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

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

3

4 Abbreviated title: Transparency in aposematic butterflies

5

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26

27 **Abstract.** Defended species are often conspicuous and this is thought to be an honest signal of
28 defences, i.e. more toxic prey are more conspicuous. Neotropical butterflies of the large Ithomiini
29 tribe numerically dominate communities of chemically-defended butterflies and may thus drive
30 the evolution of mimetic warning patterns. Although many species are brightly coloured, most are
31 transparent to some degree. The evolution of transparency from a warningly coloured ancestor is
32 puzzling as it is generally assumed to be involved in concealment. Here we show that transparent
33 Ithomiini species are indeed less detectable by avian predators (i.e. concealment). Surprisingly,
34 transparent species are not any less unpalatable, and may in fact be more unpalatable than opaque
35 species, the latter spanning a larger range of unpalatability. We put forth various hypotheses to
36 explain the evolution of weak aposematic signals in these butterflies and other cryptic defended
37 prey. Our study is an important step in determining the selective pressures and constraints that
38 regulate the interaction between conspicuousness and unpalatability.

39

40 **Keywords:** crypsis; conspicuousness; detectability; honest signal; Müllerian mimicry;
41 unpalatability

42

43 **Introduction**

44 Organisms utilize a range of defensive strategies that are effective in reducing the probability of
45 being attacked by predators [1]. These include avoiding detection, such as camouflage or crypsis,
46 or deterring predators from attacking when encountered, such as aposematism, whereby defended

47 prey advertise their unpalatability to predators using conspicuous warning displays [see 2].
48 Aposematism relies on the fact that predators learn to associate certain features that act as warning
49 signals (such as coloration, sounds, odors) with distastefulness upon attacking defended prey.
50 There is evidence that predators learn to avoid unpalatable prey more quickly and remember the
51 association between the signal and unpalatability for longer and make fewer recognition errors if
52 prey are more conspicuous [3-5]. The benefits of being conspicuous are also thought to include
53 producing stronger neophobic avoidance by naïve predators, a greater reluctance to attack and a
54 lower probability of killing an aposematic prey in an attack as predators may engage in cautious
55 sampling and/or taste rejection of unpalatable prey [6, 7].

56 Hence, defended species often have conspicuous signals that warn potential predators of
57 these defenses. Because predators that attack aposematic individuals learn to avoid prey harboring
58 a specific signal, aposematic signals are under positive frequency-dependent selection [8, 9],
59 whereby the efficiency of a signal increases with abundance. Unpalatable species exposed to the
60 same suite of predators therefore tend to converge on the same warning signals because these
61 individuals effectively share the cost of educating predators, and this is known as Müllerian
62 mimicry [10].

63 Aposematism and Müllerian mimicry occur in a range of organisms, but it has been
64 especially well studied in butterflies[11]. The Neotropical butterfly tribe Ithomiini (Nymphalidae:
65 Danainae) comprises ca. 393 species and 53 genera, and is the largest known radiation of mimetic
66 butterflies. Ithomiini numerically dominate forest butterfly communities and are thought to drive
67 convergence in wing color pattern in multiple mimicry rings [12, 13], whereby co-occurring
68 aposematic species, of both Ithomiini and other Lepidoptera lineages, converge in their aposematic
69 signal. Although several species are brightly colored, over 80% of Ithomiini have more subdued

70 color patterns as a result of transparency [13, 14], and as such, the tribe is commonly known as
71 clearwing butterflies. Transparency is likely a derived state, since many basal Ithomiini lineages
72 and most species of the sister clade Danaini are conspicuously colored and not transparent.

73 Nevertheless, all species of Ithomiini, even those that are transparent, exhibit distinct and
74 recognizable color patterns and, like other unpalatable butterflies, are similarly colored on both
75 sides of their wings and are characterized by slow flight (often described as part of a chemical
76 defense syndrome). Some species have been shown to be highly unpalatable to both vertebrate and
77 invertebrate predators [15-17] as a result of the pyrrolizidine alkaloids (PAs) they contain. Like
78 Danaini butterflies (sister-group to the Ithomiini) and arctiid moths, some basal Ithomiini species
79 sequester PAs from larval food plants (Apocynaceae), whereas other Ithomiini that have shifted to
80 Solanaceae or Gesneriaceae as hostplants obtain PA compounds as adults by feeding on flowers
81 of Asteraceae and Boraginaceae [18-20].

82 As such, the evolution of transparency in Ithomiini butterflies is very puzzling.
83 Transparency is generally assumed to be involved in concealment, but this has mostly been studied
84 in aquatic systems where it is abundant [21, 22]. If transparency has indeed evolved as a result of
85 selection for concealment, these butterflies may benefit from both crypsis and aposematism [23-
86 25]. However, because conspicuousness of unprofitable prey is thought to act as an honest
87 indicator of toxicity (see [26-29] but see also [30, 31]) and predators have been shown to
88 preferentially avoid brighter prey [3], unprofitable species that are inconspicuous are generally
89 expected to possess weaker defenses.

90 Here we test the hypotheses that transparent Ithomiini species are both (1) less detectable
91 by predators and (2) less unpalatable as a result. We estimate detectability by natural predators
92 (i.e. birds) of 33 species of Ithomiini butterflies spanning a range of transparency, from fully

93 opaque to largely transparent and unpigmented, by measuring transmittance and reflectance of the
94 wings, and using models of avian vision. We then assess the unpalatability of 10 species by
95 measuring the PA content and by recording taste-rejection of avian predators (domestic chicks).

96

97 **Methods**

98 **Specimens used**

99 Butterflies were collected in north-eastern Peru (San Martín department) in 2015-2017. Collection
100 sites included forest habitats surrounding Tarapoto (Rio Shilcayo basin: 6°27'30"S 76°21'00"W
101 alt 460 m & the Tunel ridge: 6° 27'11"S 76°17'11"W alt 1090 m), Moyobamba (6° 04'34"S
102 76°57'27"W alt 1130 m), and on Pongo-Baranquita road (6°17'53"S 76°14'38"W alt 200 m).
103 Specimens (see Table S1 for a list of species and number of individuals used) collected for optical
104 (N=33 species; see Figure S1 for photographs of the taxa used) and chemical analysis (N=10
105 species) were preserved dry in envelopes until use. Specimens to be used to measure unpalatability
106 using chicks (N=10 species) were kept in ambient conditions in 2 x 4 x 2 m outdoor insectaries
107 until use. Butterflies were used no more than a few days after capture so as to ensure that they
108 were fresh and reflected naturally occurring toxin content.

109

110 **Optical measurements, transparency and detectability**

111 To assess the relationship between detectability and transparency, optical measurements were
112 performed on 33 Ithomiini species. Although none of the species harbor obvious sexual
113 dimorphism in wing pattern, all individuals used were female when possible so as to avoid any
114 sex-related differences (see Table S1 for details). Transparent species typically possess large

115 transparent areas that can harbor different colors (e.g., yellow, orange, white or none), in addition
116 to opaque areas of varying sizes and colors (Figure S1).

117 Using spectrometry (see Supplementary Methods for details), the transmittance spectra of
118 the transparent areas and the transmittance and reflectance spectra of the opaque areas were
119 measured. The average of the measurements for transmittance (five on the forewing and five on
120 the hindwing) taken in different transparent areas of the wing was used to assess transmittance of
121 transparent patches and account for the heterogeneous properties across transparent areas.
122 Differences between individuals of the same species was found to be minimal and less than that
123 found between species (see Supplementary Methods for details). Therefore, one individual per
124 species was used in subsequent analyses, except for those species for which two individuals were
125 measured and for which we used the mean of both individuals. For each species a transparency
126 index was computed as the average of the transmittance of transparent and opaque areas in the
127 fore- and hindwing weighted by the proportion of surface occupied by those areas. This index,
128 which can vary between 0 (fully opaque) and 100 (fully transparent), therefore takes into account
129 both the proportion of transparent patches in the wing, and the degree of transparency of those
130 patches. Butterfly size was not taken into account in this index.

131 The brightness and color contrast of butterflies against a background of vegetation as
132 perceived by birds was quantified using visual modelling and Vorobyev & Osorio's
133 discriminability model [32]. Given that spectral sensitivities of the avian predator community in
134 this study system are unknown, calculations were based on the two main vision systems found in
135 birds: ultraviolet sensitive (UVS) and violet-sensitive (VS) vision (see details of model parameters
136 in SI). Light conditions used for the vision models simulated ambient light conditions in forest
137 shade, common in rainforest understory, and the reflectance spectrum of an average tropical leaf

138 as a visual background[33]. For transparent areas of the wing, the ambient light was assumed to
139 be transmitted by the wing, reflected by the leaf and transmitted again by the wing. For opaque
140 areas, the ambient light was assumed to only be reflected by the wing before reaching the observer.
141 For the semi-transparent species *Ceratinia tutia* and *C. neso*, measures of reflectance rather than
142 transmittance were used because these species had very low transmittance in any given transparent
143 areas. The color and brightness contrast of the wings were obtained by averaging the contrast
144 measured for each colorful or transparent area weighted by the surface area. All computations were
145 done using the Pavo R package [34].

