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Safety in Numbers: How Color Morph Frequency Affects Predation Risk in an Aposematic Moth*

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ABSTRACT: Polymorphic warning signals in aposematic systems are enigmatic because predator learning should favor the most common form, creating positive frequency-dependent survival. However, many populations exhibit variation in warning signals. There are various selective mechanisms that can counter positive frequency-dependent selection and lead to temporal or spatial warning signal diversification. Examining these mechanisms and their effects requires first confirming whether the most common morphs are favored at both local and regional scales. Empirical examples of this are uncommon and often include potentially confounding factors, such as a lack of knowledge of predator identity and behavior. We tested how bird behavior influences the survival of three coexisting morphs of the aposematic wood tiger moth *Arctia plantaginis* offered to a sympatric predator (great tit *Parus major*) at different frequencies. We found that although positive frequency-dependent selection is present, its strength is affected by predator characteristics and varying prey profitability. These results highlight the need to understand predator foraging in natural communities with variable prey defenses in order to better examine how behavioral interactions shape evolutionary outcomes.

Keywords: frequency-dependent selection, aposematism, warning coloration, context-dependent predation, polymorphism.

Introduction

Aposematism is loosely defined as a defense strategy that combines a primary warning signal (often bright or distinctive coloration) with some sort of secondary, gener-

ally chemical, defense (Poulton 1890). This combination allows potential predators to learn the association between signal and defense to the benefit of both predator and prey. It has been discussed as problematic in terms of its origin and historical establishment in a population, because educating naive predators about a novel and low-frequency conspicuous signal could prove to be deadly for the early carriers of the signal (Fisher 1958; Sillen-Tullberg and Bryant 1983; Guilford 1988; Alatalo and Mappes 1996; Santos et al. 2003). It has also been a puzzling topic in terms of its maintenance, as theoretical expectations suppose that within a population aposematic signals should be driven to monomorphism via positive frequency-dependent selection for efficient predator learning (Müller 1879; Endler 1988; Mallet and Barton 1989a; Joron and Mallet 1998; Sherratt 2006; Lawrence et al. 2019). Even without this selective pressure, genetic drift is expected to remove alternative morphs from small populations (Wellenreuther et al. 2014). In spite of this, there are numerous empirical examples of within-population warning signal variation or polymorphisms across a variety of taxa (Briolat et al. 2019), such as insects (O'Donald and Majerus 1984; Borer et al. 2010; Hegna et al. 2015; Rönkä et al. 2020), amphibians (Maan and Cummings 2009), and reptiles (Sanders et al. 2006). This paradox provides a good opportunity to understand the selective forces behind diversity in nature, especially in the more difficult cases where warning signal variation exists at the intrapopulation level.

There are a few explanations we can briefly describe here as to what can maintain this apparent mismatch between theory and reality in warning signal evolution, especially as it pertains to warning coloration, which we now focus on. First, the evolutionary response to selection on warning coloration can be influenced according to whether the genes involved in pigmentation show multiple pleiotropic effects, whether they are tightly linked to other selected

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genes in the chromosomal vicinity, their dominance patterns, and/or whether they form central nodes in integrated gene networks (Charlesworth and Charlesworth 1975; Joron et al. 2006, 2011; Wellenreuther et al. 2014). A clear example of this comes from the South American butterfly *Heliconius numata*, in which varying color morphs are associated with different genome arrangements that are tightly linked and inherited together as a P supergene, suggested to be maintained via variable predation (Thompson and Jiggins 2014).

Second, antipredator signals—particularly coloration—can be under multiple selective pressures, such as thermoregulation (Clusella-Trullas et al. 2008; Hegna et al. 2013), negative frequency-dependent predator or mating selection (Chouteau et al. 2017), or trade-offs between natural and sexual selection (Nokelainen et al. 2012). While each of these alternative selection pressures alone are not enough to maintain polymorphisms, spatial or temporal variation in selection on color morphs may allow the coexistence of more than one morph (Maan and Cummings 2009; Valkonen et al. 2012; Nokelainen et al. 2014) through gene flow between locally adapted populations (e.g., Mallet et al. 1990; Gordon et al. 2015; Garg et al. 2019). This can easily occur if directional selection toward the fixation of one particular warning signal shifts as prey vary in their signal efficacy over time and space or if predators vary in response to particular signals (Doktorová et al. 2019).

There is increasing evidence that spatiotemporal variation in morph survival due to habitat heterogeneity (Zvereva et al. 2002), differences in predator community (Valkonen et al. 2012; Nokelainen et al. 2014; Rönkä et al. 2020), and morphological and behavioral differences between prey can indeed affect the efficacy of the warning signal (Doktorová et al. 2019). A recent study (Holmes et al. 2017) used a simulation-based model to examine the interaction between predator perspective, migration, and genetic linkage on color polymorphism. Their results strongly suggest that predator behavior under frequency dependence can maintain geographic mosaics in coloration across space and time. At the same time, variation in the strength and direction of selection can also arise from the dynamic and often context-dependent responses of predators (Endler and Mappes 2004; Mochida 2011). Predators can vary in their decisions on the basis of their prior experience level, motivation, physiological condition, and their individual responses to prey conspicuousness or unprofitability (Langham 2004; Mappes et al. 2005; Mappes et al. 2014; Briolat et al. 2019). Resolving the conflict between theory and empirical findings in polymorphic aposematic systems may hence require specifically testing the selective advantages of varying frequencies of different morphs against their potential predators.

Laboratory studies using artificial systems have shown that warningly colored prey suffer greater costs when

rare (Lindström et al. 2001), although their per capita mortality risk may not actually decrease with increasing frequency (Rowland et al. 2010*b*). When looking at warningly colored systems in nature, studies that actually test or confirm the presence of frequency-dependent selection are rare (but see above citations). Among those that do so, much of the strongest evidence comes from artificial model experiments (Greenwood et al. 1989; Chouteau et al. 2016; Rönkä et al. 2020) or field transplant experiments (Mallet and Barton 1989*b*; Kapan 2001; Borer et al. 2010), particularly in *Heliconius* systems (Chouteau et al. 2016). For example, Mallet and Barton (1989) found that butterflies *Heliconius erato* transported into areas dominated by an alternative color morph had reduced survival and suffered higher attack rates (Mallet and Barton 1989).

