Why has transparency evolved in aposematic butterflies? Insights from the largest radiation of aposematic butterflies, the Ithomiini

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Supplementary Information

Methods

Optical measurements, transparency and detectability

All measurements of transmittance and reflectance were done using a spectrometer (Starline Avaspec-2048 L, Avantes), fiber optics (FC-UV200-2-1.5 x 40, Avantes) and a deuterium halogen lamp (Avalight DHS, Avantes) emitting in the 300-700 nm range, which covers the entire spectrum visible to birds, assumed to be the main predators. For transmittance, fibers were separated and aligned, and measurements were taken relative to a 'white' reference (no sample) and a 'dark' reference (light source turned off). For reflectance, fibers were gathered into one probe, and measurements were taken perpendicular to the sample, i.e. in a specular configuration, relative to a white reference (Spectralon WS2, Avantes) and a dark reference (light source turned off). All measurements were taken 2mm from the sample.

For 18 of the transparent species, measures of transmittance were taken for two individuals of each species so as to assess repeatability. Transmittance, which is a measure of the average amount of transparency (achromatic component), was computed as the average proportion of light

transmitted over the 300-700 nm range and was found to be repeatable within species (R=91.1%, p<0.0001). Moreover, the chroma, which is a measure of the shape (chromatic component) of the spectrum and calculated as the difference between the minimal and maximal transmittance divided by the average transmittance, was also found to be repeatable within species (R=75.1%, p<0.0001). Repeatability of common chromatic and achromatic descriptors (B2 and S8 in [1]) justifies the use of a single individual as intraspecific variation is minimal and less than that observed between transparent species, and, *a fortiori*, between transparent and opaque species. As such, one individual per species was used in subsequent analyses, except for those species for which two individuals were measured and for which we used the mean of both individuals.

For the discriminability model, we modelled both vision systems found in birds, i.e. the UVS vision and the VS vision. For UVS vision, we used the spectral data from the blue tit (*Cyanistes caeruleus)* and relative cone densities of 1:1.9:2.7:2.7 for UVS:S:M:L [2]. For VS vision, we used the spectral data from the shearwater (*Puffinus pacificus*) [3] with relative cone densities of 1:0.7:1:1.4 for VS:S:M:L [4]. For both vision types, Weber fraction was assumed to be 0.1 for chromatic vision [5, 6] and 0.2 for achromatic vision (average of the two species studied in [7]). The blue tit and shearwater were used to model UVS and VS vision type respectively, as they possess a peak in the very short wavelength range similar to that of most other species (i.e. they are good candidate representatives of both these vision types). Moreover, they are amongst the very few species for which other parameters needed to accurately model bird vision, such as relative cone densities, oil droplet transmission spectra and ocular media transmission spectra, are known [2, 3, 8].

Transparent areas reflect more in specular configurations than in diffuse configurations (the collection angle is different from the incidence angle), while opaque pigmented areas appear

matt, i.e. reflect similar amounts of light in all directions (diffuse). With the measurement configuration and contrast calculations that were used, we likely overestimate the visual contrast between transparent butterflies and their background, making them more similar to opaque butterflies than what likely occurs in nature. Hence, our measurement methods and calculations of contrast are conservative with respect to the potential trend we are testing (i.e. differences in coloration between transparent and opaque butterflies).

Experiments measuring unpalatability using domestic chicks

Domestic chicks (*Gallus domesticus*) used as model predators were obtained from a commercial hatchery and were housed in mixed sex groups of ca. 14 individuals in an outdoor metal cage measuring 2 x 2 x 1.5 m in Tarapoto (San Martín, Peru). As birds were kept outdoors, the ground consisted of natural dirt and wood chips, they had access to natural sunlight from 6AM to 6PM and they were kept at ambient temperatures in the shade. Water and naturally brown chick starter feed (Purina Avemicyn-A®) were provided ad libitum, except during training and experiments.

