A tale of 2 signals: signal mimicry between aposematic species enhances predator avoidance learning

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Müllerian mimicry, where 2 or more unrelated aposematic species resemble one another, is predicted to reduce the per capita mortality of co-mimics by allowing them to share the cost of educating naïve predators about their unpalatability. However, the specific assumptions and predictions of Müller's theory of shared resemblance have been previously unsupported; some authors have suggested that the benefits of signal similarity are undetectable or at best very small. We demonstrate clearly and unambiguously that mimicry between 2 defended forms can provide substantial protection from uneducated predators in the manner proposed originally by Müller. By utilizing prey signals that were designed and demonstrated, to be equally visible, learned with equal facility, and discriminated by our predators, we assessed the effect of the presence of signal mimicry on the survival of a Model species in a "novel world" experiment, with wild-caught great tits (*Parus major*) as predators. We found that the net effect of mimicry was mutualistic, with co-mimics showing increased survivorship through shared predator learning. Visually distinct prey showed a mortality benefit from coexistence even without signal mimicry as a result of a density-dependent dilution effect. Perfect mimicry provided an added benefit of enhanced predator avoidance learning, and our results suggest that the benefits of shared warning signals may be even stronger than Müller originally proposed. *Key words:* aposematism, avoidance learning, evolution, Müllerian mimicry, *Parus major*. [*Behav Ecol* 21:851–860 (2010)]

Secondary defenses, warning signals of these defenses, and warning signal mimicry are widespread and important components of virtually all ecological communities. Mimicry is conventionally divided into 2 separate categories: In Batesian mimicry, an undefended prey species evolves the same warning signal as a defended unprofitable species, and the survival advantages of this form of mimicry are well established both empirically and theoretically (Fisher 1930; Clarke and Sheppard 1960a, 1960b; Brower et al. 1967; Lea and Turner 1972; Charlesworth D and Charlesworth B 1975; Lindström et al. 1997, 2004; Mappes and Alatalo 1997; Pfennig et al. 2001;). Conversely, support for specific assumptions and evidence for the general predictions of Müllerian mimicry, which describes the visual resemblance between 2 or more equally defended prey species, are lacking. Müller (1878, 1879) proposed that in order for a naïve predator to learn that prey of a given appearance are unpalatable, it must sample a fixed quantity of that prey (calling this number *n*). Therefore, a shared visual signal divides the fixed cost of educating a predator equally between co-mimics, the mortality of each species is reduced, and individuals belonging to both species gain protection (though if the predator takes a fixed number of prey, then 2 prey types with different densities will have different per capita mortality costs). Theory predicts that there is strong selection on pattern monomorphism in unpalatable prey so long as the mortality costs of predator education are sufficiently high (see, e.g., comments of Wallace 1889 on Müller, p. 256). However, current experimental evidence does not offer strong support to this prediction (Beatty et al. 2004; Rowe et al. 2004). If we accept the conclusions of these studies, alternative explanations for the evolution of signal mimicry would be necessary (see Sherratt 2008 for a review).

In one laboratory study that aimed to test Müller's proposed mechanism, Rowe et al. (2004) observed the foraging behavior of wild-caught captive great tits (*Parus major*) on artificial defended prey that differed in their visual similarity to a defended model. They found that increasing signal similarity had no significant effect on prey survival. However, because the signals of each prey type used in the experiment had shared features, it is likely that signal generalization between the model and the mimetic prey explains this finding. A key assumption in Müller's original theory is that individual birds learn nonmimetic and mimetic visual signals independently of each other and without any avoidance generalization between different signals. The experiment of Rowe et al. (2004) may be more properly considered as an investigation of the survival benefits of perfect mimicry compared with imperfect mimetic resemblance. In this context, perfect mimicry provided no statistically significant benefits compared with imperfectly mimetic prey, but this does not help us to validate or test Müller's specific theory.

Beatty et al. (2004) conducted another study, this time assessing the selection for mimicry using human predators and computer-generated prey. They found that when there are only 2 unprofitable prey types, selection for mimicry was weak. One reason for the results, they suggested, was that predators

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may not be sufficiently confused to generate selection for mimicry when just 2 different forms are involved. In an explanation for the evolution of conspicuous signals, Wallace (1889, p. 255) suggested that "not only do fewer individuals of each species need to be sacrificed in order that their enemies learn the lesson of their inedibility (in cases of mimicry), but they are more easily recognized at a distance and thus escape even pursuit. There is thus a kind of mimicry between closely allied species as well as between species of distinct genera, all tending to the same beneficial end." One explanation for Beatty et al's findings is that mimicry reduces confusion in visually complex environments.

It has also been argued, in a theoretical treatment, that the mere coexistence of visually distinctive aposematic species can be mutually beneficial (Turner and Speed 1999). If predators that ingest members of one chemically defended species become risk averse with respect to further toxin ingestion, while their physiology copes with the toxins, it has been suggested that predators may heighten avoidance of species that could contain toxins, even in the absence of signal mimicry. In this way, the species mutually protect each other, in that attacks on either can protect individuals of the other species (Leimar and Tuomi 1998; Turner and Speed 1999). If true, this would limit the additional survival advantage that can accrue from signal mimicry. At present, no tests of this hypothesis exist.

There is good support from studies on natural populations of defended prey and their avian predators for the idea that signal convergence between aposematic species is beneficial (Brower et al. 1963; Benson 1972; Mallet and Barton 1989; Kapan 2001; Langham 2006). These studies often show that relatively rare novel aposematic prey forms suffer higher mortality compared with established prey (Sherratt 2008). However, in these cases, the predators were likely to be already experienced with the native defended prey morphs before encountering novel prey morphs. In other words, they were already at least partially educated, and so the mortality costs of predator education were not evaluated. Furthermore, some of the survival benefits for mimicry in the abundant established prey may be better accounted for by alternative mechanisms to Müller's hypothesis of shared learning. Most notably, it has been suggested that educated predators manifest error-prone prey recognition (MacDougall and Dawkins 1998) and in a manner similar to Wallace's distance-recognition idea described above that one way for prey to minimize recognition errors is by aposematic signal mimicry. Here, the total number of signals is reduced, and the mimetic signal becomes more common and less easily confused with other prey appearances (for examples of alternative mechanisms and tests of them, see Wallace 1889; MacDougall and Dawkins 1998; Jansson and Enquist 2003; Sherratt and Beatty 2003; Beatty et al. 2004; Sherratt and Franks 2005). It is therefore important to assess the strength of selection between naïve and experienced birds.

