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Letter

Exploring polymorphism in a palatable prey: predation risk and frequency dependence in relation to distinct levels of conspicuousness

Riccardo Poloni[1,†], Marina Dhennin[1], Johanna Mappes[2,*], Mathieu Joron[1,*], Ossi Nokelainen[2,3,*,†]

[1]Evolutionary and Behavioural Ecology Department—CEFE, Univ. Montpellier, CNRS, EPHE, IRD, Montpellier, France
[2]Organismal and Evolutionary Biology Research Program, Faculty of Biological and Environmental Sciences, University of Helsinki, Helsinki, Finland
[3]Department of Biological and Environmental Science, University of Jyväskylä, Jyväskylä, Finland

Corresponding author: Evolutionary and Behavioural Ecology Department—CEFE, Univ. Montpellier, CNRS, EPHE, IRD, Montpellier, France. Email: riccardo.poloni@gmail.com
[†]J.M., M.J., and O.N. contributed equally.

Abstract

Camouflage and warning signals are different antipredator strategies, which offer an excellent opportunity to study the evolutionary forces acting on prey appearance. Edible prey often escape detection via camouflage, which usually leads to apostatic selection favoring rare morphs. By contrast, defended prey often display conspicuous coloration acting as warning signals to predators, which usually leads to positive frequency dependence and signal uniformity. However, when two morphs of the same species vary greatly in conspicuousness, the maintenance of both cryptic and conspicuous forms in profitable prey populations remains enigmatic. Using the white and melanic morphs of the invasive box tree moth (*Cydalima perspectalis*) presented at three different frequencies, we investigate (a) the palatability of caterpillars and adult moths to birds, (b) predation rates on the less conspicuous melanic morph, and (c) the role of frequency dependence in balancing morph frequencies. Our results show that caterpillars are distasteful for birds but not adult moths that are fully palatable. We found that the less conspicuous, melanic morph, benefits from reduced predation due to its lower detectability. The more conspicuous, white morph, instead, is more predated and is best off when common, suggesting positive frequency dependence. These results offer new insights into the evolution of color polymorphism and prey defenses in a polymorphic moth species. Further investigation is required to understand the role of different predation regimes on the maintenance of the polymorphism in this species and test whether additional selection pressures operate in natural populations.

Keywords: color evolution, crypsis, evolutionary ecology, invasion ecology, predation

Lay summary

Understanding the factors influencing character variation in natural populations is a key question in evolutionary ecology. Color is one of the most studied characteristics since the beginning of evolutionary biology because it is easy to observe and is influenced by evolutionary and ecological processes. Predation is one of the main drivers of color evolution in prey communities and prey evolved camouflage or warning colors because of their role in reducing predation. Camouflage evolves because it lowers the probability of being detected by predators. Since predators are more efficient at finding prey that they are familiar with, prey that display a rare phenotype have higher survival rates (i.e., negative frequency-dependent selection). By contrast, aposematism is defined by conspicuous appearance in toxic or otherwise unprofitable prey and evolves because birds identify defended prey by learning to use their appearance as a warning signal. In this case, the most common signals are usually best identified and avoided (i.e., positive frequency-dependent selection). It is not clear, however, which form of selection predators may exert when facing cryptic and conspicuous morphs of the same species and whether predation may explain their coexistence. Here we investigate this question in a laboratory experiment, by presenting wild birds with a melanic and a white morph of the same moth., as well as caterpillars. We show that caterpillars of this species are unpalatable, whereas adults are fully palatable, showing opposite strategies of predator defense in different life stages of the same species. Unexpectedly, our results show that despite both being palatable to birds, the white morph is more predated than the melanic, but benefits from positive frequency-dependent selection, which is usually observed in species displaying warning colorations. The melanic morph instead is less predated than the white, and contrasting what is predicted by theory, it does not show negative frequency-dependent selection. Our findings help us understanding how predation acts on morph frequencies in an invasive moth species and particularly how predators influence morph ratios in a system where a conspicuous and an inconspicuous morph coexist.

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Introduction

Animal coloration has been the focus of intense research in evolutionary biology since the times of Darwin and Wallace and has played a pivotal role in our understanding of the links between ecology and evolution (Cuthill et al., 2017; Majerus, 1998; Poulton, 1890).

Crypsis evolves in response to predation as it diminishes prey detection and attack via a reduced visibility (Endler, 2006; Schaefer & Stobbe, 2006; Tinbergen, 1960). Polymorphism, the coexistence of multiple forms within a population (Ford, 1945), is frequently observed among cryptic prey, with certain taxa exhibiting remarkable variability in appearance, such as in the underwing moths (*Catocala* spp.) where 40% of the species are polymorphic (Bond, 2007). A classic explanation for polymorphism in cryptic prey rests on predator cognition. Predators tend to find cryptic prey more easily when they are familiar with their appearance, a phenomenon known as "search image formation" (Bond, 2007; Tinbergen, 1960). Consequently, more common morphs get attacked at higher rates, resulting in negative frequency-dependent (apostatic) selection that favors rarer morphs (Allen, 1974; Van Leeuwen & Jansen, 2010). This mechanism contrasts with aposematism, where the probability of an attack decreases when prey are more common. In this case, predators learn to associate prey defenses with prey appearance, avoiding prey they are familiar with, hence favoring the commonest forms (Lindström et al., 2001; Ruxton et al., 2004). Such positive frequency-dependent selection (FDS) leads to local monomorphism within populations and also operates at the community level, leading to interspecific mimicry (Chouteau et al., 2016; Kapan, 2001).