Another study examined the predation risk of common and rare morphs of wood tiger moth *Arctia plantaginis* artificial plasticine models in locations known to vary in morph frequency (from monomorphic to polymorphic sites across a wide geographical range). Here, authors also found evidence of positive frequency-dependent selection (Rönkä et al. 2020), but in different geographic locations the local signals and the strength of selection between morphs varied, likely driven by feeding tactic variation in the local predator community. For example, bird communities dominated by Paridae species, such as blue or great tits, tended to favor the survival of the yellow morph under similar frequencies in Southern Finland, while communities dominated by Prunellidae (dunnock species) favored the white morph. This result was opposite in Scotland (monomorphic yellow population), where although yellow moths have an overall survival advantage, bird populations dominated by Paridae favored the white morph, suggesting quite strongly the role that local predators may play in local morph frequencies (Rönkä et al. 2020).

Given this very interesting result, the wood tiger moth is an ideal system to explicitly test whether the patterns observed in field studies are the result of positive frequency dependence acting on the color morphs. By using real moths but under more controlled laboratory settings, we can also specifically test the selective advantages of the different morphs. We thus subject different frequencies of three coexisting morphs of wood tiger moths to predation trials involving great tits (*Parus major*), a local bird species that is a known predator of moths and other insects in general. We collect information about predator age (as a surrogate of experience level), size, and behavior along with moth warning coloration (morph) and size, and then we link these traits to the survival of common versus rare morphs in each treatment during the experiment. Specifically, our aim is to find out the following: (1) Do birds bias their decision to attack prey on the basis of differences in morph frequency on a small spatial scale? (2) What predator characteristics affect

these decisions? (3) Does the order of attack matter, that is, does preying on a particular morph influence their subsequent choices? We argue that truly understanding the maintenance of polymorphisms in aposematic systems requires confirming the presence of positive frequency-dependent selection as well as distinguishing it from other factors associated with the different warning signals involved. Doing this will give much needed evidence as to whether the ever-increasing examples of polymorphism in warning coloration should keep being considered a true paradox or rather an expected norm.

Methods

Study System

Wood tiger moths (*Arctia plantaginis*) are conspicuous and chemically defended across their life stages (Lindstedt et al. 2016; Rojas et al. 2017). Adult females exhibit variable warning signal coloration (ranging from yellow to red hind wings), whereas adult males for the most part have discrete, genetically controlled color morphs (yellow, red, white, and/or black hind wings). This polymorphism displays a global mosaic, and populations consist of a range of frequency combinations—from monomorphisms to polymorphisms—where different morphs coexist (Hegna et al. 2015) at sometimes fluctuating ratios (Galarza et al. 2014; O. Nokelainen et al., unpublished manuscript). In Finland and most of the European Palearctic regions, adult male hind wing warning signals are either white or yellow, whereas female color ranges continuously from orange to red. Crucially, experimental evidence shows that wild-caught birds do not seem to generalize between the color morphs of this species (Rönkä et al. 2020). They have two forms of chemical defense, one targeted to birds and one to insect predators (Rojas et al. 2017), of which the first is the best understood. Fluid containing pyrazines is released just behind the head of the moth when attacked; these pyrazines are produced de novo by the moth (Burdfield-Steel et al. 2018) and elicit both increased hesitation to attack and distaste responses in birds, even in the absence of color cues (Rojas et al. 2017, 2019; Burdfield-Steel et al. 2019).

Experiment

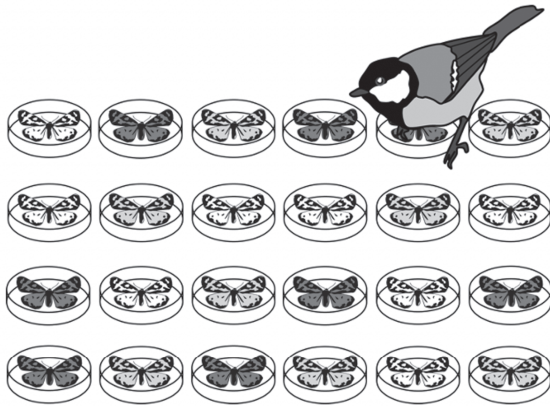
We took recently freeze-killed adult moths from a multi-year laboratory stock founded in 2011 with wild-caught individuals from mainly Central Finland. Laboratory moths were reared on a diet of lettuce and dandelion (*Taraxacum* sp.). After being killed, the moths were stored at -20°C at the University of Jyväskylä until their use in this study. The moths were then thawed and spread so that their hind wing color was visible right before each experimental trial. To account for the natural size variation present in this spe-

cies, the forewing length of each moth was measured with calipers prior to the start of the experiment. Specimens were then laid unpinned on their ventral side, with the dorsal side visible on individual petri dishes. Each experiment contained a total of 24 moths laid out in a 4×6 grid (fig. 1) in one of two enclosed indoor aviaries ($13.5 \text{ m}^2 \times 2.4 \text{ m}$ height). The floor of each aviary was covered in dark green sheeting (tarp) to approximately mimic a natural background. The four different frequency treatments were as follows: control: eight white males, eight yellow males, eight orange/red females (hereby red); red bias: six white males, six yellow males, 12 red females; white bias: 12 white males, six yellow males, six red females; yellow bias: six white males, 12 yellow males, six red females. Each bird was assigned to a single treatment. Moth position on the grid was randomized.

Wild great tits (*Parus major*) were caught from baited traps at Konnevesi Research Station (Central Finland), where this experiment took place, in October 2015. Once trapped, all birds were measured, aged, sexed, and housed individually in plywood cages ($80 \text{ cm} \times 65 \text{ cm} \times 50 \text{ cm}$) with a daily light period of 11L:13D. Birds were fed sunflower seeds, peanuts, and vitamin-enriched tallow and provided with fresh water ad lib. After the experiment, all birds were ringed for identification purposes before being released at the capture site. 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.