A further alternative explanation for Müllerian mimicry is that co-mimics may have a Batesian-like mimetic relationship, with the less well-defended prey gaining protection by resembling the better defended prey form (Marshall 1908; Huheey 1976; Speed 1993). Experiments with unequal levels of defense have provided useful insights into the function of mimicry. For example, Ihalainen et al. (2007) found an interaction between signal variation and unpalatability on defended prey survival, though inexperienced birds did not select for signal similarity. Lindström et al. (2006) showed that both the signal and the palatability of prey affect the predation pressure on Müllerian co-mimics, and Rowland et al. (2007) showed that unequally defended co-mimics benefit from coexistence, with inexperienced birds selecting for signal similarity. However, these stud-

ies did not test the central assumption of Müller's original hypothesis, such that the specific assumptions of the mathematical model proposed by Müller as well as evidence for its general predictions are still lacking.

The primary aim of the current study is to present a systematic empirical test of Müller's original hypothesis that mimicry can be substantially beneficial because it allows the costs of predator education to be shared between co-mimics. To unequivocally evaluate whether per capita attack rate between 2 defended prey is decreased by signal mimicry during the predator's period of learning, it is important to use alternative prey signals that are unlikely to be generalized by the predator. We fulfill this requirement and simulate an ecological system in which there is an aposematic species called "Model" and another called "Mimic" that can manifest 1 of 2 forms: a perfectly mimetic Mimic that completely resembles Model or a distinctive nonmimetic Mimic that has no pattern similarity with Model. We first outline the methods used to design and test the prey signals, then we evaluate the benefits of signal mimicry and prey coexistence under the selective pressure of naïve and experienced avian predators.

METHODS

We tested the predation pressure imposed by visually hunting avian predators (wild-caught great tits, *P. major*) in a laboratory-based "novel world" experiment. In this environment, the individual great tits were initially naïve predators. The predators met 2 species of prey, one called "Model" and the other "Mimic," for simplicity. Model is an aposematic species of fixed appearance and Mimic comes in 1 of 2 morphs: 1) "perfect Mimic form," which exactly resembles the Model species and 2) "distinctive form," which by design has minimal visual resemblance to the Model.

The study comprised 3 conditions: 1) Model presented without either form of the Mimic species, 2) Model accompanied by the visually distinctive Mimic form, and 3) Model accompanied by the visually perfect Mimic form (see Table 1). The number of Model species was kept constant across all treatments (and all treatments included a visually distinctive edible prey).

Predators and prey

Seventy-nine wild great tits (*P. major*) were caught at feeding stations at 3 separate but similar sites around Konnevesi Research Station, Finland, and were used as visually hunting predators in an experiment that was conducted between January and March 2008 (with permission number KSU-2007-L-687/254 from the Central Finland Regional Environmental Center and Animal Experiment Board/State Provincial Office of Southern Finland, number ESLH-2007-09311/Ym-23). Each bird was housed separately in indoor plywood cages, with a daily light period of 11.5 h and ad libitum supply of tallow, sunflower seed, and water, except for the days of training and trials (when the birds were deprived to promote food searching motivation).

The artificial prey items used in the experiment were small pieces (~0.1 g) of almond glued (UHU™ nontoxic glue) between two 8 × 8 mm pieces of paper. Onto the paper was printed a black and white signal (cross, circle, or square; see Table 1). Unpalatable prey (circles and squares) were made unpalatable by soaking the almonds in chloroquine phosphate solution for 1 h (2 g of chloroquine dissolved in 30 ml of water). The chloroquine dosage matched that used in a previous experiments (Ihalainen et al. 2007; Rowland et al. 2007). Cryptic edible (cross) prey contained untreated almond.

Table 1
Experimental setup

Treatment 1 (Model alone)			Treatment 2 (Model + distinct Mimic)			Treatment 3 (Model + perfect Mimic)		
Signal	Type	No.	Signal	Type	No.	Signal	Type	No.
⊠	Cryptic	50	⊠	Cryptic	50	⊠	Cryptic	50
■	Model	50	■	Model	50	■	Model	50
⊛	Mimic	—	⊛	Mimic	50	■	Mimic	50

or

Treatment 1 (Model alone)			Treatment 2 (Model + distinct Mimic)			Treatment 3 (Model + perfect Mimic)		
Signal	Type	No.	Signal	Type	No.	Signal	Type	No.
⊠	Cryptic	50	⊠	Cryptic	50	⊠	Cryptic	50
⊛	Model	50	⊛	Model	50	⊛	Model	50
■	Mimic	—	■	Mimic	50	⊛	Mimic	50

The types and numbers of prey presented in the experimental treatments are shown. An alternative edible prey was cryptic (a cross) as it matched the crosses on the aviary background where prey was presented. Models were a black square signal for half of the birds, and black circle for the other half of birds. Mimics were either distinct (opposite signal to Model, treatment 2) or perfect (same signal as Model, treatment 3). Numbers in the columns correspond to the number of each prey type presented at the start of a trial.

Training

Opening prey items
All birds were trained to open the artificial prey and eat the almond contained within in their home cages in 4 stages (following the procedure outlined in Ihalainen et al. 2007). We first offered birds almond slices, then 5 prey with a piece of almond glued onto one side of a white piece of paper (sized 8×8 mm), then 5 prey items with the almond sticking out of a paper parcel, and finally, 5 prey items with the almond enclosed within the paper parcel. Each bird was required to eat all prey items at each stage before moving to the next phase.

Foraging in the aviaries
Next, the birds were trained to find prey items on artificial cross backgrounds (in the small aviary) and to use the perches in the large aviary. We spread 3 cryptic prey items and 12 white prey items onto the aviary floor in groups of 3. Each bird was required to eat all prey items in order to be considered ready to participate in the main learning experiment. We also familiarized the birds with the large aviary by placing birds to overnight and feed inside the aviary. The artificial background was replaced by transparent plastic covered with peanuts and sunflower seeds.