However, despite abundant empirical evidence for positive FDS operating in aposematic prey and negative FDS in cryptic prey, polymorphism is in fact quite widespread even in unpalatable prey, and the classic associations of palatability, conspicuousness, and frequency dependence exhibit various nuances and exceptions in both cryptic and aposematic species (Kapan, 2001; Larsson et al., 2000; Rönkä et al., 2020; Ruxton et al., 2004).

The box tree moth, *Cydalima perspectalis* (Crambidae), native to subtropical Asia, has invaded Europe and part of Palearctic Asia and North America since its accidental introduction in 2007 in southern Germany (Bras et al., 2019). It displays a marked wing color polymorphism, with the coexistence of a bright, pearly white morph and a gray-brown melanic morph, found at a ratio of about 5:1 (Figure 1, Supplementary Table S1). The genetic and ecological drivers that underlie the maintenance of this polymorphism are still unknown. Bird predation is reported on both adults and caterpillars (Brua, 2014; Leuthardt et al., 2013), and although caterpillars accumulate alkaloids in their tissue, adults appear to be devoid of chemical defenses (Leuthardt et al., 2013). Adults usually rest in the foliage of shrubs and trees, often under leaves, and are mostly nocturnal, although diurnal activity is frequently observed, especially in the afternoon (personal observation, Figure 1). The coexistence of conspicuous and inconspicuous morphs in this species is still enigmatic.

Here, we use the box tree moth as a model to investigate how predators act on two color morphs of a palatable prey differing markedly in their conspicuousness. Using predation experiments with wild birds in controlled conditions, we first test whether adult

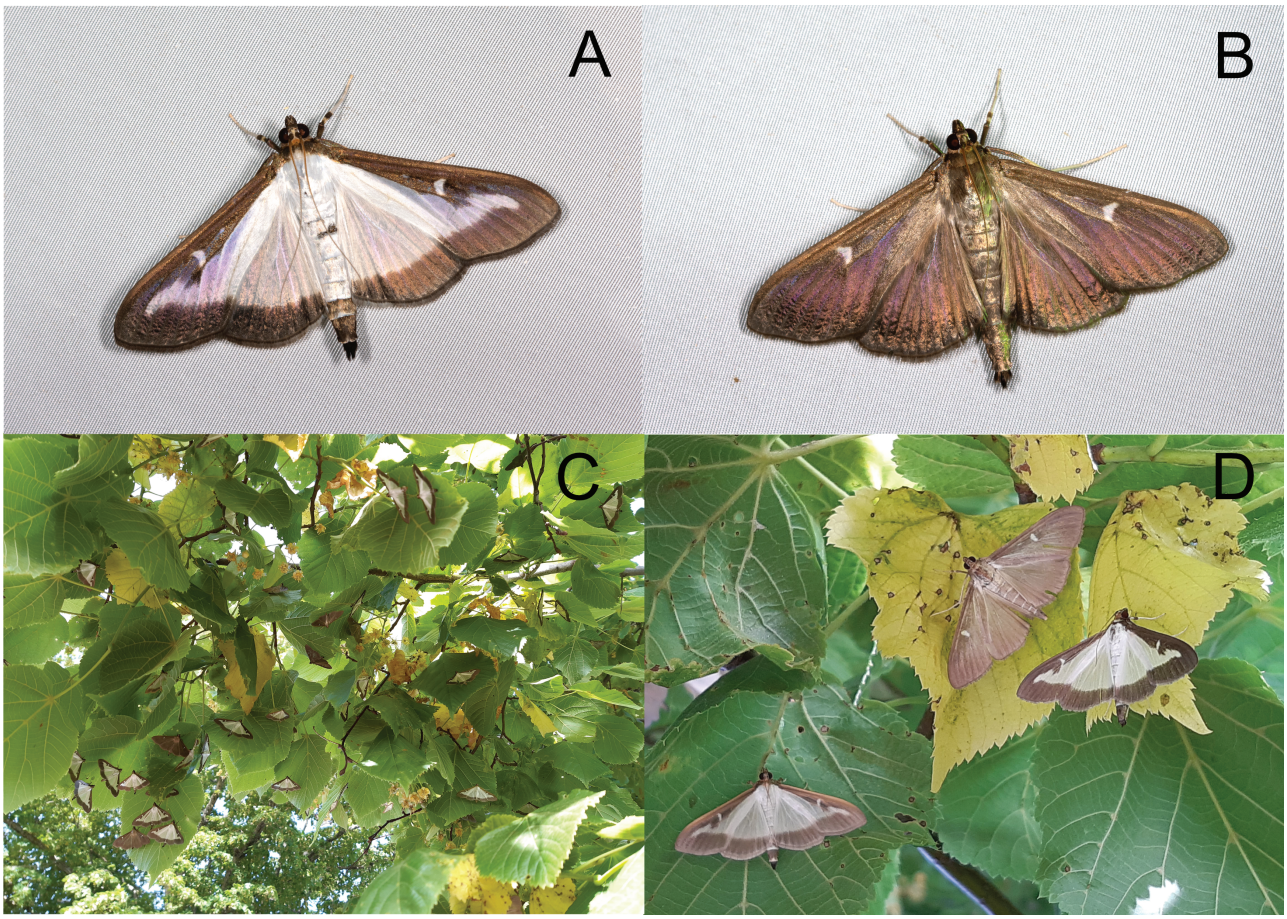

Figure 1. The two different morphs of the box tree moth: white (A), melanic (B), on natural backgrounds (C and D).

moths and caterpillars are palatable to birds or not, which would change our prediction on frequency-dependent effects. Then, we test if the putatively inconspicuous, melanic morph of the box tree moth benefits from lower detectability and negative frequency dependence, as predicted by theory. By contrast, we hypothesize that the white morph is more conspicuous to bird vision and that attack rates are less influenced by predator familiarity. Caterpillars were found to be moderately unpalatable, but both adult morphs were fully accepted. We show that the differences in conspicuousness between adults were associated with a lower predation in the melanic, inconspicuous morph, and a higher yet positive frequency-dependent predation in the white morph.

