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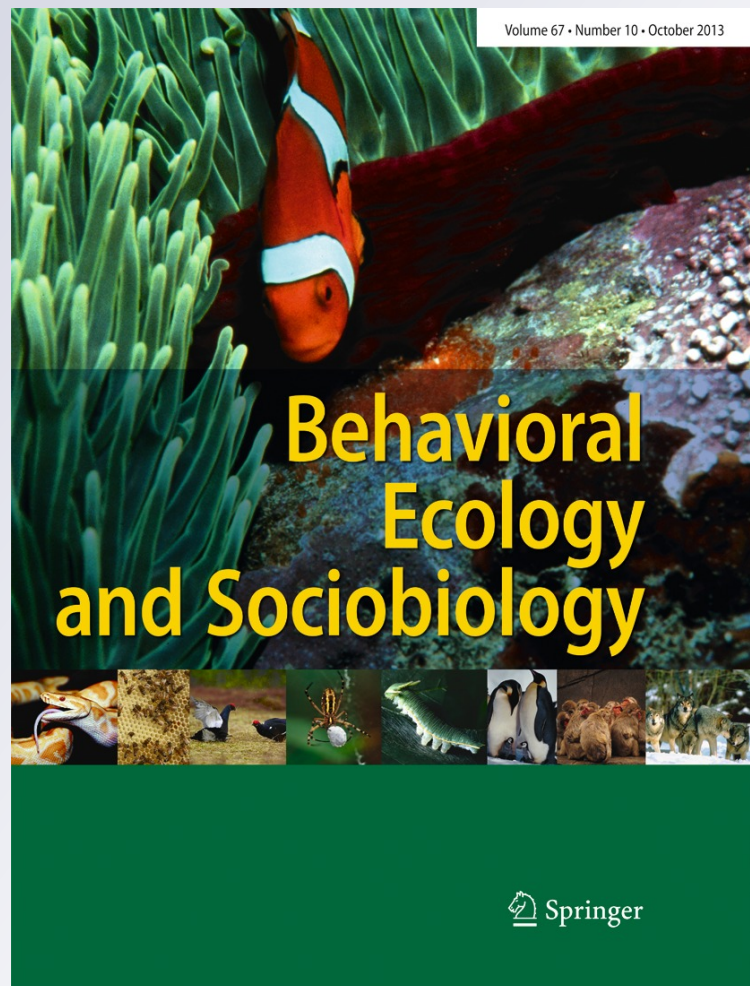
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Plant-derived visual signals may protect beetle herbivores from bird predators

Tamar Keasar · Miriam Kishinevsky · Avi Shmida · Yoram Gerchman · Nicka Chinkov · Avi Koplovich · Gadi Katzir

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Abstract Insect herbivores often use chemical signals obtained from their food plants to deter enemies and/or attract sexual partners. Do plant-based visual signals act similarly, i.e., repel consumers' enemies and appeal to potential mates? We explored this question using the pollen-feeding beetle *Pygopleurus israelitus* (Glaphyridae), a specialized pollinator of *Anemone coronaria*'s chemically defended red-morph flowers. We presented dead beetles, which had fed either on anemones or on cat food, to young domestic chicks on a red (anemone-like) or a green (leaf-like) background. We determined whether the beetles' background color and diet affected the chicks' feeding. Cuticle surface extracts from anemone-fed beetles, but not from cat food-fed beetles, contained a secondary metabolite characteristic of anemones. Latencies to the first picking up and consuming of beetles from green backgrounds were shorter than of beetles from red backgrounds. The picking up order of beetles also indicated that prey from the green background was preferred. The chicks retained this preference when re-tested, 3 days later. Handling times of anemone-fed beetles were longer than of cat food-fed beetles. A previous study showed that glaphyrids improve their mate-

finding prospects by orienting to large red anemone flowers. Here, female beetles preferred cat food-fed to anemone-fed males in mate-choice assays, thus anemone-derived chemicals did not increase mating success. Instead, the combined results indicate that *A. coronaria*'s red flowers provide a visual signal that may both deter its herbivore's predators and attract its mates. To our knowledge, this is the first experimental evidence for a potential protective role of plant-derived visual signals for insect herbivores/pollinators.