Aviaries

The experiments were conducted in 3 aviaries (1 large and 2 small). The large aviary ($57.7 \text{ m}^2 \times 3.5$ m height) had a floor covered with white A3 size paper sheets, glued together, and covered with adhesive plastic to form a grid of 15 rows and 22 columns. Between rows were wooden dividers (ca. 6-cm wide boards) to facilitate prey handling and movement of the birds. Printed onto each A3 sheets were 70 crosses and 10 fake cryptic prey items (8×8 mm pieces of cardboard with printed crosses glued on the top) glued in random positions on each sheet. The fake prey items made the background 3D, which aided camouflage of the cryptic prey. In the large aviary, there were 8 perches at a height of 0.5 m to allow prey handling.

The small aviaries ($13.5 \text{ m}^2 \times 2.4$ m height) had the same floor coverage of A3-sized paper sheets with crosses printed onto them and with fakes. The small aviary consisted of 8 rows of 10 paper sheets. Each of the smaller aviaries had 2 perches. Only 1 prey item was placed onto a single sheet to aid the experimenter in identifying the attacked items. Birds were observed through a one-way glass window. Fresh water was always available in the aviaries.

To test for any differences between predation behavior in the 2 aviary sizes, we assessed the first prey choice by birds in the visibility test (small aviary) and the main experiment, treatment 2 (large aviary). The first choices of the birds were equal for the aposematic signals we used: Wilcoxon signed-rank test $n = 24$, $Z = -0.258$, $P = 0.796$, suggesting that predatory behavior is not affected by foraging arena.

SIGNAL DESIGN

Because minor manipulations in signal design/unpalatability levels can change the results and conclusions of experiments fundamentally (Lindström et al. 2004, 2006; Ihalainen et al. 2007), it was important for us to carefully assess the various attributes of our signal designs. Given that our main aim was to accurately assess the survival benefits of signal similarity, we needed a Mimic prey form that was treated as visually distinct from the Model by the birds, so there would be minimal effect of visual generalization benefit. Additionally, it was equally important that neither Model nor Mimic were generalized to the palatable cryptic prey because the birds might then be inclined to keep attacking prey because of

confusion with edible prey. Because calculations of prey mortality can be confounded if birds prefer one of the warning signals to the other or if there is a difference in prey visibility, our signals designs needed to have equal visibility risks, no differences in initial avoidances/preferences, and no visual generalization benefit.

We used 3 separate tests of signal design to test for visibility differences, learning efficiency, and strength of visual generalization.

Visibility of the signals

We used 14 birds to test the visibility of the prey signals on the aviary background. We first familiarized the birds with all signals by allowing each of the birds to eat 5 of each type of prey item in their home cages. This was to encourage them to attack all signals according to their visibility rather than through any innate bias. Twenty prey items of each signal (square, circle, and cross) were randomly distributed on the cross background in the small aviary. Each bird was allowed to find (peck or eat) 20 prey items at which point the trial was ended. We recorded the number of each signal found and observed that there was no significant difference in the mean numbers of squares and circles found by the birds (Figure 1, paired samples test $t_{13} = 0.078$, $P = 0.939$), but fewer cryptic prey items were found on average than the other 2 prey forms (paired samples test $t_{13} = 3.663$, $P = 0.003$ and $t_{13} = 4.080$, $P = 0.001$ comparing numbers of cryptic prey found to squares and circles, respectively). A signaling prey item was around twice as visible to the birds as a cryptic prey item; on average, 8 of both signaling items were found compared with 4 cryptic items.

Learning efficiency

We tested whether the 3 prey types to be used in the main experiment were equally efficient in eliciting avoidance learning. Twenty-three birds were individually familiarized with an experimental cage (plywood, sized $50 \times 50 \times 70$ cm), during which time, 3 sets of 5 seeds were offered on a Petri dish through a doorway. Each bird was required to eat all seeds in all 3 stages before being allowed to participate in the learning trials. The bird was placed individually into the experimental cage and observed through a small net-covered window on the cage. On the day of the learning trial, we ran-
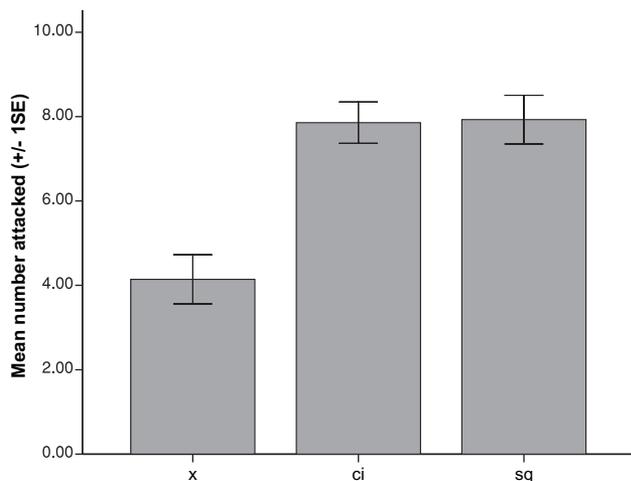
domly assigned the bird to a treatment group, according to which signal would signify unpalatability (signal learned: unpalatable circle, $n = 8$; unpalatable square, $n = 8$; and unpalatable cross, $n = 7$). In order to start the learning trial, the bird had to take and eat 3 blank prey items made from brown paper, with a small piece of almond inside. When the bird had successfully taken and eaten these 3 prey, the learning experiment commenced. The prey items (edible blank brown prey and signaling defended prey; either cross, circle, or square) were offered singly through a hatch, so that the blank and the signaling prey were presented sequentially in a random order. We offered 5 of each prey type to the bird per learning test and the bird participated in 4 learning trials during the day, with at least 30-min breaks between learning trials. The bird was allowed 1 min to eat the prey item, after which the prey was removed and classed as rejected. We offered prey every 2 min for 20 min.