Pellets of chick feed colored either orange or green were used as prey. Experimental pellets were made by mixing 2.5 g of starter chick feed, ca. 1 g of food coloring or until desired colour was obtained (powder mix, Industria Lucerico SAC), 100 ml of tap water, and 1 g of freshly killed butterflies (equal to ca. 7-20 individuals, depending on the species, of equal sex ratio) mixed into the paste. Whole butterflies were mixed directly into the chick feed so as to remove the natural visual cue (i.e. the butterfly's color pattern) and test the chicks' responses to taste only, thereby comparing the level of averseness between butterfly species. Control pellets consisted of 3.5 g of starter chick feed, water and ca. 1 g of food coloring. Mixtures were left to air dry, broken into small pellets (of ca. 1 mm^3 and ca. 20 mg) and then sieved to ensure that they were all of similar

size. A total of six chicks were tested for each butterfly species, and the chicks were never reused, such as, for example, to test another butterfly species.

Birds had no prior experience of unpalatable prey and were initially trained to eat the dyed chicken feed in a clear plastic tray placed on the bottom of the outdoor cage. No food deprivation was necessary at that time as they readily ate the colored chick feed. When chicks were ca. 10 days old, training continued for three days in the experimental set-up, which consisted of a metal chicken wire mesh cage partitioned into two sections measuring 45 x 35 x 50 cm each with a plastic bottom for easy cleaning, so as to habituate them to it. Each training period was preceded by a maximum of 2 h of food deprivation. Initially, chicks were placed either in pairs or small groups and allowed to forage from piles of both green and orange palatable pellets presented in a transparent tray with shallow wells. The number of pellets presented in the wells was gradually reduced and at the end of training and during the experiments a plastic tray with 40 pellets was presented. Half of the pellets (i.e. 20 pellets) were of each color, and pellets were placed singly on the tray in alternating colors. All chicks ate readily in the arena at the end of the training and all birds were familiar with the experimental set-up.

Experimental birds were always paired with a “buddy” in the adjacent cage partition so as to minimize stress. Birds had visual, acoustic and even some physical contact through the mesh wire. These buddy chicks did not have access to food during the experiments, so as to avoid distracting the experimental chick, but they were not food deprived prior to use. Also, chicks that were used as buddies were not later used for experiments so as to avoid having chicks with a learned bias. Both experimental birds and their buddies always had ad libitum access to water throughout training and experiments.

Chicks were first tested with all palatable pellets of both colors, and only those that showed no bias were used. Chicks were then presented with a plastic tray with 40 pellets; 20 pellets were of one color and unpalatable and the other 20 pellets were of the other color and palatable. Pellets were placed singly and the colors were alternated so as to avoid chicks attacking pellets of all the same color as a result of being grouped together. Chicks were allowed to attack (peck or eat) 20 pellets before the food tray was removed. This means that in later trials, chicks that had properly learned to avoid unpalatable pellets could eat all 20 palatable pellets and completely avoid the unpalatable ones. Chicks were tested for a total of 12 successive trials, always with the same color combination. In each session we recorded the number of palatable and unpalatable pellets attacked. By reversing the color association for half the birds that were tested (i.e. for three of the chicks tested with a given butterfly species, we used green experimental pellets, whereas we used orange experimental pellets for the other three chicks, for a total of six chicks tested for each butterfly species), we ensured that there was no color bias (i.e. that the strength of avoidance was not the result of an inherent preference for a given color). At the end of the experiments all chicks were donated to free-range homes.

To assess whether the response of chicks to a given butterfly species differed between individuals, we used the R package *rpt*R to compare the total number of experimental pellets that had been attacked by the six chicks by the end of all 12 trials. Our analysis found them to be significantly similar (Repeatability=44.9%, $p<0.001$), suggesting that different chicks found a given butterfly species similarly unpalatable. Given that individual chicks varied little in how they reacted to a given butterfly species and that this number was sufficient to be statistically valid, and as per the regulations that govern the ethical use of animals in research, which states that only the

minimum number of animals should be used (see: https://www.animalethics.org.au/three-rs/reduction for reference), no additional chicks were used to test each butterfly species.

For the analysis of whether transparent species as a group differed from opaque species as a group, the colorful but semi-transparent species *C. tutia* was classified as opaque because its transparency index is lower than that of transparent species (albeit higher than that of opaque species), perception by predators as calculated by our models is more similar to those of opaque species (i.e. although the overall transparency index is relatively high as a result of the surface area of the wing that is transparent, the individual wing areas that are transparent are effectively almost opaque; refer to figure S1 and Table 1), and it belongs to a mimicry ring formed by typically opaque species.