Birds were trained in groups overnight to forage in the experimental room and take palatable food (peanuts and sunflower seeds) from the petri dishes laid out in the grid before being returned to their home cages. Trials were run the following day after training. Prior to the start of each trial, the participating bird was food deprived for 1–2 h to ensure that they were motivated to forage. Twenty-four moths were then laid out on petri dishes in a 4×6 experimental grid (fig. 1) and a single bird was released. We observed each trial through a one-way mirror and recorded the timing, order, and outcome (i.e., eaten or rejected/dropped) of each attack as well as other bird behaviors, such as beak wiping or cleaning. Trials lasted until each bird had attacked at least 12 moths or 2 h had passed, whichever came first. Forcing the experiment to stop after a certain number of moths mimics the nonindependence of fitness that is characteristic of frequency-dependent selection, where a population is subject to a given predation pressure (proportion eaten) and the different morphs compete for not being eaten. Notably, our study did not

(A)



(B)



Figure 1: A, Schematic of experimental setup of control trial. B, Picture of actual grid setup before the experiment in the aviary (13.5 m² × 2.4 m height). Various bird perches were available around the room, and there was a water bowl in the front left corner. A color version of this figure is available online.

include any nonaposematic alternative prey because the goal was to test for frequency dependence of aposematic prey. Although we know that predators can readily attack wood tiger moths, if given a chance to choose palatable prey over them they will. Greenwood et al. (1989) showed that when model prey were all toxic, bird predators took an excess of the rare forms, whereas when prey were devoid of toxicity, they chose independently of frequency (Greenwood, Cotton and Wilson 1989). We therefore did not want to risk depleting the frequency effects in our small arena by introducing fully palatable prey items into the grid. All treatments included 10 trials and birds were used for only a single trial, resulting in a total of 40 birds used in the entire experiment. Following their use in the experiment, birds were given at least 6 h to feed in their home cages before being released.

Statistical Analyses

We performed all statistical analyses using R version 3.6.1 (R Development Core Team 2019).

Descriptive: Assessing Confounding Factors. We first assessed potential covariates by evaluating whether moth size (length), position, and the distribution of bird sexes and stages were evenly distributed across morphs and treatments. We also evaluated whether there was a potential bias

in the location of morphs across the grid, since a recent predation study using a grid system found that individuals were eaten at a higher rate when they occupied the edges rather than the middle of the grid (Arias et al. 2019). To do so, we fit linear mixed models (LMMs) where moth length was the response variable and generalized linear mixed models (GLMMs) with a binomial response and logit link for the probability of being placed on the edge. In both models, color morph, treatment, and their interaction were fixed factors. We included trial as a random factor to account for any unmeasured differences across trials or birds. We fit the model using function `lme` from R package `nlme` (Pinheiro et al. 2020) and evaluated the significance of morph and treatment on length using χ^2 ANOVAs with package `car` (Fox et al. 2019). Finally, since adult and immature birds can differ in their reaction time and behaviors, we also examined the distribution of bird age/stage across treatments. We did so by fitting binomial models for the probability that a trial contains a male or immature bird depending on treatment using function `glmer` from package `lme4` (Bates et al. 2015). The significance of model coefficients (and contrasts for factors) is evaluated using a *t*-test for normally distributed variables (LMMs) and a *Z*-test otherwise (GLMMs).

Calculating Bird Body Condition. Body condition of birds was calculated using the scaled body mass index

of birds (according to Peig and Green 2009), given the bird mass and tarsus length. In summary, this index is calculated using the following formula:

$$\text{condition} = M_i \left(\frac{L_o}{L_i} \right)^{b/r},$$

where M_i is the body mass of individual i , L_o is the average tarsus length of all birds, and L_i is the tarsus length of individual i . The term b is the regression coefficient from the linear regression between $\log(M)$ and $\log(L)$, and r is the Pearson's correlation coefficient of mass M and tarsus length L .

Testing for Fine Spatial Scale Frequency-Dependent Selection. We tested for positive frequency-dependent selection in two ways. First, we examined overall probability of survival at the end of the experiment, and then we looked at survival rates using proportional hazard modeling. For the former, we fitted a GLMM with a binomial response variable (survived vs. eaten) and a logit link. Because trials could differ in time, we added trial duration as a covariate to standardize for the different times that moths were exposed to predation. We included moth forewing length as a continuous covariate and location as a two-level fixed factor (edge vs. center) to account for any grid biases. We also added, as predator covariates, bird sex (male vs. female) and bird stage (immature vs. adult) as fixed factors and body condition as a continuous variable. We included morph (red, white, and yellow) and treatment (control, red bias, white bias, and yellow bias) as discrete fixed factors as well as their interaction. Finally, we added trial as a random effect to account for the fact that there were several moths used per trial. Models were fit using function `glmer` from package `lme4`. We performed χ^2 ANOVAs (R package `car`, function `Anova`) on this full model to determine which factors and covariates were significant and then subsequently dropped any covariate or interaction that was not significant (at $P > .1$) with the exception of treatment, morph, and trial time, which we always kept in each final model. We then evaluated estimates and significance of model coefficients on the final reduced model using function `summary`, where we could evaluate specific differences among levels of factors. We use this protocol for all GLMMs performed throughout the study. To aid interpretation of coefficients, we subsequently performed morph-specific models. The only two differences between the full model and the morph-specific ones were that (1) there were no morph effects (since there was only one morph) and (2) treatment was recoded to refer specifically to the frequency of the focal morph. For example, yellow bias and white bias treatments had the same frequency of the red morph. Therefore, there were only three treatment levels: positive bias,

negative bias, and control. The reason to pool treatments with the same frequency of the focal morph is because our interest was in the fitness of the focal morph, regardless of (or averaged over) the other morph types. In other words, selection for one morph (e.g., red) will be frequency dependent if fitness responds to its own frequency compared with the total, regardless of how many other morphs there are at different frequencies.

In all future analyses, the same explanatory variables as above were used, and in all cases unless indicated otherwise, we performed χ^2 ANOVAs, reduced the model, and estimated coefficients and significance.

To examine survival rates, we modeled proportional hazards using Cox mixed effects models. These models allow for a more fine-tuned analysis of survival using time to death (or attack) as the response variable rather than the binary outcome (survived or not). They also appropriately handle unfinished records (i.e., individuals not eaten by the end of the experiment) and different trial durations by statistically censoring nonpreyed individuals at the end of the experiment (Therneau and Grambsch 2000). We used the R package `coxme` (Therneau and Therneau 2015), which allows the incorporation of random effects. We tested the proportional hazards assumption using function `cox.zph` from package `survival` (Therneau and Grambsch 2000).