Materials and methods

Model organisms

Box tree moth specimens were obtained from a laboratory stock founded in 2020 at the Centre d'Ecologie Fonctionelle et Evolutive (France) using wild populations from Saint-Clément-de-Rivière, France (GPS coordinates: 3.84, 43.71) and Le Caylar, France (GPS coordinates: 43.86, 3.32).

Wild blue tits (*Cyanistes caeruleus*) were caught from baited traps at Konnevesi Research Station, where predation experiments took place, in February–March 2022. Once trapped, birds were measured, weighed, aged, sexed, and housed individually in plywood cages (80 cm × 65 cm × 50 cm), with food and water ad libitum (e.g., Ihalainen et al., 2012). Wild birds were used with permission from the Central Finland Center for Economic Development, Transport, and Environment, licensed from the National Animal Experiment Board (ESAVI/9114/, 04.10.07/2014) and the Central Finland Regional Environment Center (VARELY/294/2015) and used according to the Association for the Study of Animal Behavior guidelines for the treatment of animals in behavioral research and teaching. This species was chosen as a model species for behavioral studies on bird predation, with substantial background knowledge on its cognition (Reichert et al., 2021), visual system (Vorobyev & Osorio, 1998), and ecology (Blondel et al., 1991; Charmantier et al., 2016). Details on bird training and maintenance can be found in Supplementary Methods.

Palatability tests

Expectations of predator behavior differ according to the palatability of prey (Halpin et al., 2014; Ruxton et al., 2004). Therefore, before the experiment, we ran palatability tests to assess whether adult moths were palatable to birds and to what extent palatability might differ between morphs.

A total of 27 birds were involved, and each one was tested only once. Birds were placed in a plywood cubicle (80 cm × 65 cm × 50) with water ad libitum and food deprived for 1 h. The experimental design, described by Winters et al. (2021), allows detecting when the bird sees the proposed items. The prey is visible to the predator only when the predator comes to the perch (Figure 2A). Thus, this approach allows us to measure the time spent deciding to attack, without any confusion with the time spent searching (Figure 2A). Each bird was trained with crushed peanuts to get familiar with the experimental system. Next, the bird was presented with six randomly chosen prey items to be tested: three moths per morph for the tests on adult moths or six randomly chosen caterpillars for the test on larvae. Since caterpillars from the two color morphs cannot be differentiated, they were randomly chosen from the pool available.

For each prey offered, (a) prey type, (b) *hesitation time*, (c) *attack time*, and (d) eating behavior were recorded. Stress behavior like beak rubbing was also recorded. *Hesitation time* is defined as the time from

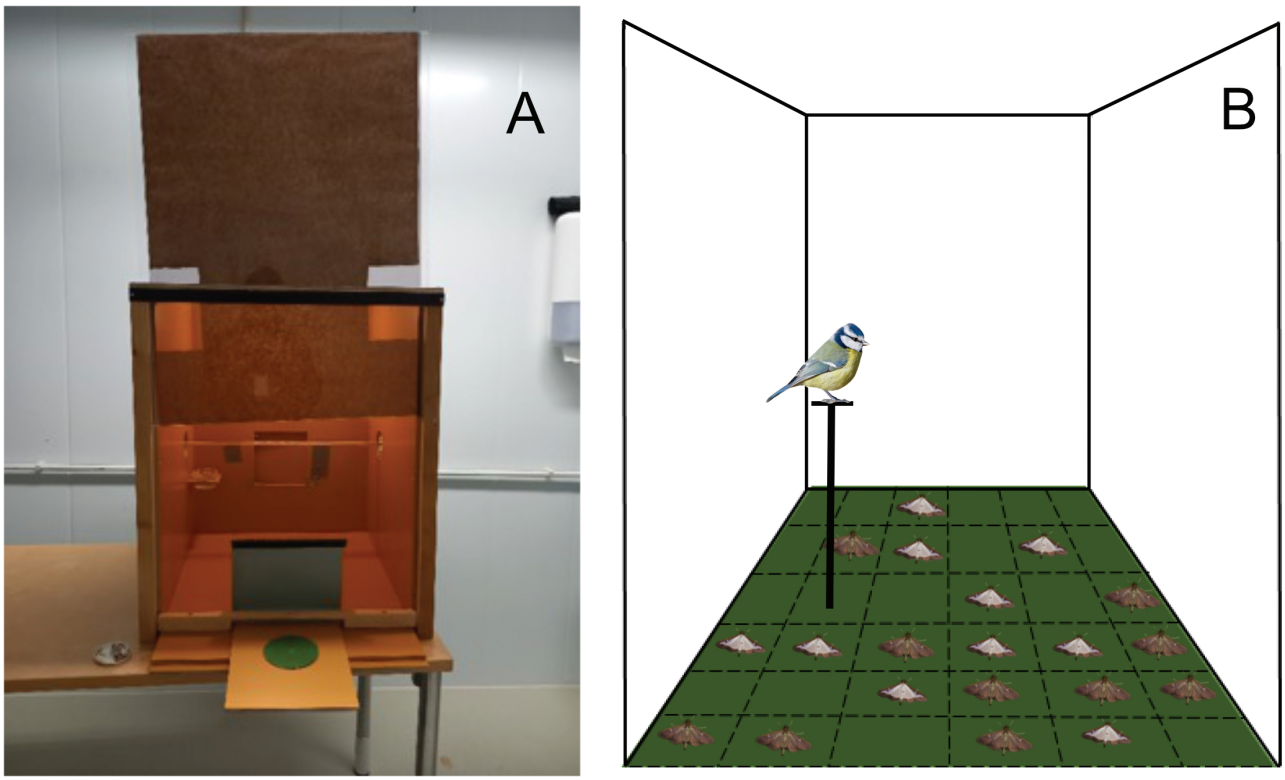

Figure 2. The experimental setup used for testing palatability (A) and predation on adults (B).