When we presented the birds with blank edible prey and one of the signaling prey, to which we added chloroquine (cross $n = 7$, circle $n = 8$, or square $n = 8$), all birds learned to avoid the signaling prey as the learning trials proceeded (Figure 2a, repeated measures analysis of variance [ANOVA] [$F_{3,57} = 13.18$, $P < 0.001$]), and the signals were learned equally efficiently (no interaction of trial and signal type [$F_{6,57} = 0.370$, $P = 0.892$]). Signal main effect $F_{2,20} = 0.471$, $P = 0.631$.

Visual generalization

The same 23 birds as used in the learning trial were then used to test for visual generalization between the prey types. It was important to determine whether the birds could discriminate between the 3 prey signals and that they would not generalize their learned avoidance of 1 signal to the 2 other signals that would be used in the learning experiment. So for a bird that had learned that squares were unpalatable, we would expect it to readily eat circles and crosses, of which it would have no prior experience, and for it to eat circles and crosses at a similar rate to one another.

We again used experimental boxes with each bird offered 3 prey types (square, circle, and cross), singly through a hatch, so that the 3 signaling prey were presented sequentially in a random order. All 3 prey types were palatable. Five of each prey type were offered to the bird per generalization test. The bird was allowed 1 min to eat the prey item, after which it was removed and classed as rejected. Prey were offered every 2 min for 30 min.

The mean number of each type of prey killed during generalization testing was analyzed separately for each of the 3 experimental groups (signal learned: unpalatable circle, $n = 8$; unpalatable square, $n = 7$; and unpalatable cross, $n = 7$) using a repeated measures ANOVA, with prey type (cross, circle, and square) as the within subjects factor. When birds that had learned to avoid circles were presented with all 3 signal types, we found that circles were eaten significantly less than novel cross and square prey, which were eaten at an equal rate to each other (Figure 2b; $F_{2,14} = 4.44$, $P = 0.032$; contrast cross vs. square $F_{1,7} = 0.00$, $P > 0.05$). Birds that had learned squares were unpalatable continued to avoid squares in the generalization test but ate significantly more circles and crosses, which were eaten at a similar rate to each other (Figure 2b; $F_{2,14} = 5.51$, $P = 0.017$; contrast circles vs. cross $F_{1,7} = 0.00$, $P > 0.05$). Furthermore, birds that had learned to avoid crosses ate significantly more squares and circles, which were also taken at a similar rate to one another (Figure 2b; $F_{2,14} = 15.00$, $P = 0.001$; contrast squares vs. circles $F_{1,7} = 1.00$, $P = 0.356$). Therefore, in all cases, the prey type that birds had learned to avoid was taken significantly less often than the
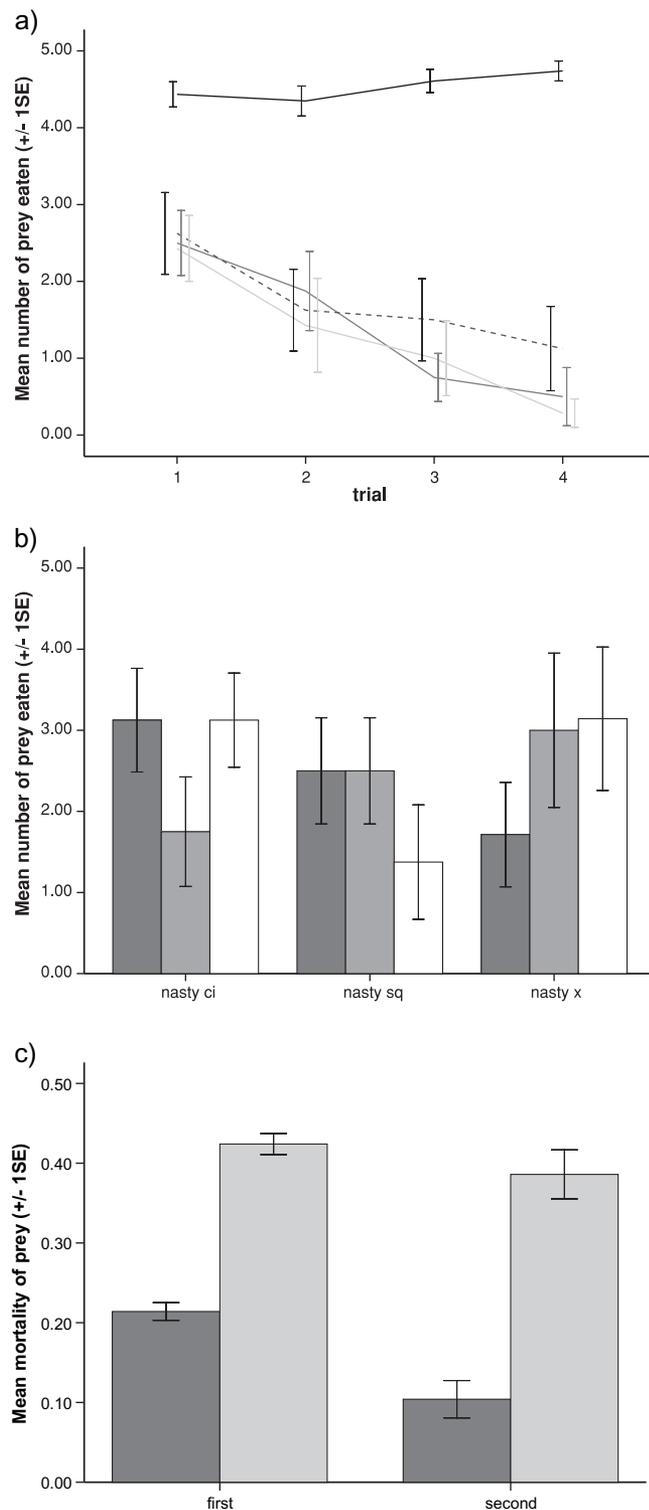

Figure 1
The mean number of prey attacked in the visibility test, when all prey were edible. Error bars represent ±1 standard error.

a)

b)

c)

Figure 2

Learning efficiency and visual generalization of prey types.
(a) Learning curves of number of each prey type eaten across the 4 learning trials (blank prey, black line; circle prey, dashed line; cross prey, light gray line; and square prey, dark gray line); (b) the mean number of cross (dark gray bar), square (white bar), and circle (light gray bar) eaten in the experimental box test, when the learned signal was circle, square, and cross (left to right); and (c) the mean number of defended Model (dark gray bar) and edible Mimic (light gray bar) prey eaten in the aviary test on day 1 (first learning trial) and day 2 (second learning trial).