Context Dependence of Attack Decisions Based on Predator Characteristics. We next examined general patterns and potentially confounding factors from the standpoint of the avian predators. We evaluated whether differences in bird stage (adult vs. immature), sex (male vs. female), body condition, or treatment affected (1) the time predators took to eat the first prey and (2) which morph was preferred first.

Time to first attack was modeled using a GLMM using function `glmer`. Since time intervals follow multiplicative processes and not a normal distribution, we modeled an exponentially distributed response variable using a gamma distribution with a shape parameter of 1 and a log link.

The analysis of which morph was eaten first required a multinomial model where the response variable—morph chosen—was a factor with three levels (red, yellow, and white). The explanatory variables used were the same as above except for morph (which was now a response variable), but models with interactions were singular and could not be included. We used function `multinom` from package `nnet` (Ripley et al. 2016) and manually calculated Z values (dividing coefficient by standard error) and P values (contrasting with a normal distribution). Because multinomial probabilities are defined by one less probability than the number of choices (morphs) because the last is redundant, one level (morph) must be chosen as

a reference against which the others are compared. We chose to use the red morph as the reference level.

We then modeled bird behavior in order to assess whether those same explanatory variables affected their avoidance response to particular morphs, as indicated by (3) beak cleaning or (4) dropping of the moth. Beak cleaning has been shown to be a clear signal of distaste in birds (Rowland et al. 2015; Burdfield-Steel et al. 2019; Doktorovová et al. 2019; Rojas et al. 2019), and dropping a moth after attack is seen as a clear sign of rejection (which moths can often survive; K. Rönkä, T. Salmi, and J. Mappes, unpublished manuscript). We analyzed these behaviors using GLMMs where the response variable was considered binary (presence or absence of the behavior) and modeled following a binomial distribution with a logit link.

Differences in Ranked Order or Consecutive Attacks. We explored attack order by analyzing whether morphs differed in how early in the trial they were attacked on average. We did so by fitting an LMM with log-transformed order of attack as the response variable and trial as a random factor. We subsequently performed separate analyses for all four treatments with the same model structure, except for the exclusion of treatment as a factor.

We next wanted to know whether the attack of a particular morph affected the chances of attacking the same morph next. To test whether there was such positive or negative reinforcement, we modeled the probability of attacking the same morph again on a consecutive attack as a function of the morph. We used morph as a fixed factor and trial as a random effect. We modeled the response as a binomial with a logit link, representing whether the same morph was attacked next. We also repeated these analyses for each treatment separately.

Results

Descriptive: Assessing Confounding Factors

Bird predators ate 12 out of 24 available moths in every trial, or 120 moths evenly across all four treatment groups. The interaction between morph and treatment did not have a significant effect on moth length ($\chi^2 = 8.109$, $df = 6$, $P = .230$) and was therefore removed from the final model. White and yellow moths were similar in size and were on average significantly longer than red moths (white vs. red: 0.581 ± 0.096 , $t_{918} = 6.767$, $P < .001$; yellow vs. red: 0.505 ± 0.086 , $t_{981} = 5.889$, $P < .001$). The yellow bias treatment had significantly longer individuals overall. For example, compared with the control treatment, they were 0.232 ± 0.105 mm longer (LMM, $t_{36} = 2.208$, $P = .034$). Because of this, moth size was included as a covariate in all analyses and removed if not significant at $P = .1$.

We fortunately found no evidence for bias in morph location on the grid across morphs ($\chi^2 = 0.949$, $df = 2$, $P = .622$) or treatments ($\chi^2 = 0.100$, $df = 3$, $P = .992$), nor was there a significant interaction between them ($\chi^2 = 6.278$, $df = 6$, $P = .393$). Neither predator stage ($\chi^2 = 2.050$, $df = 3$, $P = .562$) nor sex distribution ($\chi^2 = 1.245$, $df = 3$, $P = .742$) differed significantly across treatments as well.

Testing for Fine Spatial Scale Frequency-Dependent Selection

The full model results showed that moth length ($Z = -2.410$, $P = .016$) and location ($Z_{\text{Mid}} = 5.074$, $P < .001$) had a significant effect on the probability of moth survival to the end of the experiment. Individuals were attacked more when larger and when placed on the edge of the grid compared with the middle. Bird sex, stage, and condition were nonsignificant and thus removed from the model. There was also a significant interaction between treatment and morph, which we will clarify by the morph-specific analyses (table S1 [tables S1–S6 are available online] shows all the coefficients of the full model).

The morph-specific analyses yielded significant support for positive frequency-dependent survival in the red morph. Specifically, red individuals had a higher survival in the control and red bias treatments (positive bias treatment), where they are at higher frequency, than in the white and yellow bias treatments (here combined as negative bias because they have the same frequency of red moths; table 1; fig. 2).

The white morph also showed significant positive frequency-dependent survival, as those in the high-frequency white treatment (positive bias) survived better than in the control or negative bias trials (the combined red bias and yellow bias treatments; table 1; fig. 2). In contrast, the yellow morph showed no evidence of frequency dependence (table 1; fig. 2). They survived equally well as the other two morphs when at the same frequency (control) as when they are at a negative or positive bias. All models show that there is a survival advantage of moths placed in the middle of the grid. However, as we showed earlier, this advantage is not different for morphs across treatments.

The data did not significantly depart from the proportional hazards assumption of Cox models ($\chi^2 = 17.160$, $P = .103$). The Cox proportional hazard mixed effects models of moth rates were completely congruent with the previous binomial analyses, that is, evidence for positive frequency-dependent selection favoring the red and white morph but not yellow (fig. S1; figs. S1, S2 are available online). Because of this, we include the full summary of this model in tables S2.