the presentation of the prey on the movable tray to the moment when the bird comes onto the tray. *Attack time* is the time taken by the bird to approach and peck/eat the prey after seeing it and was used as a proxy for the time spent to decide whether to attack a prey or not, following methods developed earlier to evaluate prey palatability (e.g., Exnerová et al., 2015; Rojas et al., 2017). Four categories of eating behavior were recorded: "eat" (prey eaten completely), "half" (prey partially eaten), "catch" (prey caught but not eaten), and "refuse" (prey detected but not caught). We considered that a bird was refusing the prey if it did not attack it after 20 min.

Visual modeling

Spectrometer measurements and visual modeling were used to model how birds perceive the two morphs of the box tree moth and the experimental background (camouflage net). We measured reflectance spectra for (a) white and black wings, both fore and hind wings with different anatomical parts (see Supplementary Figure S1), (b) the camouflage net on both brown and green sides, and (c) box tree (*Buxus sempervirens*) leaves, on both sides. Measures were obtained using a spectrometer and analyzed to quantify achromatic and chromatic contrasts as seen by the blue tit visual system in units of just-noticeable differences (JND) between moths and the camouflage net, as well as between moths and box tree leaves. To predict whether the colors are distinguishable by the receiver, we used then the function "vismodel" using the discriminability model of Vorobyev and Osorio (1998). Cone density values were based on data available for the blue tit, *Cyanistes caeruleus* (Hart et al., 2000), following a standard procedure and model parameters suggested in Silvasti et al. (2021). Chromatic and achromatic distances were finally obtained using the function "bootcoldist" with the above parameters and plotted to verify that the JND was above one, suggesting that the contrasts are strong enough to see the object.

Predation tests

This part of the study aimed at assessing differential predation between the melanic and white morphs to evaluate a putative role for crypsis in different frequency treatments.

Overall, 45 birds were involved in the experiment and were tested only once. Experiments took place in an aviary (2.80 × 3.70 × 2.20 m, H × L × W) using one bird per trial. The ground was covered with a camouflage net, whose green and brown sides (spectra are visible in Supplementary Figure S2) and heterogeneous shapes (Supplementary Figure S3) were chosen to mimic a natural background. The area was divided into a grid with wooden sticks to record the position of prey items (Figure 2B). The aviary was illuminated with LED tubes (Opple LED p T8 1500 23W 4000K). For each trial, 30 moths were randomly placed on the ground with different morph ratios according to three different treatments: control: 1:1 white:melanic; melanic biased: 1:4, and white biased: 4:1. To ensure a truly random placing, grid positions were selected using the function *sample* in R (3.6.2). Each trial ended when the bird had attacked 15 moths. We chose this design with a fixed attack rate because it is considered representative of frequency-dependent predation in natural conditions, where a population is subject to a given predation pressure (proportion eaten) and the different morphs compete for not being eaten (Gordon et al., 2021).

Statistical analysis

Statistical analyses were performed using R (3.6.2).

Palatability tests

Attack time (i.e., the time taken by the bird to approach and attack the prey once seen) was used as a proxy for assessing the bird's willingness to attack the prey item. Therefore, we tested for a difference in the attack time between control (peanuts) and experimental prey (adult moths or caterpillars). All peanuts offered at the beginning of a trial, used to familiarize the bird with the experimental system, were removed before analysis. One bird (B36) refused nearly all prey items including peanuts and was removed from all analyses.

For adult moths, attack time was tested for normality, using the Shapiro–Wilk normality test. Since the data are not normally distributed, we tested for differences in attack time using a generalized linear mixed effect model with attack time as a response variable under Poisson distribution, prey type as factor, and bird ID as random effect, to account for data structure.

For caterpillars, since the refuse rate was high, and we could not compare the attack time for birds that completely refused the caterpillars, we directly compared the refuse rates between the control prey and the caterpillars using a generalized linear mixed model including bird life-history traits that could explain the refuse behavior for caterpillars (eat/refuse) and logit link. Bird age, weight at the catching date, and sex were included as fixed effects. Bird ID was included as a random factor to account for data structure. The function "dropterm" was then used to drop one-by-one nonsignificant variables ($P > 0.05$). The best model was then selected according to the Akaike information criterion.

Predation tests

To test whether color influenced the probability of attack by birds, we used a permutation analysis to compare the observed number of moths eaten for each morph to the expected value under random predation. To do so, we generated for each treatment a null distribution of the attacks, per each color morph and assuming no difference in detectability and no frequency dependence. This was run with 100,000 iterations, and the null distribution obtained was compared with the observed number of butterflies eaten, in each treatment and for each morph. Due to constraints on the availability of birds, however, treatments were not balanced between sex and age class. To investigate the effect of this in subsequent analyses, we compared the number of moths caught by each age class per morph per treatment, to detect potential biases. Permutations were also run with the original dataset, and with a reduced dataset where the age and sex classes were distributed more homogeneously.