Figures

Figure S1. Photographs of the Ithomiini taxa (N=33 species) used for optical measurements presented, from top left to bottom right, in decreasing order of transparency. The left hand side of

each photo shows the dorsal side of the wings against a dark background so as to highlight transparency, and the right hand side shows the ventral side of the wings against a white background so as to highlight colour patterns.

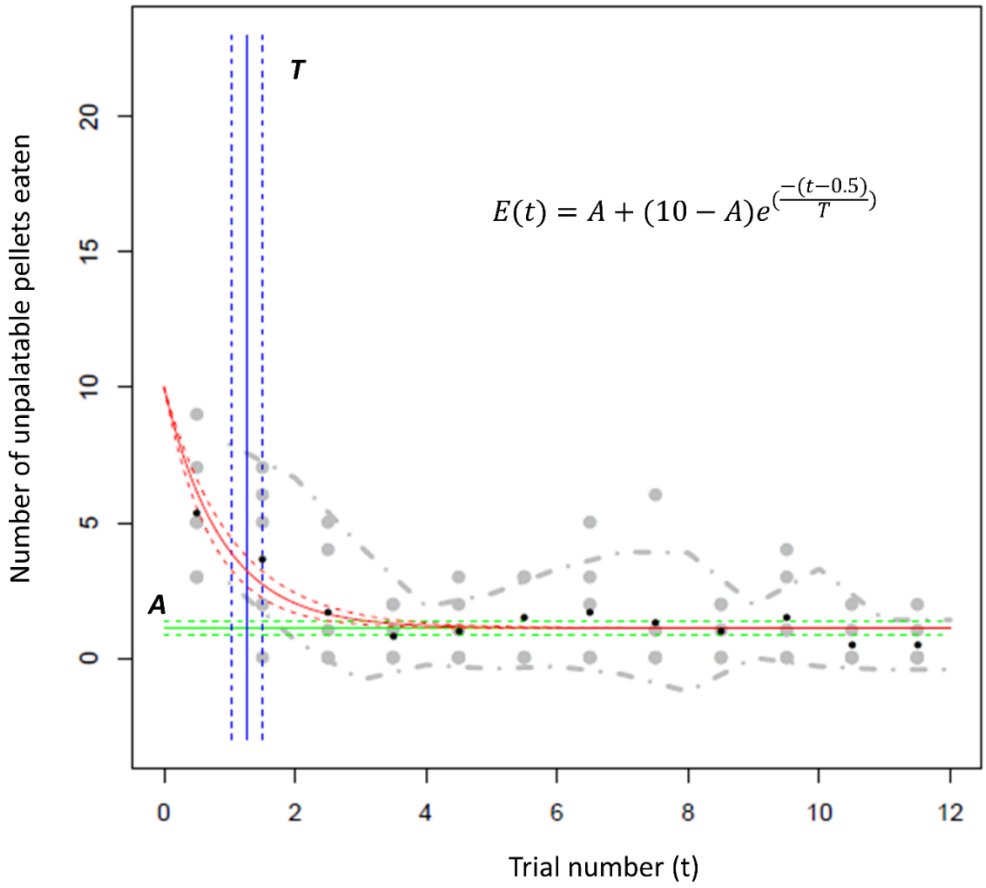


Figure S2. Exponential decrease model fitted to the behavioural experiments with chicks. The y-intercept is set to 10 (*A + (10-A)*) at the beginning of the experiment to account for the fact that chicks were feeding on both colours without bias before the first trial. The acquisition phase is illustrated by the variable *T* and indicates how many trials were necessary to learn to avoid the unpalatable pellets. The asymptote is indicated by *A* and indicates how many unpalatable pellets were still eaten at the end of the experiment.