2 novel prey types, which showed that the birds did not generalize their learned avoidance to the other prey types.

Visual generalization on backgrounds

A previous study by Ihalainen et al. (2008a) carefully assessed signal design, as we have presented here, but found that when the signals became associated with unpalatability in the main learning experiment that the signals differed in their efficiency as warning signals. For this reason, we conducted a second test of whether the birds generalized their avoidance behavior, in a setup identical to the main learning experiment, and conducted this in the large aviary. Ten new birds were used to confirm whether the birds could tell the difference between the prey when one was unpalatable.

We kept the number of Model prey (square for half the birds and circle for the remainder) constant at 50 and also presented 50 edible cryptic prey items as alternative edible prey. The Model prey were accompanied by 50 distinctive Mimics, which were edible (opposite signal to Model prey; square or circle). We released each bird singly into the experimental aviary and recorded the number and type of prey attacked. We noted a prey item as killed when the bird opened the paper shell and took a bite of the almond within. Each bird was required to "kill" 40 prey items before we ended the trial. Each bird participated in 2 trials on consecutive days, which allowed us to make exact comparison with the main experiment's naïve and experienced birds.

We found that when the birds were naïve, they could discriminate between the square and circle when one was unpalatable. The birds ate significantly more of the distinctive Mimic, which was edible, than the defended Model prey (Figure 2c; paired sample t-test $t_9 = -14.61$, $P < 0.001$). This was also true for experienced birds (paired sample t-test $t_9 = -5.98$, $P < 0.001$). Therefore, the birds could differentiate between the unpalatable Model prey and edible Mimic prey and did not generalize avoidance learning between the prey types.

Hence, we can conclude that 1) our square and circle are equally conspicuous to the predators, 2) that they are learned about with equal efficacy, and 3) that they are sufficiently distinctive that predators do not tend to generalize between them in a foraging environment.

MÜLLERIAN MIMICRY EXPERIMENT

Methods

Because we found no visibility difference, no difference in learning efficiency, and no generalization benefit between circles and squares (see "RESULTS"), we then designed the main learning experiment to determine whether signal similarity is beneficial for defended prey types or whether visibly distinct prey can mutually protect each other, simply by possessing the same defense chemical, without signal similarity. We assessed the strength of selection on these different systems by naïve and experienced predators.

Thirty-two birds participated in the main learning experiment, which comprised 3 experimental conditions 1) a defended Model prey alone, 2) a defended Model prey accompanied by an equally defended distinctive Mimic, and 3) a defended Model prey with a perfect Mimic. The signal of the Model and Mimic prey was a black square or a circle. Our design was counterbalanced, so that half of the birds received square as the Model prey type, whereas the remainder received circles.

We kept the number of Model prey constant at 50, and we also presented 50 edible cryptic prey items in all treatments as alternative edible prey. We released each bird into the large

experimental aviary and recorded the number and type of prey attacked (both killed and attacked). The position of each prey type was determined by randomly generated maps produced prior to each test, no 2 birds had the same configuration, and birds had different maps for each trial. Visibly indistinguishable prey were denoted as either Models or Mimics on the map for the authors to track.

A prey item was noted as killed when the bird opened the paper shell and took a bite or ate the almond contained within the shell. Each bird was required to kill 40 prey items before we ended the trial. Each bird participated in 2 trials on consecutive days.

Statistical analyses

We used several ways to assess the predation pressure on our prey communities. We determined the absolute numbers of prey killed by each bird and calculated the per capita mortality of each prey type by dividing the total number of each type of prey killed during the trial by the number presented. We also calculated a measure that we term "relative predation risk" by dividing the number of each prey type taken by the predicted number that would be killed if predation across the available prey forms was random, regardless of conspicuousness and defense levels. For example, in treatments 2 and 3, if predation was random, we would expect the birds to kill 13.33 crosses, 13.33 Models, and 13.33 Mimics. If the value of relative predation risk is 1 for all 3 prey types, then we can assume that the birds choose prey at net rates that are equivalent to random, whereas if the value is lower or higher than 1 for one or more prey, then we can assume that the attacks by the birds are under or over the rate predicted by random chance.

Because previous studies have sometimes found that one type of signal is more efficient in the learning context than another signal (Lindström et al. 2004, 2006; Ihalainen et al. 2008a), we also needed to determine whether the birds treated Model and Mimic prey equivalently in the main learning experiment. In order to determine whether we had equivalent signal design, we conducted a repeated measures ANOVA on the mean prey mortality, with trial (1 = day 1 or 2 = day 2) and prey type (Model or Mimic) as within subjects factors and treatment (2 and 3) as a between subjects factor.

The data did not require transformation to satisfy requirements of parametric statistics, and they satisfied the assumptions of repeated measures ANOVA, including normality (Shapiro–Wilke) and sphericity (Mauchly's). We analyzed the data using repeated measures and univariate general linear model with Tukey post hoc pairwise comparisons in SPSS v.16.0.

RESULTS

We analyzed the effect of quality of mimicry (no mimicry vs. perfect mimicry) on the survival of Model and Mimic taking into account the effects of predator experience. We ran a full factorial $2 \times 2 \times 2$ repeated measures ANOVA with prey type (Models/Mimics) and trial (first = day1/second = day 2) as within subject factors and experimental treatment (2 or 3) as a between subject factors.

We found a significant reduction in both Model and Mimic mortality when there was perfect mimicry ($F_{1,20} = 17.89$, $P < 0.001$). We found a significant difference between trials, which reflected the lower mortality of both Models and Mimics when the birds were experienced ($F_{1,20} = 62.59$, $P < 0.001$). Our signals had been designed to be distinct and nongeneralizable, which was supported by the nonsignificant interaction between the type of prey (Model or Mimic) and the treatment (distinct or mimetic prey) ($F_{1,20} = 0.619$, $P = 0.440$). The three-way interaction between prey type

(Model/Mimic) $\times$ trial $\times$ treatment was nonsignificant ($F_{1,20} = 0.013$, $P = 0.912$) (All other interactions were nonsignificant, $P > 0.1$, see Supplementary Material, see Supplementary Table 1).