To assess frequency dependence, we compared the attack rates for each color morph across treatments. To do so, we first defined the *morph-specific attack rate* as the proportion of a given morph attacked in a given trial, over the total amount of moths of the same morph available. Then, we fitted a generalized linear model under Gaussian distribution for each morph, with morph-specific attack rate as the response variable and treatments as factors. An increase in morph-specific attack rate with decreasing morph frequency (negative estimate in the model) indicates positive frequency dependence, while an increase in attack rate with increasing morph frequency (positive estimate) indicates negative frequency dependence.

Finally, as for palatability tests, we tested for an effect of different variables on the attack probability by fitting a generalized linear mixed model with the variable attacked/not attacked as a response variable under a binomial distribution and logit link. Morph and treatment were included as fixed effects as well as their interactions. Bird age, sex, and prey grid position (edge or center) were also included as fixed effects. Since trials ended when birds had eaten a fixed number of 15 moths, trial duration was added as a covariate. Bird identifier was included as a random factor to account for variance in bird behavior. First, a model

was performed including all the listed variables. Then, predictors were dropped one-by-one using the function *dropterm* to assess model performance with different configurations and to remove nonsignificant variables (Anova chi-square test, $P > 0.05$). The best model was then selected according to the Akaike information criterion.

Raw data and scripts used for this paper have been deposited in Dryad and can be downloaded at https://datadryad.org/stash/share/bN_kmjdbyAq_neVcmT-eLr6xpGgfMrYGDvhT31O0Jzc.

Results

Palatability

Palatability data for adult moths were obtained using 266 individual prey presented to 27 birds. Of 80 white and 78 melanic moths presented, 76 and 77 were eaten (respectively). Average attack time was 27 ± 67 s (SD) for peanuts, 20 ± 45 s for the melanic moth, and 14 ± 20 s for the white moth (Figure 3A). Attack time did not follow a normal distribution (Shapiro–Wilk normality test, $P < 0.001$ for all prey types). The generalized linear model did not detect any significant effect of prey type on attack time (Anova chi-square test, $P = 0.207$).

Palatability data for caterpillars were obtained using 458 prey (peanuts + caterpillars) presented to 40 birds. Of 221 caterpillars presented, 73 were refused, 40 attacked but not eaten, 22 half-eaten, and 86 eaten. Refuse rate and eating behavior varied greatly among birds (Figure 3B) and between peanuts (0%) and caterpillars (63%). The GLM with the lowest AIC included bird behavior (refused or eaten) as a response variable and sex and weight as fixed factors. The strongest effect ($P < 0.001$) was the prey type, indicating a strong difference in refuse rate between caterpillars and peanuts. No effect of bird age on attack probability was found in previous models performed with all variables, but a strong effect of weight at the catching date ($P < 0.001$) and a significant but low effect of sex ($P = 0.031$). Lighter birds were associated with a lower refuse rate ($E = -2.745$) as well as males ($E = 2.333$) (Supplementary Table S2).

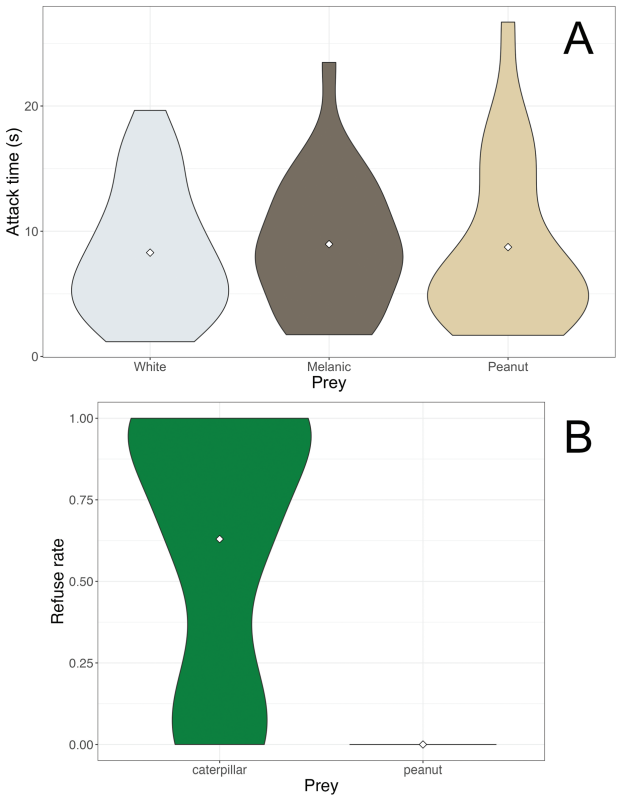

Figure 3. Violin plot showing the data for palatability experiments on adults (A) and caterpillars (B). No effect of prey type on the attack time was detected for adults (Anova chi-square test, P = 0.218, Supplementary Table S2). For caterpillars, the refuse rate significantly differed between peanuts and caterpillars (Wilcoxon signed-rank test: control prey/caterpillars, $P = 0.008$, $n = 40$).

Vision modeling

Overall, the white morph is more conspicuous that the melanic morph on all backgrounds tested (Supplementary Figure S4, Supplementary Table S3). However, the chromatic contrast for the melanic morph is higher than that for the white on the camouflage net (Supplementary Figure S4, Supplementary Table S4).

As highlighted by the JND (>1), all moths can be differentiated from the different backgrounds tested using both chromatic and achromatic contrast, except for the melanic morph, which was least detectable against the experimental background (JND = 1.297, confidence interval = 0.073 [lower], 4,209 [upper], Supplementary Figure S4). The box tree leaves also had a JND > 1 on the experimental background (Supplementary Figure S5). The white portion of the white morph has UV-reflective wings, but not the melanic morph (Supplementary Figure S4). Reflectance spectra for box tree leaves, camouflage net, and moth morphs can be found in Supplementary Figure S2, JND plots in Supplementary Figures S4 and S5 and comparisons between the contrasts for adult moths and the different background considered in Supplementary Tables S3 and S4.