146 Because species are not independent due to shared ancestry, the relationship between
147 transparency and detectability was assessed by performing phylogenetic regressions of our
148 detectability estimates against the transparency index using the R package *caper* [35]. We
149 extracted the phylogeny of our 33 species from the Ithomiini phylogeny [36], which contains 340
150 species, including all those used in this study. For each estimate of detectability, the phylogenetic
151 signal of the residuals of the regression (Pagel's lambda) was estimated and the phylogeny was
152 rescaled accordingly, such that only the amount of phylogenetic signal in the actual data was
153 accounted for [37].

154

155 **Experiments measuring unpalatability using domestic chicks**

156 Domestic chicks (*Gallus domesticus*) of ca. 10 days old were used as model predators (see
157 Supplementary Methods for further details of the methodology described below; see also [38-40])
158 to test the unpalatability of 10 different transparent or conspicuously colored Ithomiini butterfly
159 species (see Figure 1).

160 Prey consisted of orange or green colored pellets of chick feed, either with or without
161 freshly killed butterflies mixed into the paste (experimental vs control pellets; see Supplementary
162 Methods for details). A total of six chicks were tested for each butterfly species. Birds had no prior
163 experience of unpalatable prey and were initially trained to eat the dyed chick feed. Chicks were
164 tested with all palatable pellets of both colors before the start of the experiment, and only those
165 that showed no bias were used. Chicks were then presented with a plastic tray containing 20 pellets
166 of each color, presented alternatingly, for a total of 40 pellets (i.e. the chicks were given a choice-
167 test with 40 pellets, half of which were of one color and palatable, and the other half another color
168 and unpalatable). Chicks were allowed to attack (peck or eat) 20 pellets before the food tray was
169 removed. These chicks were then tested for a total of 12 successive trials, always with the same
170 color combination. By reversing the color association for half the birds that were tested (i.e. three
171 chicks were tested with green experimental pellets and three chicks were tested with orange
172 experimental pellets, for a total of six chicks tested for each butterfly species), we ensured that
173 there was no color bias in the pellets that were preferentially attacked. In each session the number
174 of palatable and unpalatable pellets attacked were recorded. Intra-individual variation in the
175 response of chicks to a given butterfly species was found to be minimal (see Supplementary
176 Methods).

177 To evaluate unpalatability of the different butterfly species, the following exponential
178 decrease model was fitted to the data pooled for all six chicks (see Figure S2) using the nls function
179 in R:

$$180 \quad E(t) = A + (10 - A)e^{\left(\frac{-(t-0.5)}{T}\right)}$$

181 where t is the trial number, $E(t)$ the number of experimental pellets eaten at trial t , and A and T are
182 constants. This is because the shape of avoidance learning curves, whereby predators reduce their

183 attack rates on defended prey over a series of test sessions, has been shown to occur in two phases
184 [41]. The first is an acquisition phase, which shows the speed with which a predator learns to
185 associate the warning signal with the defense, and is depicted by the half-life of the exponential
186 decrease model. This is illustrated in the above equation by the variable T , and the higher the value,
187 the more trials are necessary for the chicks to learn to avoid the unpalatable pellets. The second is
188 an asymptotic phase, illustrated by the variable A , where predators appear to have learned the
189 association and do not change their attack rate. A and T therefore decrease with increasing
190 unpalatability. Fully palatable pellets should lead to infinite values of T , which cannot be analyzed
191 subsequently, and we therefore arbitrarily set the upper limit to 100.

192 In the above equation, discretization of the time variable t , which is the number of trials,
193 was accomplished by adding an offset of -0.5. This was done because avoidance learning does not
194 take place at the end of trials, but during the trials. Consequently, the number of pellets recorded
195 as attacked by the chicks during a given trial t actually occurs over time between $t-1$ and t . The
196 start of each trial can therefore be set to $t-0.5$ if the attack rate is assumed to be linear between
197 these two points. Changes in attack rates are especially fast during the first few trials and the model
198 parameters are therefore very sensitive to the offset. Finally, the rate of attack by chicks was set to
199 10 before the start of the first trial so as to indicate that chicks were feeding on both colors without
200 bias, as was tested at the start of each experiment. In the equation, this is done by setting the y-
201 intercept ($A+(10-A)$) to 10 before the start of the first trial (i.e. for $E(0.5)$).

202 To test the relationship between unpalatability (estimated by both the above variables A
203 and T) and estimates of detectability, a phylogenetic regression of detectability on unpalatability
204 was performed as detailed in the previous section (correcting for the exact amount of phylogenetic
205 signal), using the R package *caper* [35]. In addition, we tested whether transparent species as a

206 group (transparency index >20%; see Figure 1) differed from opaque species as a group in their
207 unpalatability by performing a phylogenetic ANOVA using the R package *phytools* [42], and
208 implementing 1000 simulations.

209

210 **Chemical quantification of pyrrolizidine alkaloids (PAs)**

211 PA content (free bases and *N*-oxides) of the 10 species tested with the chick behavioral assays
212 above was quantitatively analyzed by colorimetric assay [43]. Individual butterflies (body and
213 wings) were weighed and pulverized using liquid nitrogen, and placed into vials containing 2 mL
214 MeOH. These vials were then stirred continuously for 3 h at room temperature. After filtration
215 using glass pipettes and cotton, 100 μ L of the liquid extract was mixed with 0.5 mL of oxidation
216 reagent (5 mg of Na₄P₂O₇ in 1 mL of H₂O₂ 30%, diluted 1:200 with MeOH) and then heated in
217 open test tubes at 100°C for 25 min, until complete evaporation of the solution. The dried samples
218 were left to cool to room temperature, and placed overnight in a desiccator so as to remove any
219 traces of residual water. Anhydrous isoamyl acetate (1.0 mL) and acetic anhydride (0.1 mL) were
220 then added to the tubes, covered so as to avoid any water contamination, and placed at 100°C for
221 2 min. Samples were again cooled to room temperature and treated with 1.0 mL of freshly prepared
222 modified Ehrlich reagent (8 mL of 20% methanolic boron trifluoride (BF₃), diluted in 72 mL of
223 absolute EtOH and 1.4 g dimethylaminobenzaldehyde). Tubes were sealed and heated at 60°C for
224 5 min and cooled to room temperature for 23 minutes. Samples were assayed photometrically at
225 561 nm against a blank solution (a mixture of isoamyl acetate, acetic anhydride and modified
226 Ehrlich reagent). An initial solution of 5 mg of crotaline and 5 mg of retrorsine dissolved in 10 mL
227 of MeOH (initial concentration: 1 mg/mL) was used to produce six different dilutions in MeOH,
228 which were then used to produce a reference curve.

229 Average PA content (per unit of weight for each butterfly) was compared between species
230 and between sexes using a non-parametric Kruskal-Wallis test done using SPSS (SPSS Inc.,
231 Chicago, Illinois). A phylogenetic regression (as above) was done to test the correlation between
232 PA content and unpalatability, using both the variables *A* and *T* calculated from the above
233 avoidance curves. Finally, the relationship between PA content and 1) detectability and 2)
234 transparency were assessed by performing phylogenetic regressions and a phylogenetic ANOVA
235 respectively, as described in the previous section.

236 Table 1 shows average transparency, detectability, unpalatability and toxin content for all
237 10 Ithomiini species tested.

238

239 **Results**

240 **Transparency and detectability**

241 The relationship between transparency (as measured by a transparency index that takes into
242 account the proportion of the different color patches and their respective transparencies) and
243 detectability by predators was assessed by measuring the transmittance (the amount of light that
244 passes through the wings) and the reflectance (the amount of light that is reflected) spectra of
245 wings using spectrometry and models of avian vision. The 33 species used in this study span all
246 major clades of Ithomiini (Figure S3) and a wide range of transparency, ranging from completely
247 opaque, with a transparency index of zero (e.g. *Melinaea mothone*, *Mechanitis messenoides*,
248 *Hypothyris mansuetus*), to highly transparent (e.g. *Heterosais nephele*, *Ithomia agnosia*,
249 *Pteronymia gertschi*) (Figure S1). Detectability (estimated by the chromatic and achromatic
250 contrasts of wing patterns against a green leaf) decreased with increasing butterfly transmittance,

251 regardless of vision type (UVS or VS), type of contrast (chromatic or achromatic), or simulated
252 light conditions (Table 2, Figure 2).