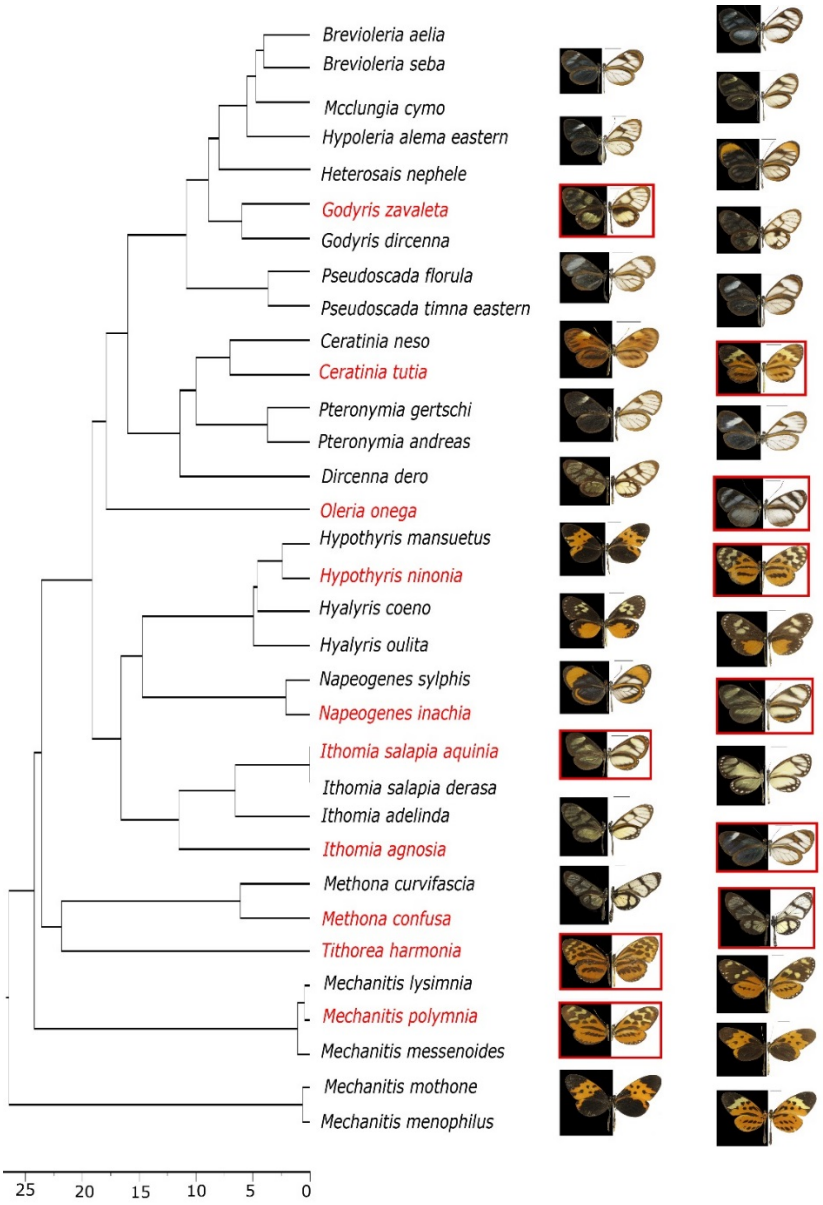
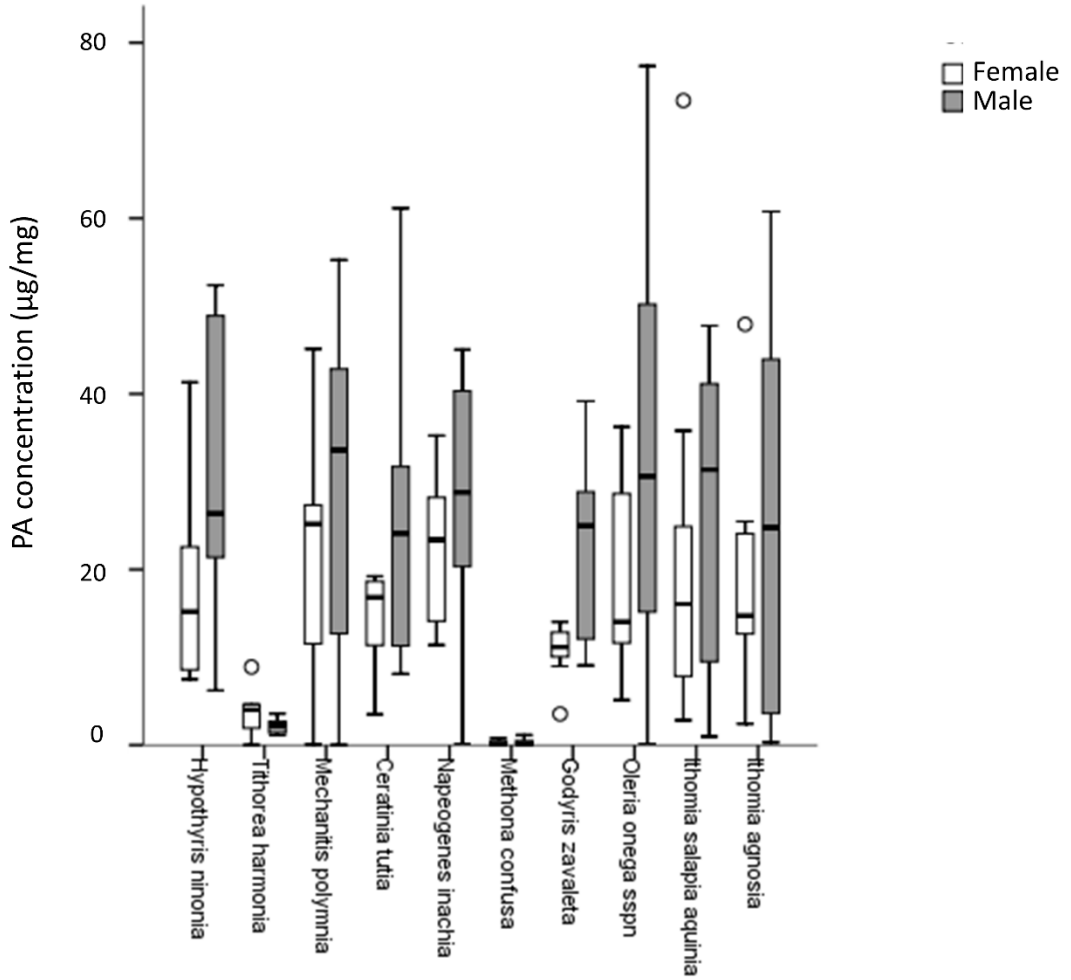