Keywords Predation · Secondary metabolite · Tritrophic interactions · Warning coloration · Domestic chick · Glaphyridae · Pollination

Introduction

Potential benefits of host plants to their insect herbivores, apart from their nutritive value, include protection from predators and facilitation of mating. Many of these benefits are associated with plant secondary metabolites that are consumed and sequestered by the herbivores, and used to produce defensive chemicals and/or sex pheromones. Examples for such chemical communication include plant pyrrolizidine alkaloids, the derivatives of which serve as mating/sexual pheromones and herbivore deterrents in insects from several orders; terpenoids produced by orchids that are collected and used by male orchid bees as sex and lek-forming pheromones; and aristolochic acids that are sequestered by swallowtail butterflies, protecting them from vertebrate and arthropod predators (reviewed by Rothschild 1984; Wittstock and Gershenzon 2002; Reddy and Guerrero 2004; Opitz and Müller 2009). A related defense mechanism involves plant-derived volatiles that are odorous but non-toxic, such as pyrazines, used by aposematic insects to deter their vertebrate predators. The pyrazine odor helps predators to associate the insects' visual signal with their noxious taste and enhances the

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avoidance of the toxic prey (Rothschild and Moore 1987; Kaye et al. 1989; Barnea et al. 2004). Pyrazines are also known to elicit hidden innate biases against novel or conspicuously colored prey (Marples and Roper 1996; Rowe and Guilford 1996; Lindström et al. 2001; Kelly and Marples 2004). Thus, plant-derived chemical cues, together with herbivore-associated visual cues, combine to repel potential predators.

Visual cues from plants could also be involved in the communication between insect herbivores and their predators. This possibility was raised by several authors: Herrera (1985) suggested that toxic fruit and aposematic insects share a common protection from bird predation through their similar color displays, in addition to their chemical defenses. Colorful and conspicuous plant galls were suggested to serve as warning signals that protect their insect inhabitants from predation (Inbar et al. 2010). Further, leaf mines produced by leaf-mining insects were proposed to visually deter herbivores from feeding on the damaged leaves (Yamazaki 2010). However, these attractive hypotheses have not been tested directly so far. Some experimental studies, on the other hand, show that birds (including domestic chicks) avoid conspicuous colors in insects but not in plants (berries; see Gamberale-Stille and Tullberg 2001; Gamberale-Stille et al. 2007).

Here, we study the roles of plant-generated chemical and visual cues in the herbivores' biotic interactions, using the chemically defended Mediterranean geophyte *Anemone coronaria* and its beetle pollinators/herbivores as a model system. The most common color morph of *A. coronaria*, and the only morph flowering in southern Israel, is the bright red. Male and female beetles of *Pygopleurus israelitus* (Glaphyridae: Coleoptera: Scarabaeoidea) spend several hours a day on the large, red bowl-shaped flowers, where they rest, feed on pollen, and mate (Dafni et al. 1990; Keasar et al. 2010). The beetles' cuticle is soft and, to the human eye, is brownish in color, with a shiny green/blue/purple pronotum and head (Baraud 1989) and no reflectance in the UV range (J. Martinez-Harms, unpublished data). Although the glaphyrids are diurnal and lack morphological defense structures, we observed no predation on these beetles by vertebrate predators in the field and only rare cases of predation by flower-dwelling crab spiders.

We set out to test whether the beetles gain chemical or visual benefits (or a combination thereof) from their anemone host plants. Anemones are avoided by several grazing mammalian herbivores (Perevolotsky et al. 2011), probably due to the toxin protoanemonin (Knight and Walter 2001). We hypothesized that this metabolite, when sequestered by the pollen-feeding beetles, renders them unpalatable to their predators, increases their mating success, or both (the *diet hypothesis*). We further hypothesized that the red flowers of *A. coronaria* provide visual signals that deter the beetles' predators and/or attract mates (the *color hypothesis*).