253

254 **Detectability and unpalatability**

255 The unpalatability of 10 of the previous 33 Ithomiini species (Figure 1) that differ in their degree
256 of transparency was tested using domestic chicks. Two facets of unpalatability were assessed in
257 our analyses (Figure S2): the speed of avoidance learning, depicted by variable T , and the number
258 of residual attacks sustained by educated predators, depicted by variable A . This last variable can
259 also be thought of as the strength and durability of avoidance learning. Both A and T decrease with
260 unpalatability (i.e., the more unpalatable a species, the more quickly predators learn to avoid them
261 (T) and the stronger the avoidance over time (A)).

262 The relationship between unpalatability and detectability was first ascertained with a
263 phylogenetic regression. In this analysis, neither the speed of avoidance learning T (Table 3, Figure
264 3a), nor the number of attacks sustained by educated predators A (Table 4, Figure 3b) were
265 significantly associated with detectability of butterflies, across both types of predators, both types
266 of contrasts and both types of simulated light conditions, despite a trend for a positive relationship
267 between chromatic contrast and variable A . However, when transparency was considered as a
268 categorical variable (i.e., largely transparent or opaque), transparent species were found to be
269 significantly more unpalatable when considering the number of attacks sustained by educated
270 predators, as estimated by variable A (transparent: $A=0.469\pm0.468$; opaque: $A=4.653\pm4.496$;
271 phylogenetic ANOVA $F=5.446$, $df=1$, $p=0.044$; Figure 3b), but not the speed of avoidance
272 learning, as estimated by variable T (transparent: $T=2.305\pm2.670$; opaque: $T=26.446\pm49.038$;
273 phylogenetic ANOVA $F=5.446$, $df=1$, $p=0.245$; Figure 3a). A single Ithomiini species, the

274 colorful and weakly transparent *Ceratinia tutia* (considered as opaque, see Supplementary
275 Methods), was found to be fully palatable to chicks.

276

277 **Chemical quantification of pyrrolizidine alkaloids (PAs), and comparison with**
278 **unpalatability and detectability**

279 PA content ($\mu\text{g}/\text{mg}$) of the 10 species tested with the chick behavioral assays was quantitatively
280 analyzed by colorimetric assay and was found to be significantly different between species
281 (Wald=62.877; $df=9$; $p<0.001$; Figure S4) but not between males and females (Wald=2.021; $df=1$;
282 $p=0.155$; Figure S4), although content appears to be more variable within males. Surprisingly, no
283 PAs were detected in any individual of the species *Methona confusa*. PA content was also low for
284 the species *Tithorea harmonia*, known to sequester PAs from their hostplants as larvae [20].

285 Surprisingly, PA content did not predict unpalatability and there was no relationship
286 between PA content and the speed of avoidance learning T (Table S2a; Figure S5a) nor the number
287 of attacks sustained by educated predators A (Table S2b; Figure S5b). For example, *Methona*
288 *confusa*, which contained little to no PAs, were found to be highly unpalatable to chicks, and
289 *Ceratinia tutia*, which possessed average quantities of PAs, were found to be palatable to chicks.

290 There was no significant relationship between PA content and detectability (Table S3).
291 When transparency was considered as a categorical variable (i.e., largely transparent or opaque),
292 there was no significant difference in PA content between groups (free PA content of transparent
293 species= 19.2 ± 9.8 and of opaque species= 17.8 ± 10.1 ; phylogenetic ANOVA $F=0.0496$, $df=1$,
294 $p=0.836$. N-oxides PA content of transparent species= 18.6 ± 9.5 and of opaque species= 17.5 ± 10.0 ;
295 phylogenetic ANOVA $F=0.0378$, $df=1$, $p=0.850$).

296

297 **Discussion**

298 Transparency has likely evolved in clearwing butterflies (Ithomiini) from a brightly colored
299 aposematic ancestor, and this study shows that it has resulted in a significant decrease in
300 detectability by avian predators (i.e. improved concealment). This is consistent with results from
301 four Ithomiini species that showed that transparency decreases detectability for both human
302 observers and blue tits [44]. Surprisingly, we did not find a concurrent decrease in unpalatability
303 with increasing transparency. Despite considerable empirical support that conspicuousness
304 promotes the effectiveness of aposematic signals, and thus that selection should favor conspicuous
305 signals in well-defended individuals, there are many examples of unprofitable species that are
306 rather cryptic [30, 45].

307 Various reasons have been put forward to explain weak aposematic signals. Some studies
308 have shown that in addition to greater conspicuousness, greater toxicity of prey can also increase
309 the speed and efficiency of predator avoidance learning [9, 39], and this could potentially reduce
310 selection on brightly colored visual patterns [30]. Here we found that the most transparent species
311 were all highly unpalatable. In fact, transparent species were found to be significantly more
312 unpalatable than opaque species, the latter spanning a larger range of unpalatability, including one
313 fully palatable species. Indeed, although the speed of avoidance learning (T) was not significantly
314 different for both transparent and conspicuously colored species, the number of residual attacks
315 sustained by educated predators (A) was significantly reduced for transparent species in the
316 absence of a visual cue. This suggests that some aspects of taste, rather than conspicuousness, may
317 improve the memorability of unpalatability in transparent species. However, it should be noted
318 that as a result of the relatively small number of species tested, the difference in unpalatability
319 between transparent and opaque species may not be accurately estimated. This does not refute the

320 findings that all transparent species tested are highly unpalatable, but it may be premature to
321 conclude that transparent species are systematically more unpalatable than conspicuous species.
322 Reduced conspicuousness in aposematic species can be advantageous for several reasons. Some
323 studies have shown that high levels of conspicuousness can potentially lead to aposematic prey
324 experiencing higher attack rates than cryptic prey because conspicuousness increases prey
325 visibility to predators, especially when the prey community is exposed to a large number of naïve
326 predators [46-48]. In addition to naïve predators, educated predators may also, at times, exert
327 significant selection pressures on aposematic prey [49]. This is because chemically-defended prey
328 contain not only toxins, but also nutrients, and such prey may be profitable to attack, for example
329 when alternative prey are scarce [50, 51], or when predators do not have a high toxic load [52, 53].

330 Furthermore, although clearwing butterflies may not be very conspicuous, they are also not
331 entirely cryptic [see 44] as they possess bold and colorful markings on their wings. This is unlikely
332 to be disruptive coloration, as the pattern elements appear to enhance the outline of the wings, and
333 pattern edges have been shown to improve predator avoidance learning [54]. Moreover, Ithomiini
334 engage in mimetic interactions with other unpalatable butterfly species. Because Müllerian
335 mimicry is the result of convergent selection on a warning display, this suggests that the color
336 pattern in this system is both recognized and avoided by predators. The coloration of transparent
337 Ithomiini butterflies, or lack thereof, could therefore have a cryptic function from a distance and
338 prevent the individual from being seen by predators, but also have an aposematic function once
339 the individual has been seen [23-25]. It is also possible that maximum conspicuousness is not
340 important as long as the signal is distinguishable from the undefended and edible prey, which are
341 typically cryptic, and/or that the signal contrasts with the background [28, 55-57]. This explanation
342 has been suggested for the zigzag pattern of European vipers (*Vipera*), which is not very

343 conspicuous, but appears to be a compromise between crypsis and aposematism [58]. As such, a
344 less detectable signal, although potentially less efficient in educating predators, could be beneficial
345 due to its lower detectability, particularly in a situation where predators may kill defended prey.

346 Chemical defenses in most Ithomiini butterflies are believed to be the result of pyrrolizidine
347 alkaloids (PAs) acquired solely pharmacophagously as adults [15], and this would certainly
348 explain the large variation in PA content observed in this study. However, our study suggests that
349 PAs may not be the only chemicals responsible for unpalatability in clearwing butterflies. For
350 example, no PAs were found in *Methona confusa*, despite being strongly unpalatable, and some
351 species may therefore also sequester other chemical compounds from their Solanaceae or
352 Gesneriaceae hostplants. In support of this, larvae of another *Methona* species (*M. themisto*) were
353 found to elicit aversive reaction in chicks, suggesting that they contain chemical defences that are
354 potentially sequestered from their hostplants [59]. In those species that sequester other toxic
355 compounds, collecting PAs as adults may provide additional protection. Similarly, *Ceratinia tutia*
356 was the least unpalatable species despite PA concentrations similar to those of other more
357 unpalatable species, suggesting that PA sources may differ in their ability to cause aversion in
358 predators. Whatever the reason, large variations in PA contents such as those observed in our study
359 suggest that individuals within the population may differ in their level of chemical defenses and
360 that in some species younger butterflies may be more palatable as they would not have
361 accumulated sufficient toxins. This may have important repercussions on predator-prey dynamics
362 as the presence of potentially more palatable individuals (i.e. automimics) has been shown to
363 increase sampling by predators [40, 60].