The original theory presented by Müller (1878, 1879) assumed the consumption of a predicted "n" individuals of each prey type in the total process of predator education. Therefore, we first analysed the overall mortality, where the number of Model prey killed over the 2 learning periods was combined. The addition of distinct Mimics (treatments 2) significantly reduced Model mortality (Figure 3a; $P = 0.008$; overall ANOVA general linear model $F_{2,32} = 23.52$, $P < 0.001$), as did the addition of perfect Mimics ($P < 0.001$). We found that the Model species had significantly lower mortality when presented with the perfect Mimic form than with the distinctive nonmimetic form of the Mimic species (Tukey post hoc; $P = 0.005$).

We conducted separate analyses to follow up these results, focusing on Model prey mortality for brevity (because mimic prey mortality is equivalent in results). We pooled signal design (circle and square) because signals were counterbalanced and equally efficient. We analyzed Model prey mortality split by trial (naïve and experienced) using univariate ANOVA.

Naïve predators

Birds were tested in 2 learning periods spread over 2 days. When the birds were naïve, in the first learning period, we found that the addition of Mimics (either perfect or distinct) significantly reduced Model mortality (Figure 3b, dark gray bars; $F_{2,32} = 40.53$, $P < 0.001$). It has been suggested that aposematic prey may benefit by mere coexistence with other aposematic species even without signal mimicry (Turner and Speed 1999). We confirmed this possibility with the finding that the mortality of Model prey presented alone was significantly higher than Model species accompanied by distinct Mimics ($P < 0.001$).

Because the Model species benefits from coexistence with even a distinctive Mimic, it is important to evaluate whether perfect signal mimicry between Model and Mimic adds any further per capita survival benefit. We found that the Model species had significantly lower mortality when presented with the perfect Mimic form than with the distinctive nonmimetic form of the Mimic species (Tukey post hoc, $P = 0.016$). Models with perfect Mimics also had lower mortality than Model prey presented alone, $P < 0.001$. We were able to estimate the extent to which signal mimicry adds survival benefit to that already delivered by simple defensive mutualisms by calculating effect sizes; the observed decrease in Model prey mortality when accompanied by perfect Mimics compared with Models with distinctive Mimics had an effect size (r) of 0.61 when the birds were naïve.

Because the total density of prey increased when Mimics were added to the setup, the risk of predation for a member of the population of Models (the likelihood of being eaten by chance) decreased. To control for this dilution effect, we calculated the predation risk for the Model relative to that predicted assuming random predation across prey forms (see "METHODS" and Rowland et al. 2007). Model species did not show a decrease in relative predation risk with the addition of distinctive Mimics (Figure 3c; Tukey post hoc test: Model alone compared with Model accompanied by distinctive mimic, $P = 0.994$, main ANOVA $F_{2,32} = 7.97$, $P = 0.002$). Only the addition of perfect Mimics reduced the relative probability of a Model prey species being attacked (Tukey post hoc test: Model alone compared with Model accompanied by perfect Mimic, $P = 0.005$ and Model accompanied by distinctive Mimic compared with Model accompanied by perfect Mimic, $P = 0.006$).
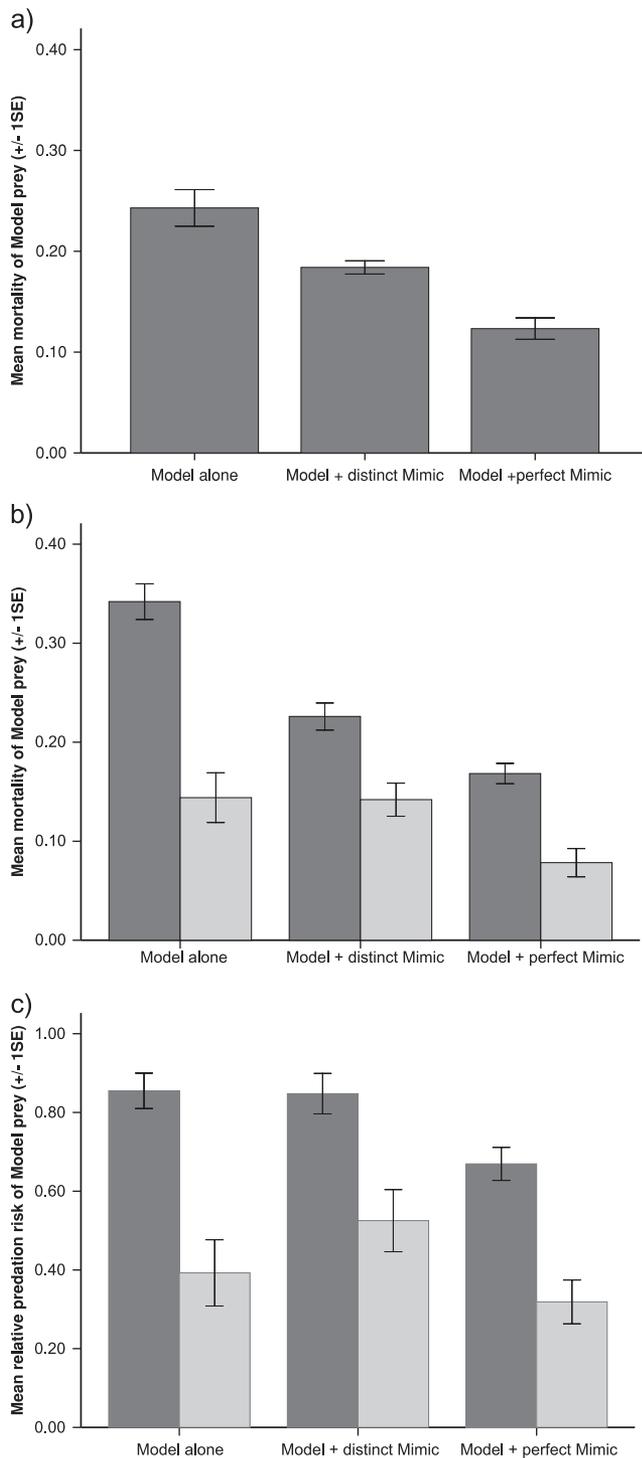
Figure 3
The mortality and relative predation risk for Models. (a) The total mortality of Models combined for both learning periods combined. (b) The mean number of Model prey eaten in the first learning trial (dark gray bar) and second learning trial (light gray bar) in treatments were a defended model prey was presented alone (Model alone), accompanied by visually distinct, equally defended mimic prey (Model + distinct Mimic), or accompanied by visually mimetic equally defended prey (Model + perfect Mimic). (c) The mean predation risk in the first learning trial (dark gray bar) and second learning trial (light gray bar) in treatments were a defended Model prey was presented alone (Model alone), accompanied by visually distinct, equally defended Mimic prey (Model + distinct Mimic), or accompanied by visually mimetic equally defended prey (Model + perfect Mimic).