Predation

Prey position on the grid and in the attack sequence

Predation data were obtained using 1350 individual prey tested with 45 birds. Moths were positioned randomly in the arena, and no bias in moth positioning (edge vs. center) was confirmed before the trials (chi-squared test, $P = 0.935$, $n = 741$ [edge] and $n = 609$ [center]). In the linear model fitted with raw data, no effect of cell number on attack probability was found (Anova chi-square test, $P = 0.74$). However, cells in the center of the arena received markedly more attacks than cells at the edge (chi-square test, $P < 0.001$ and Table 1). The order of attack (i.e., whether a moth is attacked first, second, etc.) did not explain attack probability for both morphs in the linear model (*F*-statistic $P = 0.863$). Searching time increased significantly with order (i.e., toward the end of the experiment) (*F*-statistic $P < 0.001$).

Attack rate of different color morphs

In the treatment with equal morph ratios, the melanic morph was attacked less often than expected under random predation, whereas the white morph was attacked more often than expected

Table 1. Generalized linear model fixed effects for predation experiment

	E	SE	*Z*	*P*(>|*z*|)
(Intercept)	0.736	0.152	4.848	<0.001
Morph (melanic)	−0.536	0.128	−4.185	<0.001
Treatment (control treatment)	−0.158	0.141	−1.119	0.263
Treatment (white-biased)	−0.328	0.156	−2.096	0.036
Grid position (edge)	−0.556	0.111	−4.989	<0.001

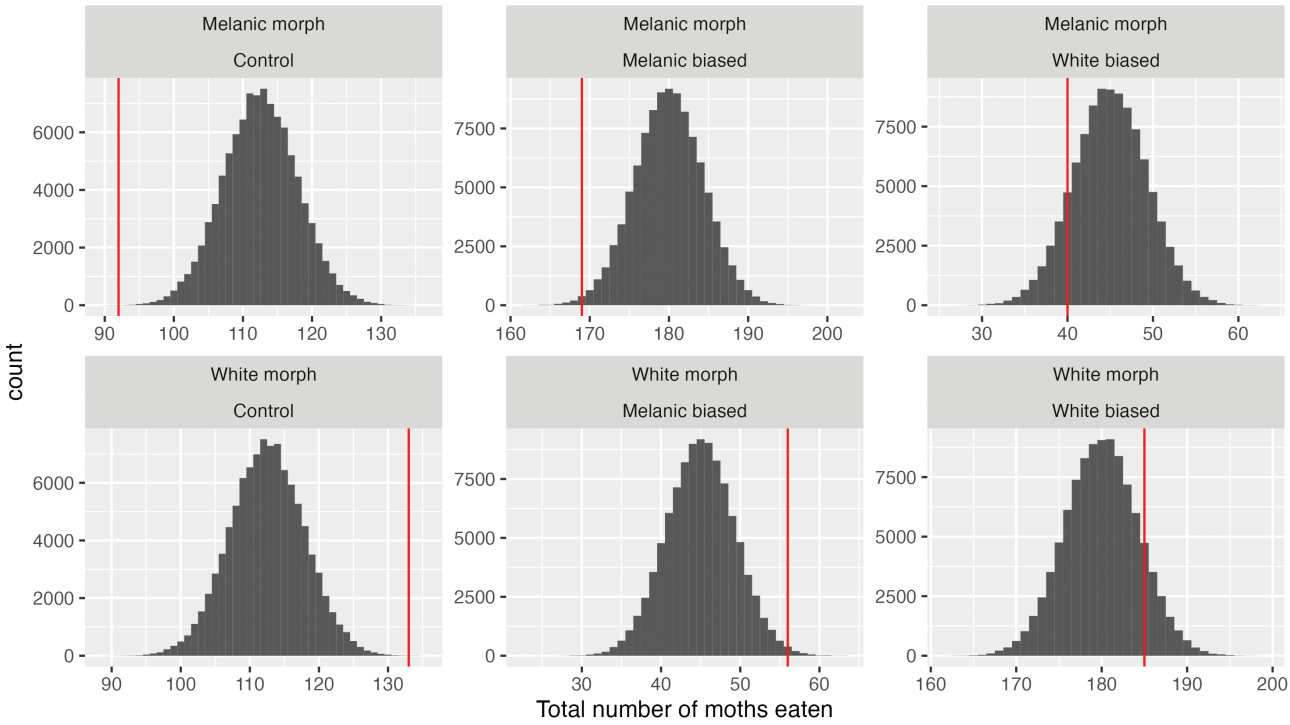

Figure 4. Results of permutation analysis performed for predation data (100k iterations). The distribution (gray bars) represents the random predation simulated by randomly sampling half of the moths from all the moths used in each treatment, to reproduce the design used. The red line represents the observed predation.

Table 2. Anova table with fixed effects for the generalized linear model performed on predation data

Factor	LR Chisq	df	Pr(>Chisq)
Morph	17.74	1	<.001
Treatment	4.42	2	.110
Grid position	25.11	1	<.001

($P < 0.001$, Figure 4). In the melanic-biased treatment, the melanic morph was also attacked less often than expected under random predation, and the white morph was attacked more often ($P = 0.008$, Figure 4). In the white-biased treatment instead, the melanic morph was attacked slightly less often than expected, and the white morph was attacked more often, but there was no significant difference between the observed attack rate and the null distribution ($P = 0.148$, Figure 4).