364 In conclusion, we found that transparent Ithomiini butterflies, or clearwing butterflies, are
365 less detectable than their brightly colored counterparts. Although less conspicuous, transparent

366 clearwing butterflies are not any less unpalatable, and may in fact be more unpalatable than opaque
367 species. It seems likely that the color pattern of transparent Ithomiini butterflies is sufficiently
368 distinguishable from palatable prey to trigger recognition and avoidance learning by predators.
369 The decrease in conspicuousness of transparent wing patterns thus allows butterflies to benefit
370 from both limited detection by predators (crypsis), but also predator deterrence when detected
371 (aposematism). This may be the result of a shift in microhabitat [61], which may alter signal
372 properties or result in differences in the predators that are present or their propensity to attack
373 defended prey. The fact that the transparent species tested were all highly unpalatable suggests
374 that strong unpalatability may decrease selection for conspicuousness and favor the evolution of
375 some forms of crypsis under certain conditions. Strength of unpalatability and potentially some
376 aspects of taste appear to improve memorability in the advent of reduced conspicuousness.
377 Whether this is also true for the other clades of aposematic butterflies with transparent species that
378 engage in mimicry with Ithomiini species (e.g. Pericopina and Diopini) is unknown. Further
379 studies focusing on the selective pressures acting on aposematic signaling will improve our
380 understanding of the factors that regulate the interaction between conspicuousness and
381 unpalatability.

382 **Author contributions**

383 ME designed the study. MM designed the behavioral experiments with the chicks, and MM, CC,
384 MC, LBH and JB performed the experiments in the field and analyzed the data. MM, ME and DG
385 collected specimens for optical and chemical measurements. CD, DG, and CP performed optical
386 measurements and analyses, under the supervision of CA, SB and ME. AM performed chemical
387 measurements, under the supervision of BN, ME and VL. CH performed color pattern imaging
388 and analyses. MM, DG and ME wrote the paper with contributions from all authors.

389

390 **Competing interests.** We have no competing interests.

391

392 **Data accessibility.** Data available from the Dryad Digital Repository:
393 <https://datadryad.org/review?doi=doi:10.5061/dryad.d2h629q> [62]

394

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408

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547

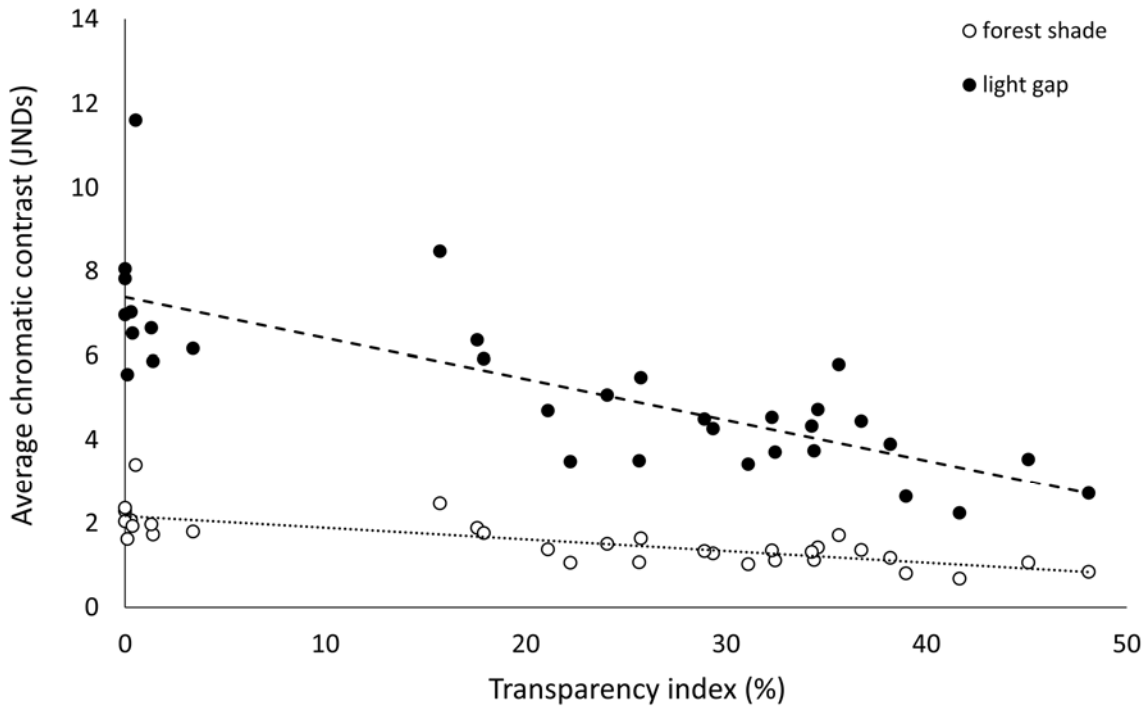
548 **List of Figures**



549

550 **Figure 1.** The 10 taxa of Ithomiini butterflies that were used to quantify toxicity and unpalatability,
551 ranked by decreasing transparency from top left to bottom right. Species on the top row are *Ithomia*
552 *agnosia*, *Ithomia salapia aquinia*, *Oleria onega*, *Godyris zavaleta*, *Methona confusa*. Species on
553 the bottom row are *Napeogenes inachia*, *Ceratinia tutia*, *Mechanitis polymnia*, *Tithorea harmonia*,
554 *Hypothyris ninonia*. The transparent group (transparency index >20%) consisted of the six first
555 species (the entire top row + *Napeogenes inachia*) and the opaque group consisted of the four last
556 species on the bottom right. The left hand side of each photo shows the dorsal side of the wings
557 against a dark background so as to highlight transparency, and the right hand side shows the ventral
558 side of the wings against a white background so as to highlight colour patterns.