Considering predation across prey forms in this manner, signal mimicry rather than the simple addition of nonmimetic aposematic prey was the only beneficial strategy for prey.

Experienced predators

When birds were experienced (but had not necessarily completed learning), we found that the addition of distinctive Mimics to the Model community was no longer beneficial, with the mortality of Model prey presented alone not significantly different to that of Model prey accompanied distinctive Mimics (Figure 3b, light gray bars: Tukey post hoc, $P > 0.05$, overall ANOVA, $F_{2,32} = 4.19$, $P = 0.025$). The addition of perfect Mimics significantly lowered the mortality of Model prey compared with when Model prey were presented alone ($P = 0.046$). However, when we control for the dilution effect of adding Mimics to the system, we found that the addition of the distinctive Mimic did not significantly change the mean relative predation risk on the Model prey (Tukey post hoc test: Model alone compared with Model accompanied by distinctive Mimic, $P = 0.131$, overall ANOVA, $F_{2,32} = 4.32$, $P = 0.023$; Figure 3c, light gray bars) nor did the presence of signal mimicry significantly reduce the relative attack probability of the Model prey (Tukey post hoc test: Model alone compared with Model accompanied by perfect Mimic, $P = 0.706$), suggesting that Model prey accompanied by perfect Mimics only benefited from a density-dependent decrease in mortality through dilution of the prey community.

Assessing the quantitative predictions of Müller's original Model

In addition to our measures of predation pressure on our prey communities, we tested whether our results matched the quantitative predictions of Müller's original model, which stated that if n is the number of individuals of a distinct species that are eaten in the course of predator education, then in a situation where 2 or more equally defended and equally abundant species resemble one another, the fixed cost of educating the predator is divided between the co-mimics ($n/2$). Furthermore, when presented together, dissimilar looking species should each lose n individuals through predation and gain no survival benefit from coexistence.

Müller's original model is based on the entire period of predator learning. We therefore estimate one value of n, based on both learning trials combined. For treatment 2, dissimilar looking species lost $n = 35.6 \pm 4.22$ individuals through predation. For treatment 3, we followed Müller's equation (i.e., na1/(a1 + a2)), where $n = 35.6$, and a1 was the number of Models (i.e., 50) and a2 the number of mimics (50). The predicted number of individuals eaten in the course of predator education was $n = 17.8$. We then tested whether the observed number of visually mimetic prey killed matched the predictions based on this values of n (see Supplementary Material section iii).

When we compared the mean number of visually mimetic co-mimics eaten, to those predicted from Müller's equation, we found that the observed numbers eaten were higher than those predicted by Müller's original model ($t_{11} = 3.308$, $P = 0.0070$; see also Greenwood et al. 1989).

We then calculated 2 estimates of n to determine any differences between naïve and experienced predatory behavior. We again calculated the mean total number of prey taken in treatment 2. When the birds were naïve in the first learning trial, $n = 21.9$ prey ± 3.142. When the birds were experienced in the second learning trial, $n = 13.7$ prey ± 4.00.

In the first learning trial, the observed numbers of visually mimetic prey eaten was higher than predicted by Müller's equation, ($t_{11} = 5.284$, $P < 0.001$). In the second learning trial, we found that the birds attacked visually mimetic prey in accordance with the prediction from Müller's equation ($t_{11} = 0.752$, $P = 0.468$. Hence, in the second learning trial, the mortality benefit from converting the Mimic from a distinctive to a perfectly mimetic form was as predicted by Müller's equation.

DISCUSSION

Müllerian mimicry, where 2 or more unrelated aposematic species resemble one another, has been supported comparatively (Dumbacher and Fleischer 2001), through mathematical modeling (Mallet 1999) and biogeographically (Mallet and Barton 1989). Müller originally proposed that the mortality cost to prey of educating predators would be reduced if the prey shared a warning signal. However, evidence for strong selection on pattern monomorphism in unpalatable prey is lacking: Beatty et al. (2004) did not find selection for signal monomorphism when model and mimics were equally abundant, or in simple prey communities, and Rowe et al. (2004) found that predator avoidance learning was not significantly faster when one pattern signaled unpalatability than when there were 2 different patterns. We argue that prior to our study presented here, no experiment existed that specifically assessed the benefit of signal similarity in the way that Müller (1878, 1879) proposed. We evaluated the benefits of mimicry by utilizing prey signals that were designed and demonstrated, to be equally visible, learned with equal facility, and discriminated by our predators. With these important criteria met, we clearly and unambiguously demonstrated that signal mimicry between 2 defended forms provides protection from predation by sharing out the costs of learning, as Müller originally proposed. Alongside recently published work, our present experiment raises some important questions about signal mimicry, and we deal with these in turn.

Is there strong selection for perfect mimicry through predator learning?