Overall, the model with the lowest AIC was the GLM including morph, treatment, and grid position (edge or center) as fixed factors (AIC = 1835, Tables 1 and 2). Morph color emerged as highly significant in the model (Anova chi-square test, $P < 0.001$, Table 2), together with grid position (Anova chi-square test, $P < 0.001$, Table 2). Both morph (melanic) and position (edge) had a negative estimate value in the model (Table 1), suggesting that melanic moths are attacked less heavily than white and that prey positioned at the edge of the grid received fewer attacks. These findings agree with the preliminary analysis of the spatial distribution of attacks and with the permutation analysis.

Age and sex effect

Age and sex were found to be nonsignificant in the GLM. In effect, juveniles and adults displayed nearly identical attack behavior, eating on average the same number of white and melanic moths

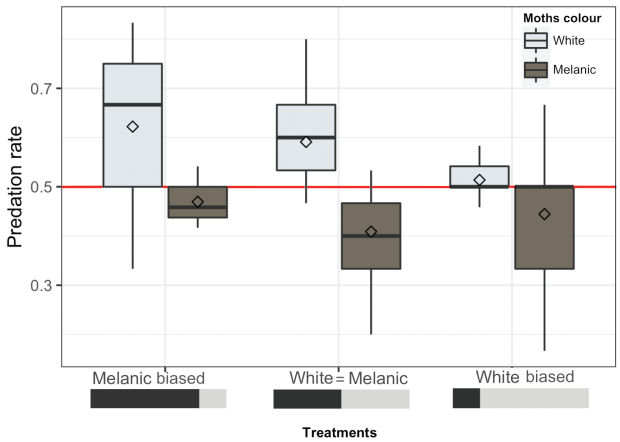

Figure 5. Box plot showing morph-specific attack rate (i.e., the number of attacked moths from one morph over the total moths of the same morph) for the predation experiment. White squares represent the median, black bars the mean, and the red line the morph-specific attack rate expected under a random scenario.

in each treatment (Supplementary Figure S6), and the same was observed for males and females (Supplementary Figure S7). The permutations performed with the subsampled dataset also gave similar results to those obtained with the original dataset (only with higher *P*-values, owing to the subsampling) (Supplementary Figures S8 and S9). This suggests that variation in bird age and sex did not affect our results and that treatments were not confounded by age or sex effects.

Frequency dependence

In the case of positive FDS, a difference should be observed between treatments in the *morph-specific attack rate*. For the melanic

morph, no significant effect of treatment was found on the overall variation of morph-specific attack rate (Anova chi-square test, $P = 0.284$). For the white morph, treatment significantly explained the variation in attack rate, with a negative estimate for the treatment at higher frequency indicating positive frequency dependence (Anova chi-square test, $P = 0.029$, white-biased treatment $E = -0.108$). This is also visible in Figure 5, where the white morph exhibits decreasing predation toward higher frequencies. This is not the case for the melanic morph (Anova chi-square test, $P = 0.284$) that does not display a monotonous effect across the treatments and an equal predation either. Indeed, when comparing the control treatment with the melanic-biased treatment, we do observe a significant difference in the attack rate (Anova chi-square test, $P = 0.023$), but not comparing the white-biased treatment with the control ($P = 0.436$) or the white-biased treatment with the melanic-biased treatment ($P = 0.535$).

Discussion

Our experiments suggest that the wing pattern polymorphism in the box tree moth is associated with clear differences in predation patterns. Melanism is associated with lower predation rates owing to being inconspicuous, while the conspicuous white morph suffers higher predation. Unexpectedly, for a palatable prey, the conspicuous form experiences lower predation rates when it is encountered more frequently by predators. The melanic morph, on the other hand, does not show clear frequency-dependent predation.

Two life stages, two different strategies against predation

Adult box tree moths were highly palatable to blue tits, in agreement with the reported absence of alkaloids in their tissues (Leuthard et al., 2013). Caterpillars, by contrast, appeared moderately unpalatable to blue tits in our experiments, suggesting that the two stages may be exposed to different predation regimes. However, bird avoidance of caterpillars was highly variable.

In our experiment, we found no difference in age, but male birds were more likely than females to attack caterpillars. This partly contrasts with previous findings, reporting that females and adult birds are more likely to attack unpalatable prey (Gordon et al., 2021; Winters et al., 2021). Birds might also balance unpalatability with nutrition capacity (Barnett et al., 2007; Halpin et al., 2014), and we found that lighter birds attacked defended caterpillars at a higher rate, in agreement with previous literature (Gordon et al., 2021; Winters et al., 2021).

The maintenance of polymorphism in the box tree moth

Prey polymorphisms attract attention in evolutionary ecology because they provide insight into the functional role of character ecology in evolution (Alatalo & Mappes, 1996; Bond, 2007). Much attention has been given to the operation of selection imposed by predators, accounting for phenotypic variation in cryptic prey, and the evolution of conspicuousness in the context of aposematism, warning signals, or mimicry (Merilaita et al., 2017; Ruxton et al., 2004). Our study brings an interesting example of an intermediary situation by evaluating the effect of predation on coexisting morphs that differ markedly in their conspicuousness but are all highly palatable.

According to our experiments, the melanic morph of the box tree moth enjoys a survival advantage in the face of predation by an insectivorous bird. This advantage likely owes to the melanic morph being less visible than the white morph. Beyond the direct, and fairly strong, effect on detectability, frequency treatments give a more nuanced yet unexpected picture of selection acting on the two morphs. Under selection on crypsis, acting on the melanic morph, a stronger survival advantage at low frequency might have been predicted (Bond & Kamil, 2002), yet our findings do not fully support this. Similarly, the white morph appears to be best off at high frequency, yet positive frequency-dependent predation is usually associated with aposematic, defended prey, not with palatable prey. So, predators in our experiment do not display frequency-dependent predation in the directions predicted for palatable, cryptic prey.