559

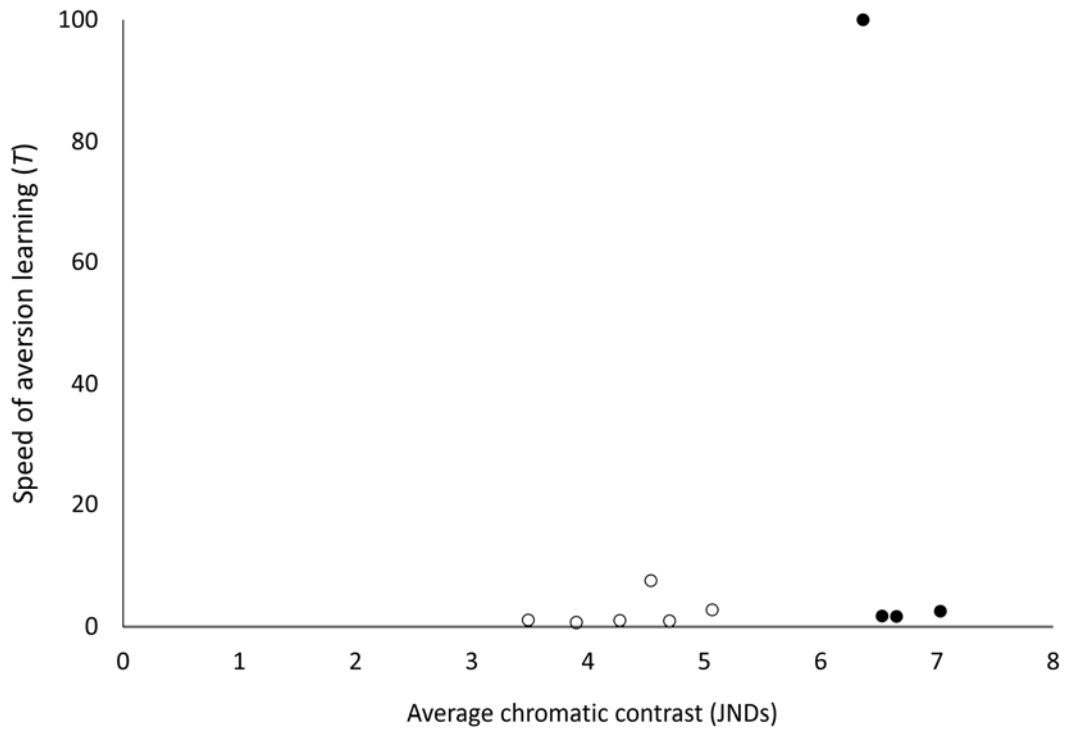


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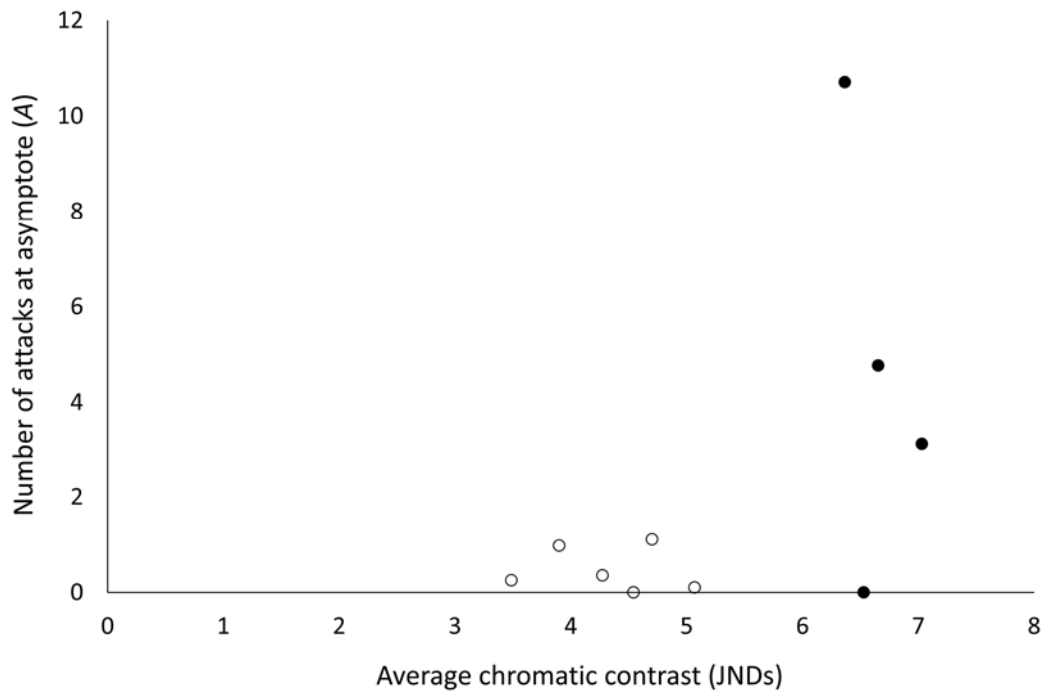
561 **Figure 2.** Relationship between detectability of Ithomiini butterflies by birds (chromatic contrast
 562 in just noticeable difference units [JNDs] for UVS-vision), in forest shade (white circles) and large
 563 gaps (black circles), and butterfly transparency as measured by the transparency index. Lines were
 564 fitted by the method of least squares linear regression for forest shade (dash line: $y=-0.028x+2.170$;
 565 $R^2=0.607$) and large gap (solid line: $y=-0.097x+7.378$; $R^2=0.616$). Results of the phylogenetic
 566 regressions are shown in Table 2.

567

568 **a**



569
570 **b**



571

572 **Figure 3.** Relationship between unpalatability of Ithomiini butterflies, shown as a) the speed of
573 aversion learning (T) and b) the number of attacks by educated predators (A), both of which
574 increase with increasing palatability, and detectability by birds (chromatic contrast in just
575 noticeable difference units for UVS-vision in large gap light conditions). Transparent species are
576 shown as white circles and conspicuous/non-transparent species are shown as dark circles.
577

578 **Table 1.** Average transparency, detectability by avian predators (chromatic contrast in just
579 noticeable difference units [JNDs] for UVS-vision in large gap light conditions), unpalatability,
580 shown as the speed of aversion learning (*T*) and number of attacks by educated predators (i.e.
581 residual attacks *A*), both of which increase with increasing palatability, and PA concentration for
582 the 10 taxa of Ithomiini butterflies that were used. The transparent group consist of species with
583 transparency index >20% (i.e. the six last species). The colorful but semi-transparent species
584 *Ceratinia tutia* was classified as opaque because measures of transmittance were low in any given
585 transparent areas (i.e. although the overall transparency index is relatively high as a result of the
586 surface area of the wing that is transparent, the individual wing areas that are transparent are
587 effectively almost opaque; refer to figure S1)

Species	Transparency index (%)	Average chromatic contrast (JNDs)	Speed of aversion learning (<i>T</i>)	Residual attacks (<i>A</i>)	PA concentration (µg/mg)
<i>Hypothyris ninonia</i>	0.29	7.03	2.47	3.14	23.55
<i>Tithorea harmonia</i>	0.37	6.53	1.70	0.00	2.83
<i>Mechanitis polymnia</i>	1.30	6.65	1.61	4.77	23.98
<i>Ceratinia tutia</i>	17.57	6.37	100.00	10.71	20.83
<i>Napeogenes inachia</i>	21.10	4.70	0.88	1.11	25.13
<i>Methona confusa</i>	22.22	3.49	1.03	0.25	0.32
<i>Godyris zavaleta</i>	24.06	5.07	2.72	0.10	17.76
<i>Oleria onega</i>	29.35	4.27	0.97	0.36	27.23
<i>Ithomia salapia aquinia</i>	32.28	4.54	7.54	0.00	23.22
<i>Ithomia agnosia</i>	38.19	3.90	0.68	0.99	21.69

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589

590 **Table 2.** Butterfly average transparency in relation to average detectability by bird predators for
 591 both UVS and VS vision, in both forest shade and large gaps, and for chromatic and achromatic
 592 contrasts. Relationships were assessed by performing phylogenetic regressions of detectability on
 593 transparency index, accounting for the phylogenetic signal of the residuals (λ).

Bird vision	Light environment	chromatic/achromatic	λ	model <i>p</i> -value	adjusted R ²	<i>F</i> -stat	<i>df</i>	intercept estimate	coefficient estimate
UVS	forest shade	chromatic	0.43	<0.001	0.53	36.17	30	2.23	-0.03
UVS	forest shade	achromatic	<0.001	<0.001	0.80	123.46	30	6.86	-0.08
VS	forest shade	chromatic	0.44	<0.001	0.53	36.23	30	2.068	-0.03
VS	forest shade	achromatic	<0.001	<0.001	0.80	122.49	30	6.83	-0.08
UVS	large gap	chromatic	0.44	<0.001	0.54	37.36	30	7.58	-0.11
UVS	large gap	achromatic	<0.001	<0.001	0.79	117.90	30	11.65	-0.14
VS	large gap	chromatic	0.45	<0.001	0.54	37.36	30	7.50	-0.11
VS	large gap	achromatic	<0.001	<0.001	0.79	116.64	30	11.58	-0.14

594

595

596 **Table 3.** Speed of aversion learning (*T*) in relation to butterfly detectability by bird predators for
 597 both UVS and VS vision, in both forest shade and large gaps, and for chromatic and achromatic
 598 contrast. Relationships were assessed by performing phylogenetic regressions of *T* on detectability,
 599 accounting for the phylogenetic signal of the residuals (λ).

Bird vision	Light environment	chromatic/achromatic	λ	model <i>p</i> -value	adjusted R ²	<i>F</i> -stat	<i>df</i>	intercept estimate	coefficient estimate
UVS	forest shade	chromatic	<0.001	0.38	-0.02	0.86	8	-28.94	26.15
UVS	forest shade	achromatic	<0.001	0.92	-0.12	0.01	8	6.31	1.10
VS	forest shade	chromatic	<0.001	0.46	-0.05	0.60	8	-21.16	23.42
VS	forest shade	achromatic	<0.001	0.92	-0.12	0.009	8	6.49	1.07
UVS	large gap	chromatic	<0.001	0.28	0.04	1.33	8	-48.89	11.34
UVS	large gap	achromatic	<0.001	0.97	-0.12	0.002	8	17.59	-0.62
VS	large gap	chromatic	<0.001	0.43	-0.04	0.68	8	-31.45	8.32
VS	large gap	achromatic	<0.001	0.96	-0.12	0.0039	8	19.05	-0.78

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601

602 **Table 4.** Number of attacks by educated predators (*A*) in relation to detectability for both UVS and
 603 VS vision, in both forest shade and large gaps, and for chromatic and achromatic contrast.
 604 Relationships were assessed by performing phylogenetic regressions of *A* on detectability,
 605 accounting for the phylogenetic signal of the residuals (λ).