Table 1: Generalized linear mixed model fixed effects for survival probability of moth morphs

	Estimate	SE	Z	P
Red morph:				
Intercept	2.468	1.995	1.237	.216
Trial duration	.012	.007	1.686	.092
Length (mm)	-.165	.119	-1.395	.163
Location (middle)	.708	.245	2.886	.004*
Treatment:				
Positive bias	-.414	.304	-1.363	.173
Negative bias	-.914	.322	-2.841	.005*
Yellow morph:				
Intercept	2.153	1.974	1.091	.275
Trial duration	-.001	.008	-.131	.896
Length (mm)	-.147	.113	-1.301	.193
Location (middle)	.950	.249	3.819	<.001*
Treatment:				
Positive bias	.315	.293	1.077	.281
Negative bias	-.029	.316	.093	.926
White morph:				
Intercept	2.632	1.901	1.385	.166
Trial duration	-.011	.007	-1.589	.112
Length (mm)	-.159	.110	-1.450	.147
Location (middle)	.504	.249	2.027	.043*
Treatment:				
Positive bias	.685	.292	2.346	.019*
Negative bias	.246	.309	.796	.426

* $P < .05$.

Context Dependence of Attack Decisions Based on Predator Characteristics

The average time to eat the first moth across all four treatments was 6.3 min (fig. 3). Analyzing the factors affecting the time to first attack showed that the only significant factors were bird stage (immature birds take longer to attack), bird sex (males take longer to attack), the interaction between them (immature females take longer to attack), and the interaction between bird sex and treatment (male birds in the yellow bias treatment show shorter times to attack than in other treatments; table 2; fig. S2A). Bird condition did not influence the time to first attack.

The multinomial model examining which morph was chosen first yielded only two significant findings. Birds in both the red bias and the yellow bias treatments appear to have a lower probability of choosing white moths as their first attack (red bias: red vs. white: -2.974 ± 1.454 , $Z = -2.046$, $P = .041$; yellow bias: red vs. white: -4.650 ± 2.320 , $Z = -2.004$, $P = .045$). All other factors were nonsignificant ($P > .05$; table S3).

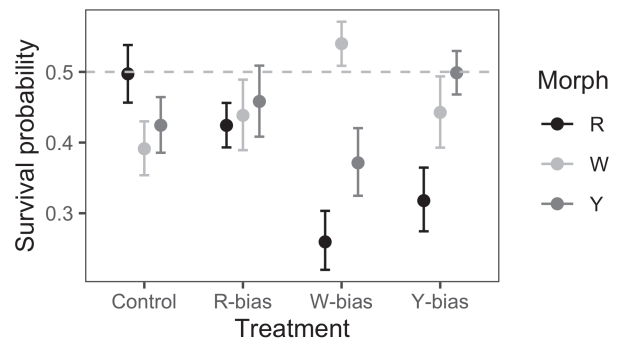
The red morph elicited more beak cleaning than the other morphs across all treatments (red vs. white: estimate = 0.794 ± 0.202 , $Z = 3.938$, $P < .001$; red vs. yellow: esti-

mate = 0.576 ± 0.194 , $Z = 2.966$, $P = .003$; fig. 4A). Beak-cleaning behavior also depended on bird sex by stage: immature males beak cleaned less (-0.845 ± 0.380 , $Z = -2.224$, $P = .026$). Overall, males either cleaned more when the red morph was common (control and red bias) or cleaned less when the red morph was rare (white bias and yellow bias, both $P < .05$) and showed no difference in stage ($\chi^2 = 4.947$, $df = 1$, $P = .026$; table S4; fig. S2). This was also explained by the significant treatment \times sex interaction ($\chi^2 = 11.923$, $df = 3$, $P = .008$; table S4).

Results were different for the model examining moths that were attacked and then promptly dropped. White moths elicited marginally more drop behaviors (e.g., white vs. yellow: estimate = 0.379 ± 0.200 , $Z = 1.892$, $P = .059$). Adult male birds dropped fewer moths overall (estimate = -13.226 ± 3.769 , $Z = -3.509$, $P < .001$). This was less pronounced for immature male birds (estimate = 0.958 ± 0.460 , $Z = -2.082$, $P = .037$). There was a treatment \times stage interaction such that immature birds dropped more in the red bias treatment than the control (estimate = 1.20 ± 0.574 , $Z = 2.099$, $P = .036$; fig. S2). There was a significant interaction between bird condition and treatment ($\chi^2 = 10.105$, $df = 3$, $P = .018$) such that condition had a negative effect in the white bias treatment (birds in better condition were less likely to grab and then drop moths; estimate = -0.898 ± 0.373 , $Z = -2.410$, $P = .016$) but no effect on the other treatments (table S5). Condition also interacted with bird sex ($\chi^2 = 11.880$, $df = 1$, $P = .001$) such that males in better condition were more likely to drop a moth than females (0.682 ± 0.198 , $Z = 3.449$, $P = .001$).

Differences in Ranked Order or Consecutive Attacks

The full model analyzing whether morphs differed in how early in the trial they were attacked across all treatments

**Figure 2:** Probability of surviving (not being attacked) to end of trial by moth morph and treatment. Error bars represent standard errors. R = red; W = white; Y = yellow.

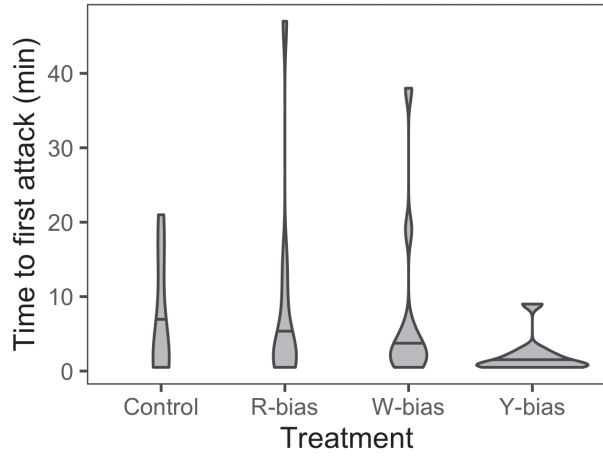


Figure 3: Violin plots showing treatment differences in distribution of time birds took to first attack a moth. Horizontal lines in the plots indicate the median time of first attack for that treatment. R = red; W = white; Y = yellow.

yielded no significant results overall ($\chi^2 = 1.05, P = .591$), except that red moths were chosen later than yellow moths (estimate = $0.371 \pm 0.168, t_{468} = 1.892, P = .028$). Analyzing separately by treatment, we found that this result was significant only in the control treatment, where there is a balanced morph ratio; red moths tended to be eaten later than white moths (nonsignificant; estimate = $0.279 \pm 0.123, t_{117} = 1.712, P = .090$) or yellow moths (significant; estimate = $0.371 \pm 0.166, t_{117} = 2.234, P = .027$). All bird-related covariates (sex, stage, and condition) had nonsignificant effects on the models (all $P > .90$) and were hence removed.