Figure S3. Phylogeny of the taxa (N=33 species) used in this study, which span all major clades of Ithomiini, adapted from [9]. The axis at the bottom represents time, in millions of years. Pictures of the different species are shown on the right of the figure in the same order as in the phylogeny. The left hand side of each photo shows the dorsal side of the wings against a dark background so as to highlight transparency, and the right hand side shows the ventral side of the wings against a white background so as to highlight colour patterns. Taxa highlighted in red (N=10) are those that were used to quantify toxicity and unpalatability.



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149 **Figure S4.** Quantification (µg/mg) of PA content for females (white) and males (grey) of the 10
150 Ithomiini taxa tested. Species are in order of least transparent (on the left) to most transparent (on
151 the right).

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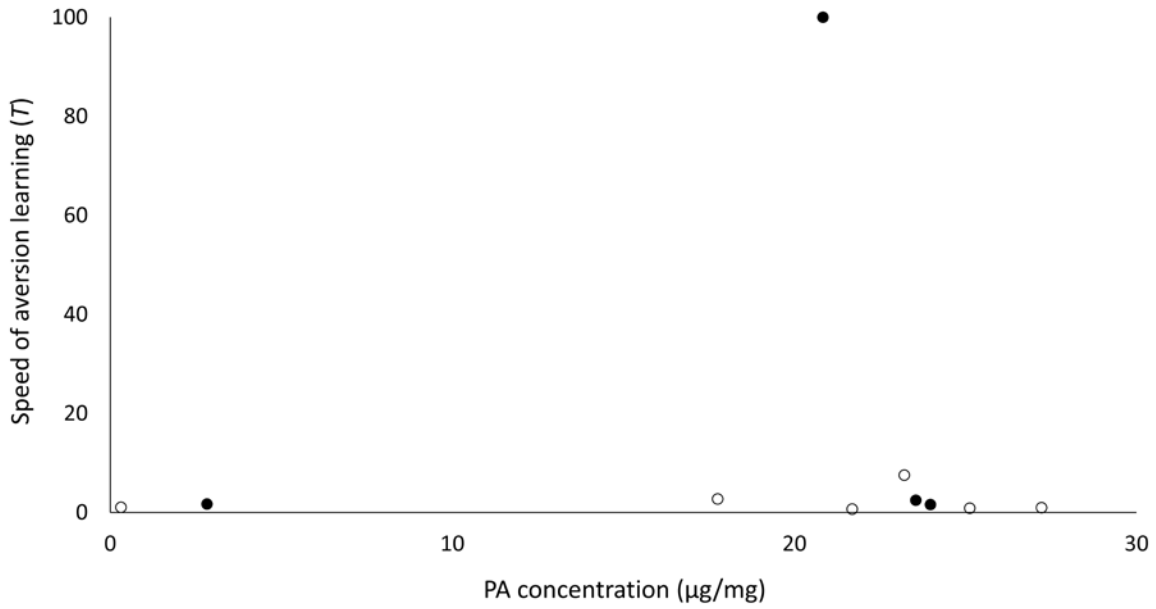
a)



b)