Bird vision	Light environment	chromatic/achromatic	λ	model <i>p</i>-value	adjusted R^2	<i>F</i>-stat	<i>df</i>	intercept estimate	coefficient estimate
UVS	forest shade	chromatic	<0.001	0.10	0.21	3.46	8	-5.76	5.06
UVS	forest shade	achromatic	<0.001	0.41	-0.03	0.75	8	-2.96	0.10
VS	forest shade	chromatic	<0.001	0.15	0.15	2.54	8	-4.62	4.78
VS	forest shade	achromatic	<0.001	0.41	-0.03	0.74	8	-2.94	0.10
UVS	large gap	chromatic	<0.001	0.08	0.25	3.98	8	-8.03	1.90
UVS	large gap	achromatic	<0.001	0.87	-0.12	0.03	8	-0.29	0.27
VS	large gap	chromatic	<0.001	0.15	0.14	2.51	8	-6.16	1.59
VS	large gap	achromatic	<0.001	0.88	-0.12	0.02	8	-0.04	0.24

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610 **Supplementary Information**

611 **Methods**

612 **Optical measurements, transparency and detectability**

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

622 For 18 of the transparent species, measures of transmittance were taken for two individuals
623 of each species so as to assess repeatability. Transmittance, which is a measure of the average
624 amount of transparency (achromatic component), was computed as the average proportion of light
625 transmitted over the 300-700 nm range and was found to be repeatable within species ($R=91.1\%$,
626 $p<0.0001$). Moreover, the chroma, which is a measure of the shape (chromatic component) of the
627 spectrum and calculated as the difference between the minimal and maximal transmittance divided
628 by the average transmittance, was also found to be repeatable within species ($R=75.1\%$, $p<0.0001$).
629 Repeatability of common chromatic and achromatic descriptors (B2 and S8 in [1]) justifies the use
630 of a single individual as intraspecific variation is minimal and less than that observed between
631 transparent species, and, *a fortiori*, between transparent and opaque species. As such, one

632 individual per species was used in subsequent analyses, except for those species for which two
633 individuals were measured and for which we used the mean of both individuals.

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

646 Transparent areas reflect more in specular configurations than in diffuse configurations
647 (the collection angle is different from the incidence angle), while opaque pigmented areas appear
648 matt, i.e. reflect similar amounts of light in all directions (diffuse). With the measurement
649 configuration and contrast calculations that were used, we likely overestimate the visual contrast
650 between transparent butterflies and their background, making them more similar to opaque
651 butterflies than what likely occurs in nature. Hence, our measurement methods and calculations of
652 contrast are conservative with respect to the potential trend we are testing (i.e. differences in
653 coloration between transparent and opaque butterflies).

654

655 **Experiments measuring unpalatability using domestic chicks**

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

662 Pellets of chick feed colored either orange or green were used as prey. Experimental pellets
663 were made by mixing 2.5 g of starter chick feed, ca. 1 g of food coloring or until desired colour
664 was obtained (powder mix, Industria Lucerico SAC), 100 ml of tap water, and 1 g of freshly killed
665 butterflies (equal to ca. 7-20 individuals, depending on the species, of equal sex ratio) mixed into
666 the paste. Whole butterflies were mixed directly into the chick feed so as to remove the natural
667 visual cue (i.e. the butterfly's color pattern) and test the chicks' responses to taste only, thereby
668 comparing the level of averseness between butterfly species. Control pellets consisted of 3.5 g of
669 starter chick feed, water and ca. 1 g of food coloring. Mixtures were left to air dry, broken into
670 small pellets (of ca. 1 mm³ and ca. 20 mg) and then sieved to ensure that they were all of similar
671 size. A total of six chicks were tested for each butterfly species, and the chicks were never reused,
672 such as, for example, to test another butterfly species.

673 Birds had no prior experience of unpalatable prey and were initially trained to eat the dyed
674 chicken feed in a clear plastic tray placed on the bottom of the outdoor cage. No food deprivation
675 was necessary at that time as they readily ate the colored chick feed. When chicks were ca. 10 days
676 old, training continued for three days in the experimental set-up, which consisted of a metal
677 chicken wire mesh cage partitioned into two sections measuring 45 x 35 x 50 cm each with a plastic

678 bottom for easy cleaning, so as to habituate them to it. Each training period was preceded by a
679 maximum of 2 h of food deprivation. Initially, chicks were placed either in pairs or small groups
680 and allowed to forage from piles of both green and orange palatable pellets presented in a
681 transparent tray with shallow wells. The number of pellets presented in the wells was gradually
682 reduced and at the end of training and during the experiments a plastic tray with 40 pellets was
683 presented. Half of the pellets (i.e. 20 pellets) were of each color, and pellets were placed singly on
684 the tray in alternating colors. All chicks ate readily in the arena at the end of the training and all
685 birds were familiar with the experimental set-up.

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

693 Chicks were first tested with all palatable pellets of both colors, and only those that showed
694 no bias were used. Chicks were then presented with a plastic tray with 40 pellets; 20 pellets were
695 of one color and unpalatable and the other 20 pellets were of the other color and palatable. Pellets
696 were placed singly and the colors were alternated so as to avoid chicks attacking pellets of all the
697 same color as a result of being grouped together. Chicks were allowed to attack (peck or eat) 20
698 pellets before the food tray was removed. This means that in later trials, chicks that had properly
699 learned to avoid unpalatable pellets could eat all 20 palatable pellets and completely avoid the
700 unpalatable ones. Chicks were tested for a total of 12 successive trials, always with the same color

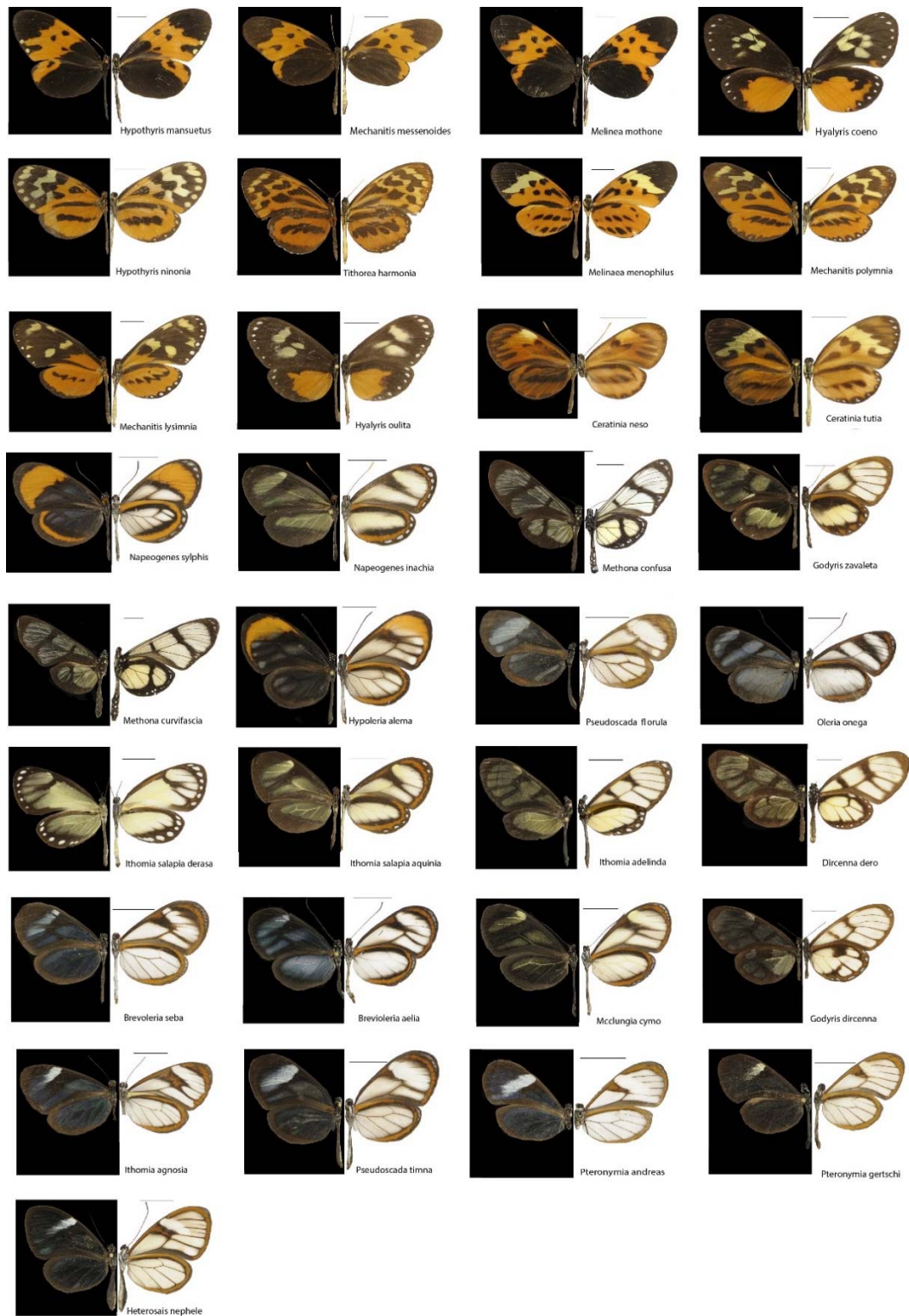
701 combination. In each session we recorded the number of palatable and unpalatable pellets attacked.
702 By reversing the color association for half the birds that were tested (i.e. for three of the chicks
703 tested with a given butterfly species, we used green experimental pellets, whereas we used orange
704 experimental pellets for the other three chicks, for a total of six chicks tested for each butterfly
705 species), we ensured that there was no color bias (i.e. that the strength of avoidance was not the
706 result of an inherent preference for a given color). At the end of the experiments all chicks were
707 donated to free-range homes.