Model results addressing whether eating a given moth affected the probability of repeating the same moth showed a significant interaction between morph and treatment ($\chi^2 = 94.524, df = 6, P = .000$). Analyzing each treatment separately, unsurprisingly we find that each morph was significantly more likely to be repeatedly attacked in the treatment where it was more frequent, but this was not found in the control treatment with the balanced morph ratio (fig. 5; table S6). Again, all bird covariates (sex, stage, and condition) were nonsignificant; $P > .09$ were removed.

Discussion

This study joins the ranks of a small number of experimental studies that have examined the complex role of predator-driven selection for uniform aposematic warning signals. Specifically, we empirically tested, at a small spatial and temporal scale, the theoretical assumption of positive frequency-dependent selection in three naturally

co-occurring color morphs of an aposematic moth. Our results show that positive frequency-dependent selection operates on a local scale as expected but surprisingly differs in strength between the three morphs. By using real individuals and a spatial scale that allowed for clear observations of predator attacks and behavior, we found that wood tiger moths with white and red hind wing warning coloration showed a strong survival advantage when common, while the survival of the yellow color morph was not significantly affected by its frequency. Our findings support a recent study using moth plasticine models in the field (Rönkä et al. 2020), a previous spatial model (Gordon et al. 2015) in the system, and others outside of the system (e.g., Chouteau et al. 2016), which suggest that warning signals are subject to positive frequency-dependent selection. However, our study also highlights the short-term and fine-scale predator responses that underlie these results, thus adding mechanistic insight. We will now discuss our findings in depth by expanding on our main questions and end with suggested future directions for studies in this topic.

Do Birds Bias Their Decision to Attack Prey Based on Small-Scale Morph Frequencies?

Although we have found clear evidence for frequency-dependent selection in the wood tiger moth, this was not consistent across the three color morphs. Positive frequency-dependent survival, as stated earlier, is present in the white and red color morphs, with the yellow morph showing a nonsignificant trend in the same direction. Additionally, for the red morph, even though there was an apparent

Table 2: Generalized linear mixed model fixed effects affecting time to eat first moth across treatments

	Estimate	SE	Z	P
Intercept	-3.249	3.342	-.972	.331
Bird age (immature)	1.682	.485	3.469	.001*
Bird sex (male)	2.030	.784	2.589	.010*
Treatment:				
Red bias	1.003	.759	1.322	.186
White bias	.622	.742	.839	.401
Yellow bias	.171	.852	.201	.841
Bird condition	.181	.165	1.094	.274
Bird age (immature) ×				
bird sex (male)	-1.913	.671	-2.850	.004*
Bird sex (male) ×				
treatment (red bias)	-1.285	.961	-1.337	.181
Bird sex (male) ×				
treatment (white bias)	-.848	.967	-.877	.380
Bird sex (male) ×				
treatment (yellow bias)	-2.408	.995	-2.420	.015*

* $P < .05$.

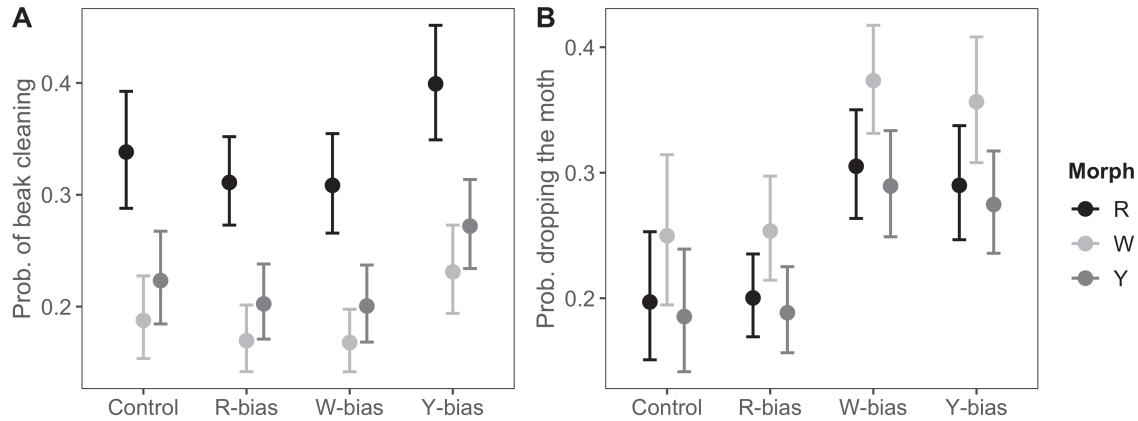


Figure 4: Effect of morph and morph frequency (treatment) on bird distaste behavior as measured by probability of beak cleaning after attacking a moth (A) and probability of dropping moth after attacking it (B). Estimates were extracted from a binomial generalized linear model. Error bars represent standard errors of the estimates. R = red; W = white; Y = yellow.

benefit to being common, unlike the other morphs it was only in comparison to their much lower survival rate when rare. The red morph also suffered very high proportional attack probabilities even though across all treatments they were often attacked later in the trials, suggesting that their visual signal is perhaps more initially aversive to birds (see also Lindstedt et al. 2011; Rönkä et al. 2018). These overall results may be attributed to a variety of explanations that are system specific and/or morph related.

First, morph frequency naturally fluctuates in the wild in this system. Therefore, selective pressure by predators may be more dynamic than we think; that is, wild populations may be quite affected by small scale frequencies. While red hind wing coloration in Finland is limited to females, males can be either white or yellow. In Central Finland, where this particular experiment was held, local populations show an overall stable white bias from year to year. This is known from yearly population censuses over a span of 10 years (Nokelainen et al. 2014; Rönkä et al. 2020) and an intensive 2-year mark-recapture study (S. Gordon et al., unpublished manuscript) showing that approximately 50% of the population has white hind wing coloration compared with 27% yellow and about 23% red. Additionally, while there is an overall bias toward white morph frequencies in Central Finland, the frequencies of each morph fluctuates throughout the summer flying season each year (S. Gordon et al., unpublished manuscript). It is not uncommon to have a calling female surrounded by multiple male morphs of varying frequencies at peak season and in certain locations. This contrasts from Southern Finland, for example, which shows a fluctuating biannual frequency of yellow and white morphs (Galarza et al. 2014). Indeed, a study by Nokelainen et al. 2014 in Southern Finland showed that the yellow morph had a survival advantage over the white

morph in the year that it was the common morph, while a second study (Rönkä et al. 2020) performed in Southern Finland a few years later in a year dominated by the white morph showed opposite results.