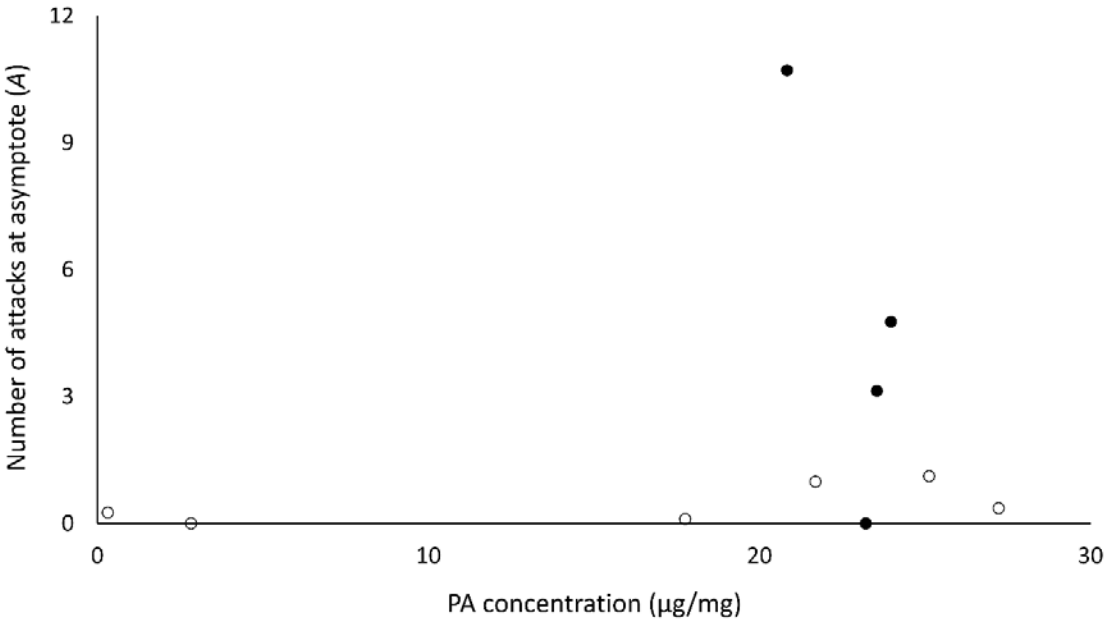


Figure S5. Relationship between unpalatability of Ithomiini butterflies, shown as a) the speed of aversion learning (*T*) and b) the number of attacks at the end of the experiment (*A*), both of which increase with increasing palatability, and the PA content (µg) per mg of butterfly tissue.

Transparent species are shown as white circles and conspicuous/non-transparent species are shown as dark circles.

Table S1. The number of males and females of each Ithomiini taxon used for optical measurements and PA quantification. * indicates species for which two individuals were used to test the repeatability of the optical measurements and for which the mean of both individuals was used in subsequent analyses.

		Optical measurements	PA quantification
*Brevioleria aelia**	Male		
	Female	1	
*Brevioleria seba**	Male		
	Female	1	
Ceratinia neso	Male		
	Female	1	
Ceratinia tutia	Male		9
	Female	1	7
*Dircenna dero**	Male		
	Female	1	
*Godyris dircenna**	Male		
	Female	1	
Godyris zavaleta	Male		9
	Female	1	7
*Heterosais nephele**	Male		
	Female	1	
Hyalyris coeno	Male		
	Female	1	
Hyalyris oulita	Male		
	Female	1	
*Hypoleria alema eastern**	Male		
	Female	1	
Hypothyris mansuetus	Male		
	Female	1	
Hypothyris ninonia	Male		8
	Female	1	10
*Ithomia adelinda**	Male		
	Female	1	
*Ithomia agnosia**	Male		7
	Female	1	10
*Ithomia salapia aquinia**	Male		6
	Female	1	11
*Ithomia salapia deresa**	Male	1	