708 To assess whether the response of chicks to a given butterfly species differed between
709 individuals, we used the R package *rptR* to compare the total number of experimental pellets that
710 had been attacked by the six chicks by the end of all 12 trials. Our analysis found them to be
711 significantly similar (Repeatability=44.9%, $p<0.001$), suggesting that different chicks found a
712 given butterfly species similarly unpalatable. Given that individual chicks varied little in how they
713 reacted to a given butterfly species and that this number was sufficient to be statistically valid, and
714 as per the regulations that govern the ethical use of animals in research, which states that only the
715 minimum number of animals should be used (see: [https://www.animaethics.org.au/three-
rs/reduction](https://www.animaethics.org.au/three-
716 rs/reduction) for reference), no additional chicks were used to test each butterfly species.

717 For the analysis of whether transparent species as a group differed from opaque species as
718 a group, the colorful but semi-transparent species *C. tutia* was classified as opaque because its
719 transparency index is lower than that of transparent species (albeit higher than that of opaque
720 species), perception by predators as calculated by our models is more similar to those of opaque
721 species (i.e. although the overall transparency index is relatively high as a result of the surface area
722 of the wing that is transparent, the individual wing areas that are transparent are effectively almost

723 opaque; refer to figure S1 and Table 1), and it belongs to a mimicry ring formed by typically
724 opaque species.
725

726 **Figures**



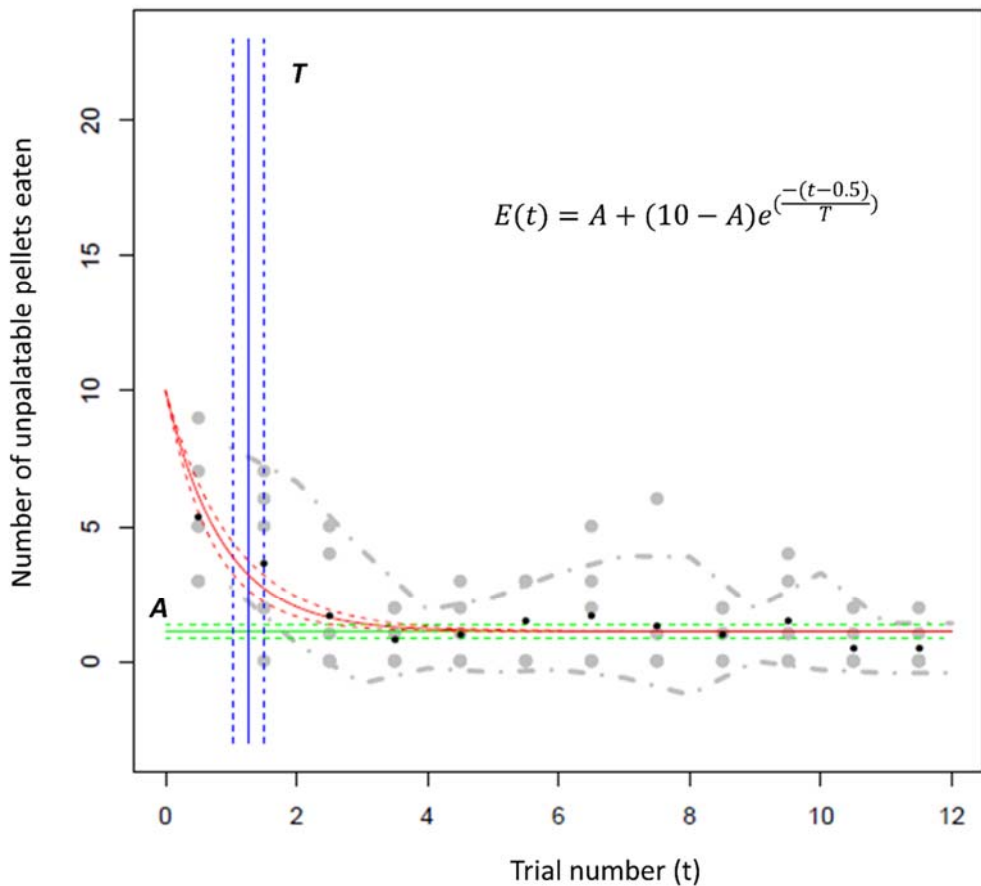
727

728 **Figure S1.** Photographs of the Ithomiini taxa (N=33 species) used for optical measurements

729 presented, from top left to bottom right, in decreasing order of transparency. The left hand side of

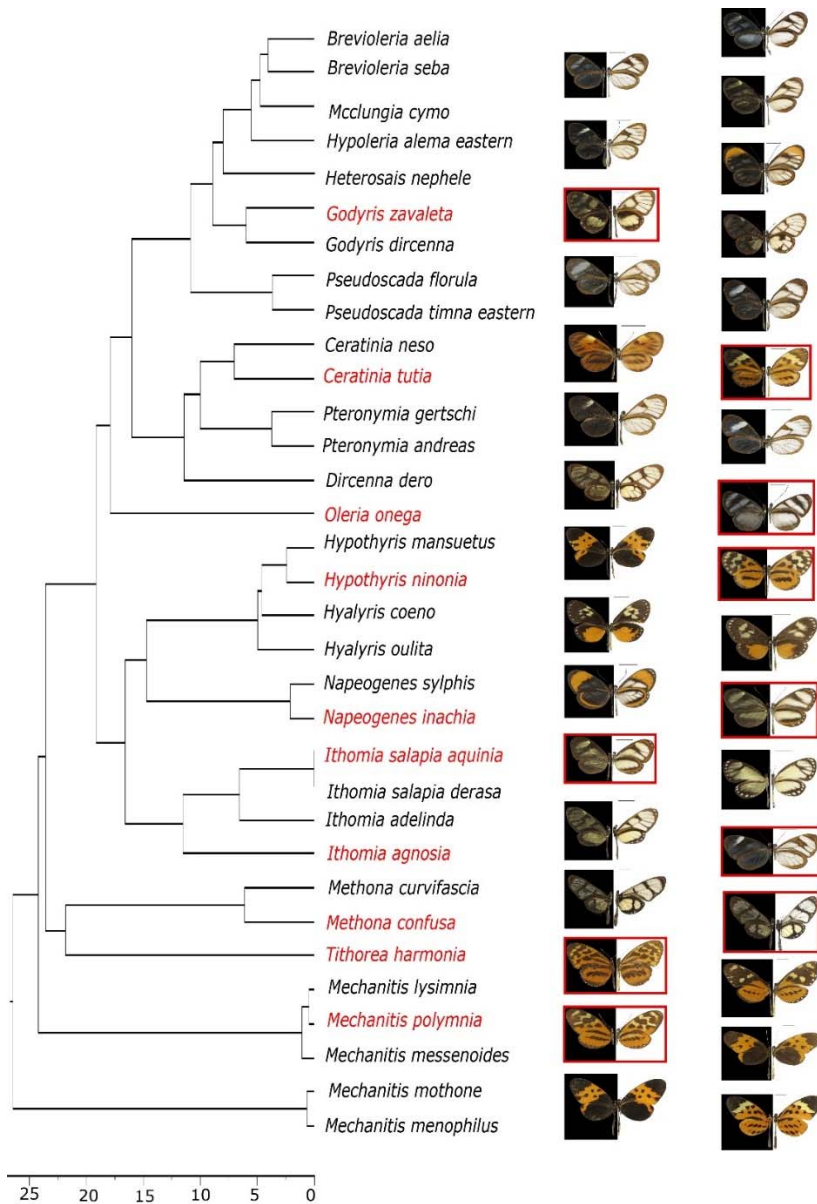
730 each photo shows the dorsal side of the wings against a dark background so as to highlight
731 transparency, and the right hand side shows the ventral side of the wings against a white
732 background so as to highlight colour patterns.