Second, differences in toxins between the male morphs may have caused variation in their attack rates. Although in our study we have used real individuals (which still present chemical defense), they were frozen and thus could not actively deploy their defense. A previous study suggests that while white moths seem to induce greater learning of their unpalatability to blue tits (*Cyanistes caeruleus*), yellow moths seem to promote stronger initial adverse reactions probably as a result of having a more aversive odor (Rojas et al. 2017). Without the active release of the pyrazines upon attack, yellow moths may have suffered a greater disadvantage if they have invested more in having an aversive odor rather than taste. Indeed, a recent study comparing models that included both color and chemical defense found an overall advantage of the white morph over the yellow (Rojas et al. 2019). This apparent difference in distaste between the two morphs may explain why white moths show a greater likelihood of being dropped than yellow moths after attack (fig. 4B). The same study also found that when visual and chemical cues were combined, models with white wings elicited significantly longer attack latencies, suggesting that experiments using models that contain only the visual component of the moth's defense may underestimate the aversiveness of the white morph compared with the yellow morph (Rojas et al. 2019). Regardless of chemical differences between the male morphs, our results seem to suggest that both may be less toxic than the red female morph, where birds hesitated more in attacking them and employed more beak-cleaning aversive behavior.

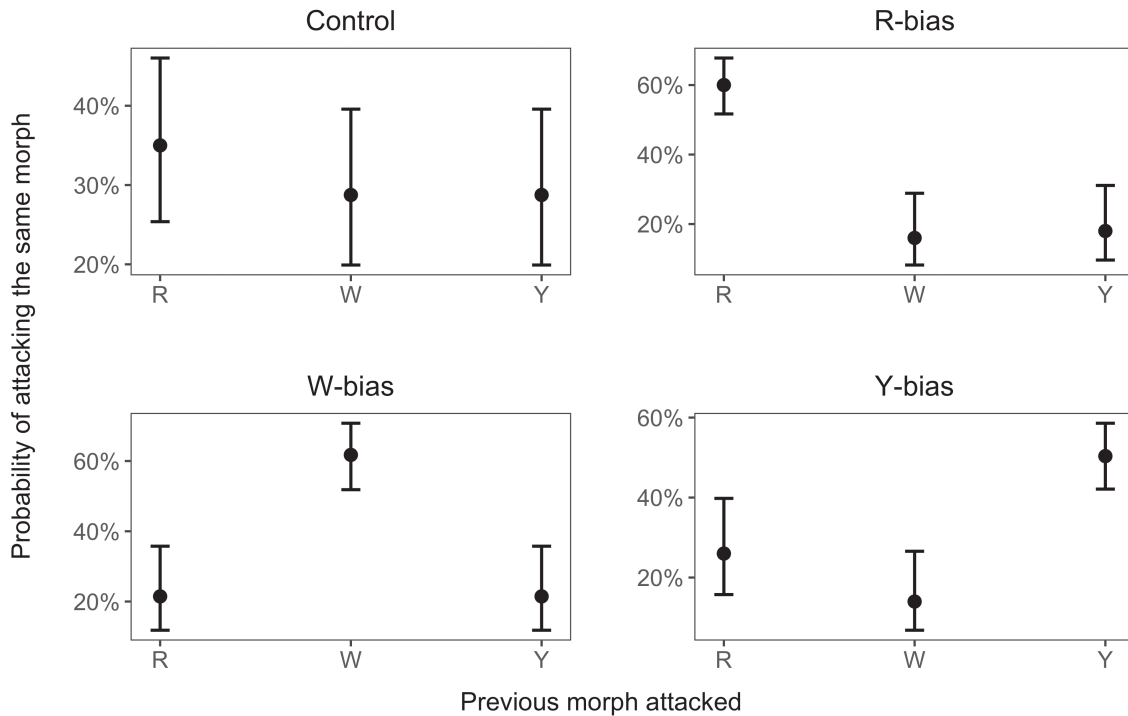


Figure 5: Probability of bird attacking the same morph on a consecutive attack for each morph and each of four treatments. R = red; W = white; Y = yellow.

Initial bias, their level of toxicity, and nutrition content may explain these results between males and females. Many studies highlight that birds and other organisms have an initial bias against red coloration (Ham et al. 2006; Teichmann et al. 2020), as many warning colors are red. However, although many organisms have innate aversions to the color red, it has also been shown that red coloration can be used as information for food quality (e.g., signaling ripeness in fruit; Albrecht et al. 2012). Therefore, it is possible that birds make context-dependent choices (depending on prior exposure, dietary needs, physiological condition, and food profitability) for whether to prefer or avoid prey with red coloration (Gamberale-Stille and Tullberg 2001; Teichmann et al. 2020). In our experiment, given that the red morph was attacked later, it is likely that the birds had an initial aversion that they then learned to quickly get over—to the extreme detriment of overall survival when there are few reds. This fits with previous studies in this system that show that blue tits learned to avoid red-colored wood tiger moth models faster than white or yellow moths (Rönkä et al. 2018) and that birds hesitated longer to attack red moths in comparison with more orange and yellow moths (Lindstedt et al. 2011). Notably, Lindstedt et al. (2011) found no corresponding survival advantage to red models in the field, mirroring our findings.