	Female		
Mechanitis lysimnia	Male		
	Female	1	
Mechanitis messenoides	Male	1	
	Female		
Mechanitis polymnia	Male	1	7
	Female		11
Melinaea menophilus	Male		
	Female	1	
Melinaea mothone	Male	1	
	Female		
*Mcclungia cymo**	Male		
	Female	1	
*Methona confusa**	Male		7
	Female	1	3
*Methona curvifascia**	Male		
	Female	1	
Napeogenes inachia	Male		7
	Female	1	8
Napeogenes sylphis	Male		
	Female	1	
*Oleria onega**	Male		10
	Female	1	8
*Pseudoscada florula**	Male		
	Female	1	
*Pseudoscada timna eastern**	Male		
	Female	1	
*Pteronymia andreas**	Male		
	Female	1	
*Pteronymia gertschi**	Male		
	Female	1	
Tithorea harmonia	Male		10
	Female	1	8

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Table S2. Average PA content (amount free bases and N-oxides per unit of butterfly weight) in relation to a) speed of avoidance learning *T* and b) number of attacks sustained by educated predators *A*. Relationships were assessed by performing phylogenetic regressions of *T* or *A* on PA content, accounting for the phylogenetic signal of the residuals (λ).

a)

PA content (µg/mg)	λ	model *p*-value	adjusted R^2	*F*-stat	*df*	intercept estimate	coefficient estimate
free bases	<0.001	0.8	-0.12	0.07	8	6.27	0.31
N-oxides	<0.001	0.89	-0.12	0.02	8	8.78	0.18

b)

PA content (µg/mg)	λ	model *p*-value	adjusted R^2	*F*-stat	*df*	intercept estimate	coefficient estimate
free bases	<0.001	0.48	-0.05	0.55	8	0.43	0.09
N-oxides	<0.001	0.52	-0.06	0.46	8	0.57	0.09

Table S3. Butterfly average PA content of - a) amount free bases and b) N-oxides, per unit of butterfly weight - in relation to average detectability by bird predators for both UVS and VS vision, in both forest shade and large gaps, and for chromatic and achromatic contrasts. Relationships were assessed by performing phylogenetic regressions of PA content on detectability, accounting for the phylogenetic signal of the residuals (λ).

a)

Bird vision	Light environment	chromatic/ achromatic	λ	model *p*-value	adjusted R^2	*F*-stat	*df*	intercept estimate	coefficient estimate
UVS	forest shade	chromatic	1	0.62	-0.09	0.26	8	8.93	1.39
UVS	forest shade	achromatic	1	0.73	-0.11	0.12	8	12.10	2.58
VS	forest shade	chromatic	1	0.62	-0.09	0.26	8	8.91	1.41
VS	forest shade	achromatic	1	0.82	-0.12	0.05	8	13.63	1.79
UVS	large gap	chromatic	1	0.59	-0.08	0.31	8	8.47	0.88
UVS	large gap	achromatic	1	0.72	-0.11	0.14	8	11.98	0.79
VS	large gap	chromatic	1	0.59	-0.08	0.31	8	8.44	0.89
VS	large gap	achromatic	1	0.81	-0.12	0.06	8	13.57	0.51

b)

Bird vision	Light environment	chromatic/ achromatic	λ	model *p*-value	adjusted R^2	*F*-stat	*df*	intercept estimate	coefficient estimate
UVS	forest shade	chromatic	1	0.55	-0.07	0.40	8	7.11	1.67
UVS	forest shade	achromatic	1	0.69	-0.10	0.17	8	11.15	2.94
VS	forest shade	chromatic	1	0.54	-0.07	0.40	8	7.09	1.68
VS	forest shade	achromatic	1	0.78	-0.11	0.08	8	12.63	2.23
UVS	large gap	chromatic	1	0.52	-0.06	0.46	8	6.69	1.04
UVS	large gap	achromatic	1	0.67	-0.10	0.19	8	11.02	0.90
VS	large gap	chromatic	1	0.52	-0.06	0.46	8	6.66	1.05
VS	large gap	achromatic	1	0.77	-0.11	0.09	8	12.56	0.64

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