We tested the hypotheses with regard to predators by presenting very young domestic chicks, *Gallus gallus*, with beetles that were fed on either anemones or on cat food. The beetles were sacrificed and placed on either a red or a green background. This allowed us to separate chemical cues that the beetles could have obtained from their diet from visual signals that simulated the color displays of anemones vs. their surrounding foliage. The *diet hypothesis* predicts that beetles that had fed on *A. coronaria* will be less attractive to predators than beetles that had fed on an alternative food source. The *color hypothesis* predicts that beetles on red, anemone-flower-like, backgrounds will be better protected from predation, compared with beetles on green, leaf-like background because the red background may serve as a warning signal to potential predators.

In the context of mate attraction, the *color hypothesis* is supported by previous studies showing that glaphyrids prefer red displays over other colors (Dafni et al. 1990), particularly when these displays are large (Dafni 1997). A common preference of males and females for large red displays may help them locate potential mates. This common preference was observed in field surveys, in choice experiments with plastic models, and in greenhouse choice assays with different-sized flowers (Keasar et al. 2010). In the present study, we complemented these findings with a mate-choice assay that tested whether feeding on anemone confers mating advantages, as predicted by the *diet hypothesis*.

Methods

Beetles and birds

Glaphyrid beetles (*Pygopleurus israelitus*) were collected from anemones in February and March. Individuals for mating and predation tests were collected in 2010 in the Lahav forest (31°23'N, 34°52'E), and in 2011 in the vicinity of Mt. Carmel (32°44'N, 35°03'E), Israel, respectively. The age of the beetles and their reproductive status were unknown. Male beetles, identified by the red color of their last abdominal segment, were separated from the females immediately after collection.

Domestic chicks (*Gallus gallus domesticus*, $n=30$) were used as predators. They were obtained from a commercial hatchery within a few hours of hatching and housed in two large cages when not tested. The chicks received water for drinking and bathing ad libitum until the morning of the experiment. They fed on a commercial poultry seed mix, augmented by mealworm beetles (*Tenebrio molitor*), and their body mass was recorded before each experiment. Before testing, they were food deprived for 1–3 h. For the predation experiments, the chicks were moved into a wire cage (44×28×22 cm) within the experimental room (4.60×3.75×2.16 m). The cage housed four

individuals, separated from each other by a wire mesh that allowed visual and vocal interactions. In each test, the behavior of one focal individual was recorded, while the others served as companions only. The companion chicks were not used as focal individuals in later observations, to prevent potential effects of social learning. The room was lit by four white-light neon tubes and two incandescent yellow-light bulbs with a 12L:12D cycle. Predation experiments were conducted during 1 week in August 2012 at room temperatures of between 27 and 30 °C. The experiments were recorded with a video camera on a tripod, in the absence of a human observer.

Predation experiment

Beetles for this experiment were kept at room temperature and under ambient light conditions after collection. One half of the individuals were fed freshly picked anemones and the other half provided with dry cat food and fresh banana during this period. The cat food served as a protein source (30 % protein, main ingredients: commel, dried chicken, and dried fish), while the banana provided the beetles with water and carbohydrates. The aim of this diet was to sustain the beetles for several days without exposing them to the anemone's secondary metabolites. After a week, the beetles were frozen and kept at –80 °C until the experiments.

The chicks were arbitrarily allocated to one of two experimental treatments. In the “anemone-red” treatment, chicks were offered anemone-fed beetles in a red dish and cat food-fed beetles in a green dish. In the “anemone-green” treatments, the chicks were offered anemone-fed beetles in a green dish and cat food-fed beetles in a red dish. To produce the two dish colors, we glued a colored (red or green) circular (6 cm in diameter) cardboard to the bottom of each of the two Petri dishes. Color reflectance was measured with an Ocean Optics spectrometer, Red Tide, Ocean Optics, USA, QP-600-025-SR/BX under the experimental room conditions, according to the protocol of Chittka and Kevan (2005).