Learned color biases alone, however, cannot explain why the red morph suffered such a high attack rate despite being initially avoided. When looking more closely at the birds' behavior, we found that the red morph elicited, across all treatments, more beak cleaning, a characteristic response to distasteful prey (fig. 4A). This suggests that not only do females possess a stronger primary signal, but also they are more distasteful and likely toxic. Yet they still suffer high attack rates when rare. The answer to this may lie in the nutritional content of red females. Wood tiger moths are capital breeders, and females emerge as adults prepared to lay hundreds of eggs. This heavy investment in egg production means that females are a much more nutritious meal than male moths of both colors, with a much greater ratio of body mass to wing size. This effect can be seen quantitatively in the control treatment, where we can focus solely on predator choice without the effect of frequency. It shows that birds have an initial hesitation to eating red individuals (table 2) but a greater repeatability of eating a red morph after they have tried their first (table S6). This corresponds with the findings of several studies showing that predator attack motivation can be influenced by trade-offs between prey toxicity and nutrition (Barnett et al. 2007; Rowland et al. 2010a; Sandre et al. 2010; Marples et al. 2018), including Halpin et al. (2014), who show that European starlings

increase their intake of toxic prey when the prey's nutritional content is increased. Recognizing that warningly colored species may in fact lie on a shifting scale between profitable and unprofitable, depending on both the context and the experience of the predators, may help to explain how signal polymorphisms can persist.

Although we have here explained various reasons accounting for slight yet likely important differences between the morphs, overall the results are similar: moth warning coloration is under or trending toward positive frequency-dependent selection. What then are the local implications of these morph-specific frequency effects for the maintenance of polymorphism in the wood tiger moth? Why do we not see fixation of a particular wood tiger moth morph in populations across Central Finland, especially for the more common white male morph? While a generalist feeder, the wood tiger moth often shows a patchy distribution, and patches of different morph frequencies can occur on the local scale by chance. We know from prior experiments that positive frequency-dependent mating selection (Nokelainen et al. 2012; Gordon et al. 2015), thermoregulation (Hegna et al. 2013), and predator community structure (Nokelainen et al. 2014; Rönkä et al. 2020), among other factors, have been found to directionally shift selection favoring either white or yellow male morphs in different patches. A spatial model parameterized with positive frequency dependence and varying predator communities—combined with small levels of gene flow between the spatial patches favoring one morph over another—showed for the first time that hind wing polymorphism can indeed persist under these conditions in this system (Gordon et al. 2015). This gives even more weight to these fine-scale results.

What Predator Characteristics Affect Morph Survival, and Does Attack Order Matter?

Although the bird characteristics measured did not by themselves affect overall moth survival or the first moth chosen, it is important to note that the moths in our experiment were recently frozen; hence, they could not attempt to avoid a predation attempt behaviorally. Predator hesitation to attack a particular moth can be enough time for a live moth to drop into the foliage and escape predation attempts (personal lab and field observations). Therefore, even with a lack of an effect on survival in this experiment, predator characteristics and behavior are still vital topics to discuss in terms of biological relevance and morph fitness.

Our results show evidence of behavioral differences between the sexes and developmental stages of the predators used in this study, although their distribution between the treatments was balanced. In particular, adult great tit females are the most willing to rapidly attack prey across the trials,

while males at both stages and juvenile females take longer before making their first attack. Thus, it may be that female and male birds employ different foraging or antipredator strategies, potentially linked to the social environment that individuals encounter in the wild (Krams et al. 2010). Studies predict that in flocking populations, dominant individuals have greater access to restricted resources, such as food or habitat (e.g., Kaufmann 1983; Hegner 1985), and subordinate birds compensate for this through the use of unsafe foraging habitats (Ekman and Askenmo 1984), reduced cautiousness (Koivula et al. 1995), and faster resumption of foraging after the appearance of a predator (Hegner 1985). However, while adult females are generally protected from predators by adult males when they feed (I. Krams, personal communication), juvenile females—which may need energy resources the most (Krams et al. 2010)—are at a greater risk of attack from both predators and conspecifics. Therefore, juvenile females may hesitate more in attacking and/or make more mistakes in attacking toxic prey, as seen in these results.

Conclusion

Warning signal polymorphisms challenge the theoretical assumption that predation should impose frequency-dependent selection on warning coloration, favoring more common signals. In setting out to test that assumption in the wood tiger moth, we found strong evidence for its existence while also highlighting several potential mechanisms that may allow the coexistence of more than one morph in aposematic populations. Though expected to lead to purifying selection or fixation of a particular warning signal, there are instances where positive frequency-dependent selection can result in mosaic systems or intra- and interpopulation diversity (Holmes et al. 2017). For example, variability in predator behavior or motivation, variation in predation risk (or community) across temporal and spatial scales, morph-specific trade-offs in prey, and other forces (such as negative frequency-dependent sexual selection) can all lead to instances where multiple morphs can coexist, leading to the occurrence of multiple adaptive peaks across the fitness landscape. Our study found aspects of the first three processes occurring even when examining the responses of only a single predator species. Great tits show behavioral differences according to both stage and sex, particularly in their hesitation to attack certain aposematic prey. We also found that frequency variation on a fine scale influenced the attack risk on the different morphs and that the survival of different color morphs did not respond equally to these changes in frequency.

There are a few future directions that can follow from our study. As discussed above, the local population of wood tiger moths in the study area show a stable frequency bias toward the white male morph, potentially explaining the

strong positive frequency-dependent selection favoring its survival. However, separating cause and effect can be difficult: is the white morph more prevalent in central Finland because it has an advantage against the local predators, or has the previous experience of the predators with the white bias population preconditioned them to show greater avoidance of the white morph when common? Evidence has been found for population-level differences in great tit behavior toward aposematic prey (Exnerová et al. 2015), suggesting that even within a single predator species, predator variation may affect the outcome of studies such as ours. One way to examine this would be to repeat this experiment at other locations known to vary in morph frequencies. Future studies can also expand to include interactions between more than one predator, which can also provide valuable information as to the full dynamism of selective pressures and constraints under real-world conditions.

In conclusion, our findings highlight that while positive frequency dependence is important in many aposematic systems, it is not the only process shaping survival outcomes. Characterizing frequency-dependent selection under more conditions is therefore an important necessity toward understanding the evolution of warning signals under dynamic environments.

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Statement of Authorship

S.P.G., E.B.-S., and J.M. conceived and designed the study. S.P.G., E.B.-S., and J.K. performed the experiment and collected the data. S.P.G. analyzed the data. S.P.G. and E.B.-S. wrote the first draft of the manuscript, and all authors contributed substantially to revisions.

Data and Code Availability

Data have been deposited in the Dryad Digital Repository (<https://doi.org/10.5061/dryad.c866t1g69>; Gordon et al. 2021).

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