One of the key points made in some recent papers on Müllerian mimicry is that selection for mimicry may be weak when the prey are abundant relative to the number of naïve predators in a habitat (Mallet and Joron 1999; Beatty et al. 2004). The argument here is that if predators learn relatively quickly (i.e., n is small), but the total prey number (N) is large, then the per capita risk of death from predator learning (n/N) is itself relatively small. Mallet and Joron (1999) use this result to explain the persistence of mimetic polymorphisms in some lepidopteran species. In contrast, Beatty et al. (2004) used a similar argument to show that Müller's mechanism may be of relatively trivial importance in large prey populations, and they argued that visual complexity of prey appearances within a habitat instead fosters the evolution of mimicry. Superficially, our data appear to support the idea that the costs of predator education, though present, may be quite small. In terms of absolute change in per capita survival probability, the effect of perfect mimicry on prey survival is quite small (0.0252, i.e., [survival frequency for perfect mimic − survival for distinctive nonmimic = 0.9392 − 0.914]). However, our data are for one average predator foraging for a total of 80 from 300 prey in total (100 Mimic, 100 Model, and 100 edible cryptic prey). If we had more predators, however, the selective difference would soon become quite substantial. We can estimate this if selection (s) against the nonmimetic form is measured as: $1 − ([yn_d/N]/[yn_p/N])$, where y is the number

of predators, n_d the number of distinctive Mimics attacked in treatment 2, and n_p the number of perfect mimics attacked in treatment 3 (and N is the total number of Mimics put out). For one predator, selection is present but comparatively weak, $s = 0.027$, whereas multiplying upward for 4 predators, $s = 0.133$, a substantial force against the nonmimetic prey would pertain. Measures of selection by predators against novel aposematic forms in natural habitats tend to generate selective values similar to, or larger than, this (Sherratt 2008). Having established that costs of learning in relation to mimicry can be demonstrated and quantified, a really key ecological question is then the ratio of naïve predators to prey within each habitat.

Previously, an experiment by Rowe et al. (2004) found no detectable benefit from improving the quality of mimetic resemblances from imperfect to perfect. Although the results of their experiment could arguably explain the occurrence of imperfect mimicry and polymorphisms in Müllerian mimicry complexes (Turner 1987; Joron and Mallet 1998; Mallet 1999), there are nonetheless many examples of very high quality mimicry observed between unpalatable species (Benson 1972; Mallet and Barton 1989; Dumbacher 1999; Kapan 2001; Symula et al. 2001). How then might perfect mimicry evolve if selection for perfection is weak as Rowe et al. (2004) reported? One argument that may be presented is that variation in the signals presented in Rowe et al. fell short of some threshold point at which mimicry becomes highly effective. We can, however, again use the argument that over the course of a season, there may be much greater pressure from naïve predators per individual mimetic prey in the real world compared with those often simulated in laboratory investigations, such that the costs of imperfect mimicry are likely to be much higher in the real world. Additionally, most previous studies (Rowe et al. 2004; Ihalainen et al. 2007; Ihalainen et al. 2008a though see Ihalainen et al. 2008b) have focused on the initial learning phase of predation. However, as Ihalainen et al. (2007) noted, natural prey populations are exposed not only to predation by naïve predators but also experienced hunters, which may pay more attention to subtle differences in visual signals because they have more knowledge of these prey, and selection may be even more intense (see Langham 2004). However, we are not yet able to quantify the role that fully educated predators play in the selective pressure for visually mimetic prey. As Wallace (1889) and later MacDougall and Dawkins (1998) hypothesized, if fully experienced predators manifest error-prone prey recognition, prey may reduce the cost of mistaken identity by aposematic signal mimicry. A further experiment, where birds are trained so that they become fully experienced, is necessary to assess the selective pressure imposed by fully educated predators.

Our experiment and Müller's theory

A key assumption in Müller's original theory is that individual birds must learn nonmimetic visual signals independently of each other and without any avoidance generalization between different signals, which leads to the general prediction that perfect mimicry is at a selective advantage over no visual resemblance between prey. We find clear support for this hypothesis. During predator learning, signal mimicry reduced the per capita mortality of Model prey. Importantly, we can refute the possibility that the benefit from mimicry can be explained by simple density-dependent dilution of mortality because the visually mimetic prey were also less likely to be attacked by predators during learning (measured as relative predation risk).

When we tested whether our results matched the quantitative predictions of Müller's original model (see supplementary material), by estimating the value of n based on

treatment 2 (Model + distinct Mimic), we found that overall, a greater number of mimetic prey were eaten than would be predicted if dissimilar looking species lost a combined *n* individuals through predation. Exploring this more closely, we found that naïve predators attacked more prey than predicted, whereas experienced predators attacked according to theory. This may occur because there is more to lose, for a naïve predator, if some of a numerous unpalatable prey form turns out to be palatable (Beatty et al. 2004; Ruxton et al. 2004).

Mutualisms without mimetic resemblance

It has been argued, in a theoretical treatment, that the mere coexistence of visually distinctive aposematic species can be mutually beneficial, thereby limiting the additional survival advantage that can accrue from signal mimicry (Turner and Speed 1999). They argued that toxin intake from one aposematic species would suppress attacks on another toxin-containing species because predators would be monitoring their own toxin levels and cease attacks on toxin-containing prey when the costs of dealing with toxins additional to those already in a predators' body outweigh the benefits of the nutritional components in those prey. In essence, this is an argument for a dilution effect in which the prey's defensive toxins cause rapid "satiation" in the appetite for aposematic animals in the predators. There is growing evidence that predators can make this kind of a trade-off (Skelhorn and Rowe 2007) and indeed in our experiment, the Model prey gained advantage when the distinctive Mimic was added to the system. Though it looks like support for hypothesis of Turner and Speed (1999), analysis of relative predation risk indicated that this was a result of a general dilution effect (i.e., there are more prey in the system, so the probability of attack on Models diminished) rather than the one specifically outlined by Turner and Speed (1999). It seems likely that an approach taken recently by Barnett et al. (2007) and Skelhorn and Rowe (2007), in which predators are prefed on a toxin before encountering defended prey will be a better method to evaluate this hypothesis.

Conclusions

Our results clearly and unambiguously show that signal mimicry can benefit co-mimics during a predator's period of education. In fact, we illustrate that visually distinct aposematic prey can be at a greater disadvantage (relative to those that share a signal) than predicted by Müller's theory. This consequently has important implications for how we think about the evolution of the many examples of perfect mimicry observed between unpalatable species (Benson 1972; Mallet and Barton 1989; Dumbacher 1999; Kapan 2001; Symula et al. 2001).

SUPPLEMENTARY MATERIAL

Supplementary material can be found at http://www.beheco.oxfordjournals.org/.

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