However, frequency treatments included only six prey items for the rare morph. This low number causes a large variance in sampling across birds, and asymmetry in the maximum sampled proportions of the rare and common morph because of the 15-item limitation design. Those limitations reduced the power to compare and detect frequency effects when considering the rarer morph. Time-limited trials could provide useful insight into the role of frequency dependence at low frequencies.

Given that sex and age were not uniformly distributed through the treatments, we verified that this factor did not impact our conclusions. Moreover, the birds classified as juveniles due to their 1-year plumage are not naive birds, but 8- to 9-month-old birds that endured a winter season and fully independent. In similar experiments, no difference was found in prey detection between more- or less-experienced birds; instead, age affected aversion toward defended or new prey (neophobia; e.g., Exnerová et al., 2015; Gordon et al., 2021). Here, both morphs of the box tree moths were new to the birds, but no aversion for adult moths was found in the palatability experiment (see above and Figure 3A). Thus, age and sex classes are highly unlikely to play a role in the frequency dependence revealed by the different treatments.

Positive FDS usually arises in the context of aposematic, warning coloration, whereas negative FDS arises for palatable species because predators are more familiar with the most common phenotype. In the context of our experiment, the white morph is both palatable and conspicuous and shows a positive frequency-dependent survival. This result was not predicted because predator familiarity is not expected to affect the detection probability for conspicuous prey (Bond & Kamil, 2002). Positive FDS is known for conspicuous prey living in aggregations (Cresswell & Quinn, 2011; Lindström et al., 2001). In this case, detection risk for prey groups increases less rapidly than group size (Bond & Kamil, 2002; Riipi et al., 2001) causing a decrease in per capita predation risk (Lehtonen & Jaatinen, 2016; Wrona, 1991). In our experiments, the arena was relatively small and the rate of prey encounter was high compared with the natural conditions for a foraging blue tit, so a density effect on predation could perhaps explain this pattern. Similarly, in natural conditions, very high densities are reported in the invasive populations of the box tree moth in Europe, with no evidence of difference in group behavior between morphs. This high concentration of individuals is perhaps providing the conditions for a dilution of predation risk by individual predators in the more conspicuous of the two forms. Our experiments show that positive FDS can arise for a palatable prey independently of group living.

Are birds driving frequencies of the box tree moth in nature?

Predation by birds plays a significant role in shaping the evolutionary dynamics of butterfly and moth coloration (Bowers et al., 1985; Chouteau et al., 2017; Cook & Saccheri, 2013; Nokelainen et al., 2012).

The blue tit, which was used for our experiments, is a generalist predator largely feeding on Lepidoptera (Blondel et al., 1991; Nokelainen et al., 2012). Therefore, it represents a realistic predator for the box tree moth, in terms of nutrition and search behavior. Morph frequencies in the box tree moth are relatively stable across Europe, with the melanic morphs being found at a frequency of 15%–25% with, generally, subtle variation over its continental distribution (Supplementary Table S1). This pattern suggests that some processes may stabilize frequencies. In our experiment, we found evidence for reduced predation in the melanic morph and for positive frequency dependence in the white morph. Our findings alone do not provide an obvious balancing mechanism that may explain the long-term coexistence of both color morphs. Combined with those mechanisms, as in other color polymorphic prey, other forces must come into play, like mate preferences (Chouteau et al., 2017), deleterious effects of melanization (Michie et al., 2010), and local adaptation to environmental conditions (Harris et al., 2012; Huey et al., 2000).

Vision modeling and higher predation rate suggest that the white morph is indeed more conspicuous than the melanic morph. Achromatic contrast is known to provide a more reliable detection channel for a foraging bird in motion than chromatic contrast (Nokelainen et al., 2022). The relatively low chromatic contrast of both morphs on the background is therefore unlikely to significantly change the relative conspicuousness of the morphs given their large differences in achromatic contrast (Schaefer et al., 2006).

Invasive species may reach spectacular densities. During population outbreaks, the box tree moth becomes so numerous that predators are unlikely to influence their morph frequencies (Ledru et al., 2022; Poloni, unpublished data). However, once caterpillars have defoliated all available hosts, moth populations drop and persist at lower densities according to the recovery of food plants (Suppo et al., 2020). During such periods, visual predators may be expected to operate selection on morph frequencies. It may be interesting to ask, in future work, whether morph frequencies change according to population density. However, because predatory selection on morphs is unlikely to be strong during moth outbreaks, morph frequencies are unlikely to change significantly unless other selective forces operate.

Taken together, our study shows, with a natural system, that morphs with different levels of conspicuousness may be influenced differently by predation. A palatable conspicuous morph appears to show positive FDS, whereas its less conspicuous counterpart enjoys benefits via crypsis but does not seem to be under negative FDS. This is not predicted by theory, expecting palatable prey to be inconspicuous and influenced by negative FDS. Elucidating the genetic and ecological factors associated with this polymorphism will provide a better understanding of how a balance of mechanisms within and between populations produces equilibrium frequencies and fosters the maintenance of diversity in this invasive insect.

Supplementary material

Supplementary material is available online at *Evolution Letters*.

Data and code availability

Raw data and scripts used for this article have been deposited in Dryad and can be downloaded at https://doi.org/10.5061/dryad.b8gtht7kp

Author contributions

R.P. and M.J. conceived the study; R.P., J.M., M.J., and O.N., designed the experiments; R.P., M.D., and O.N. collected data for predation experiments; R.P. and M.D. analyzed the data; R.P. and M.J. led the writing with substantial contributions from J.M. and O.N. All authors reviewed and edited the manuscript. All authors contributed to the discussion of the results and provided their contribution to the manuscript.

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