733



734

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



741

742 **Figure S3.** Phylogeny of the taxa (N=33 species) used in this study, which span all major clades

743 of Ithomiini, adapted from [9]. The axis at the bottom represents time, in millions of years. Pictures

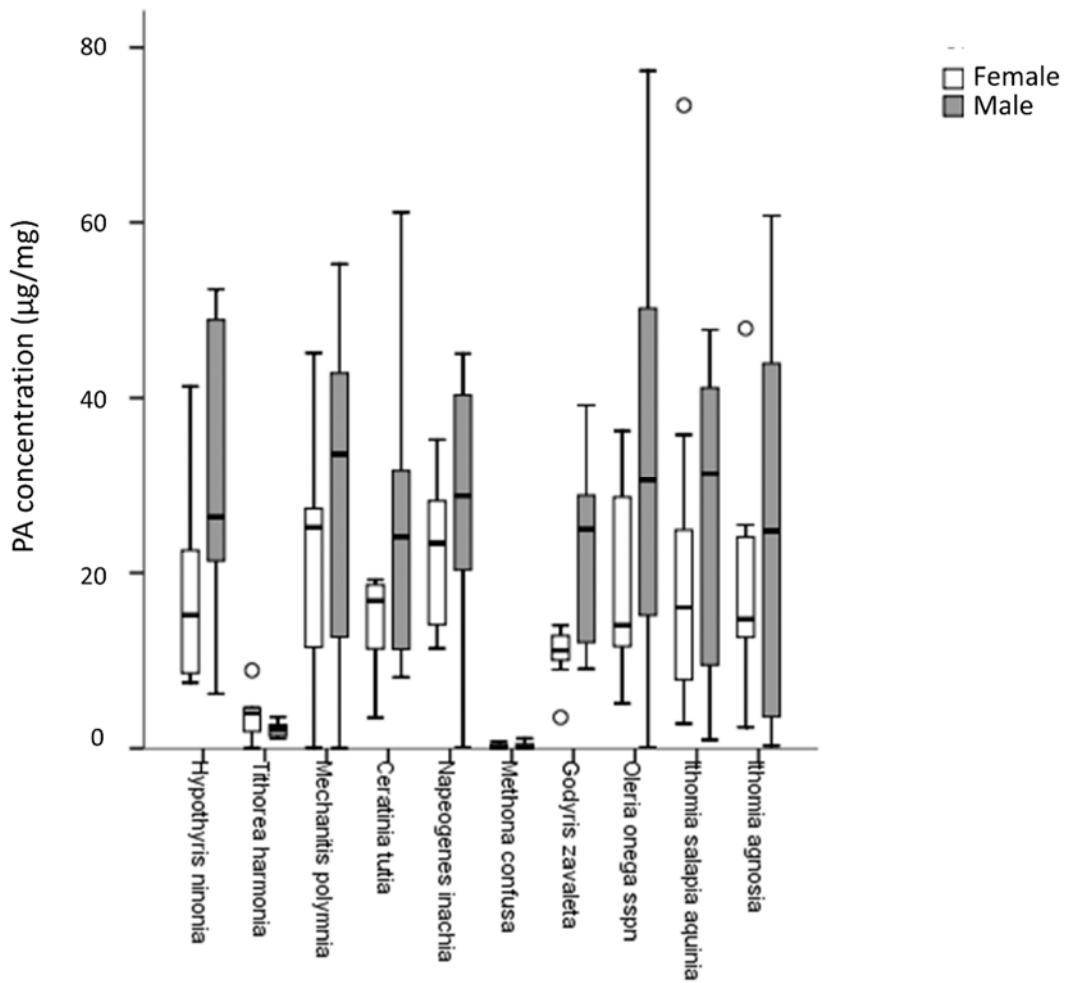
744 of the different species are shown on the right of the figure in the same order as in the phylogeny.

745 The left hand side of each photo shows the dorsal side of the wings against a dark background so

746 as to highlight transparency, and the right hand side shows the ventral side of the wings against a

747 white background so as to highlight colour patterns. Taxa highlighted in red (N=10) are those that

748 were used to quantify toxicity and unpalatability.

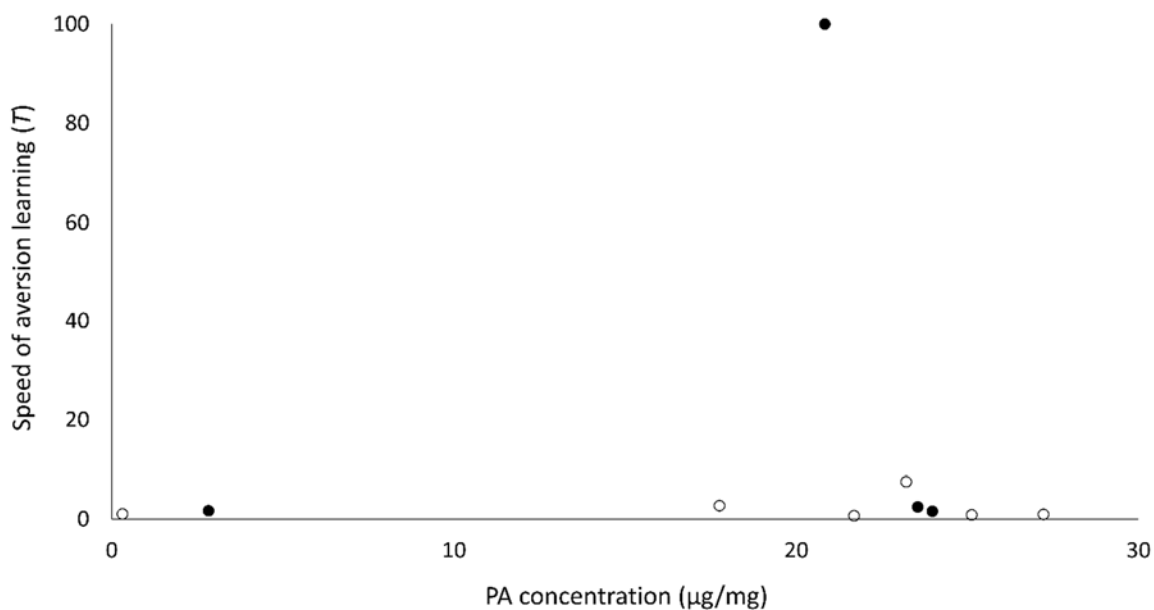


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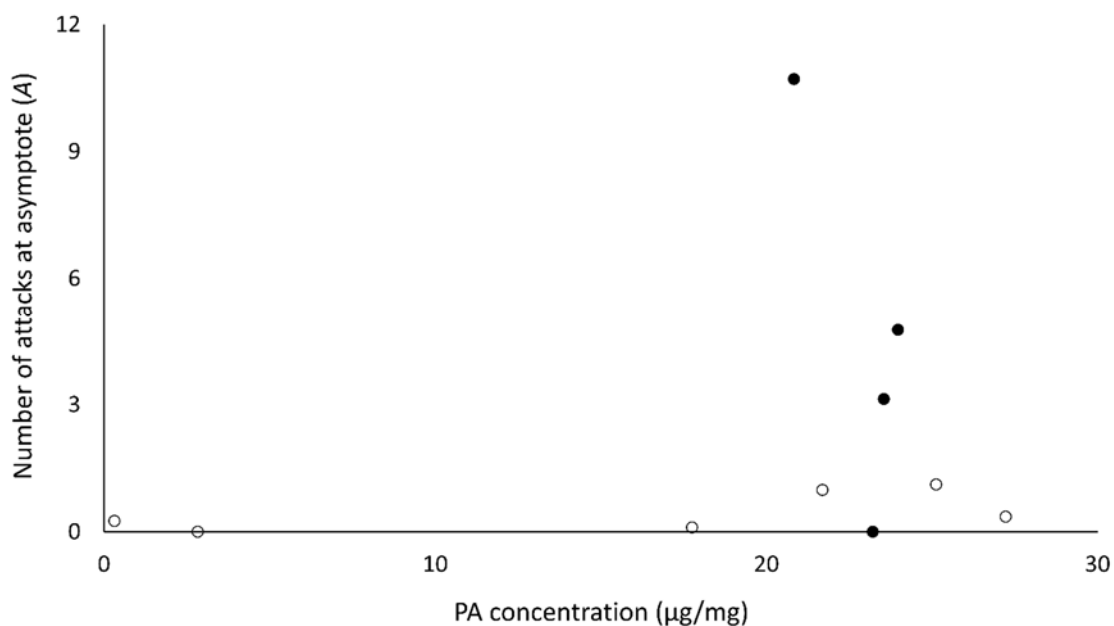
750 **Figure S4.** Quantification ($\mu\text{g}/\text{mg}$) of PA content for females (white) and males (grey) of the 10
 751 Ithomiini taxa tested. Species are in order of least transparent (on the left) to most transparent (on
 752 the right).

753

754 a)



755 b)
756



757

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

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

763

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

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

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

768

769

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

774 a)

PA content ($\mu\text{g}/\text{mg}$)	λ	model <i>p</i> -value	adjusted R ²	<i>F</i> -stat	<i>df</i>	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

775

776 b)

PA content ($\mu\text{g}/\text{mg}$)	λ	model <i>p</i> -value	adjusted R ²	<i>F</i> -stat	<i>df</i>	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

777

778

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

784 a)

Bird vision	Light environment	chromatic/achromatic	λ	model <i>p</i>-value	adjusted R^2	<i>F</i>-stat	<i>df</i>	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

785

786 b)

Bird vision	Light environment	chromatic/achromatic	λ	model <i>p</i>-value	adjusted R^2	<i>F</i>-stat	<i>df</i>	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

787

788

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