damselfly wings might also participate in inter- and intraspecific communication (Schultz & Fincke, 2009; but see Palacino-Rodríguez et al., 2016). The wax filaments produce broadband light scattering between 300 and 700 nm (Schultz & Fincke, 2009), creating the reported UV-reflective white pigmentation. High-resolution imaging of wing colouration in the damselfly mimic species of the 'clear wing complex', *E. fasciata*, suggests that white patches involve longer and thicker rods than other regions of the wing. This agrees with a previous study in the helicopter damselfly *Megaloprepus caerulatus*

(Drury, 1782), where wax filaments were longer and thicker in white wing patches compared to other non-white patches on the wings (Schultz & Fincke, 2009). Interestingly, images of the transparent wings of the sister genus *Cora* sp. and of *Hetaerina* sp., a member of the family Calopterygidae (a sister clade to the family Polythoridae under the Superfamily Calopterygoidea), showed that the wax rods are much thinner than in any wing region of *E. fasciata*. Therefore, Polythoridae mimic damselflies of the 'clear wing complex' might have evolved the UV-reflective white wing patches by further development of the wax rods, nanostructures already present in other non-white regions of the wings and in other closely related species of damselflies. Given the high salience of the UV-reflective white patch for avian predators, it is plausible that the presence of larger concentration of wax across and within species might have provided a selective advantage and facilitated the parasitism of the 'clear wing complex'. However, formal comparative tests are still necessary to evaluate the evolution of white patches in damselflies, in relation to the white patches in the butterflies in this system.

### 4.3 | Final remarks and future directions

Our field investigations on the selective pressures driving the multi-component aposematic signal observed in the 'clear wing complex' provide a robust match to experimental evidence suggesting a central role of predator cognitive processes in the evolution of imperfect mimicry (Chittka & Osorio, 2007; Kazemi et al., 2014). The salience of the butterfly UV-reflective white patch for the main predators of this system has probably driven the generalization of palatable damselfly mimics as unprofitable prey.

In parallel, the results of our study leave open questions on the evolution of the multicomponent aposematic signal previously found in two Polythoridae damselflies that are Batesian mimics of the 'clear wing complex' (Outomuro et al., 2016). In addition to the morphology of the wing studied here, flight characteristics presented advergence between butterfly models and damselfly mimics in our previous study (Outomuro et al., 2016). Indeed, flight mimicry is not uncommon in mimic species mainly preyed by birds (Kitamura & Imafuku, 2015; Skowron Volponi et al., 2018; Srygley, 1999). Due to the logistical challenge to incorporate movement to prey replicas used in our field investigation, we are unable to provide further information on the role that this feature might play in combination with wing morphology and colouration. Future experimental designs that incorporate movements that resemble those of the models in field experiments with this and similar systems, might provide crucial answers on how resemblance in multiple features, both alone and when combined, might facilitate associative learning of the aposematic colour signal.

Other ecological variables might have been in play in the evolution of the multicomponent signal previously described in this system. Previous studies have found that multicomponent signals can confer higher protection to mimics under higher complexity in the ecological community, for instance under higher diversity of predators (Arias, Davey, et al., 2020; Kikuchi et al., 2016). Similarly, a

lower frequency of mimic species at the community level can confer a higher protection of a particular aposematic signal (Finkbeiner et al., 2018). Furthermore, in recent years there is accumulating evidence for an important interaction between mimicry and sexual selection shaping warning signal evolution (Finkbeiner et al., 2014; Rojas et al., 2018). Our study cannot provide further depth into the evolutionary relevance of such ecological variables in this system. Nonetheless, the wide distribution of species within the 'clear wing complex' system in Neotropical habitats and the expectable variation of community structure among them encompass exciting avenues of future research in signal evolutionary patterns.

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### AUTHORS' CONTRIBUTIONS

A.C.-L. and D.O. conceived the experiment ideas and design the methodology; A.C.-L., J.E.V. and D.O. made the artificial replicas; all authors participated in collecting data for some or all of the experiments; A.C.-L. and D.O. analysed the data and lead the writing. All authors contributed critically to the drafts, gave final approval for publication and declare no conflict of interests.

### DATA AVAILABILITY STATEMENT

The raw data for the predation experiment, the reflectance and transmittance spectral curves and the dimensions of the cuticular wax rods are accessible from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.n2z34tmvk> (Outomuro et al., 2020).

### ORCID

Alberto Corral-Lopez  <https://orcid.org/0000-0001-7784-0209>

Javier Edo Varg  <https://orcid.org/0000-0002-7895-4563>

Emilio Realpe  <https://orcid.org/0000-0002-7024-2879>

David Outomuro  <https://orcid.org/0000-0002-1296-7273>

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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