In a test, each chick was presented with three thawed beetles on a red dish and three beetles on a green dish, for 10 min, at the end of which it was returned to the group cage. Chicks that did not pick up any beetle during the 10-min period were excluded from the experiment and from data analysis. This reduced sample sizes to 12 in the anemone-red and 13 in the anemone-green treatments. Each chick was observed twice using this protocol, 1 day and 4 days after hatching. This allowed us to test whether chicks innately respond to their prey's diet and background color, or whether this response requires learning. Innate responses should be similar in naïve and experienced chicks, while learned responses are expected to occur only in individuals with previous exposure to the beetles. Figure 1 provides a scheme of the experimental design.

Preliminary mating observations

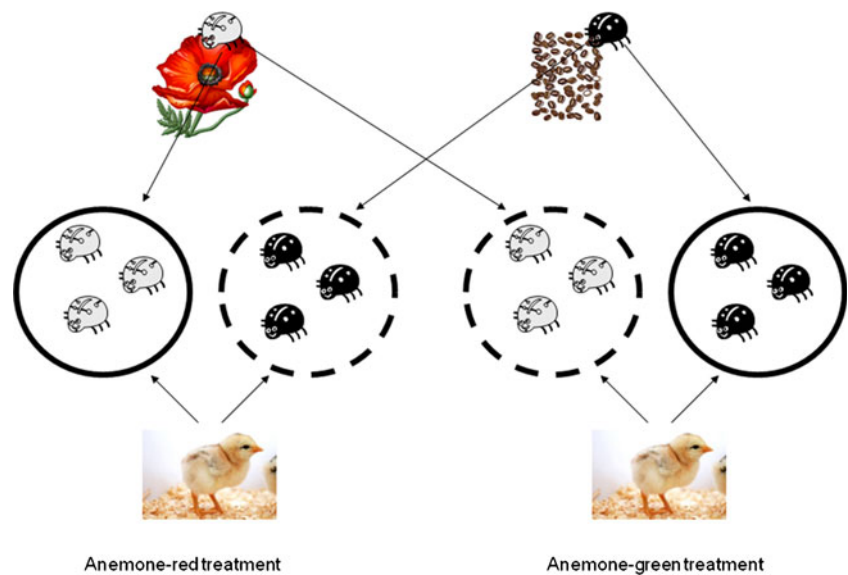
We conducted preliminary observations in March and April 2009 (a) to find a non-anemone food source for the beetles; (b) to test whether the beetles mate more than once; and (c) to measure mating durations in captivity for the design of the main mating experiment. We deprived the beetles of food for 2–3 days, and kept them at 4 °C until the beginning of the first observation session. Pairs, one male and one female ($n=36$), were then placed each in a 500-ml plastic container at 27 °C, and were provided with water-moistened dry cat food and fresh banana as food sources. We observed each pair for the occurrence of mating at 10-min intervals. Copulating individuals were observed until they separated after mating, while observations of non-mating pairs were terminated after 30 min. The male and female were then put each into a 500-ml container and kept at room temperature until the second observation session, 3 days later. All individuals received cat food and banana ad lib during this time. In the second observation session, we used the same individuals and formed the same 36 pairs as in the first session, and used an identical protocol to record their rematings.

Mate-choice experiment

Males and females were collected in the field, and kept in separate containers for 48 h to increase sexual receptivity, until the beginning of the mate-choice experiment. During this time, 58 of the males were provided with freshly picked anemones, 42 were provided with dry cat food and fresh banana, and 16 were provided with a water-soaked cotton pad, without food. The females were allowed to feed on anemones before the beginning of the experiment. All food types were provided ad lib.

A male that had fed on anemone and a male that had fed on cat food were placed with a female in a 500-ml plastic container. One male in each container, randomly chosen, was marked with a color dot for identification. The containers were inspected every 10 min for 8 h, and the identity of any copulating male was noted. When copulations were observed, we recorded which male was the first to mate and the duration of copulations. Observations were repeated with 30 pairs of males on two observation dates. In 16 additional replicates, we housed a female with one male that had fed on cat food and another male that had been exposed to water only. We used the same protocol as above to score their copulations. This control tested whether starved males mate less often than fed males. If this were the case, differences in palatability between the food sources offered to the males (i.e., anemone vs. cat food) might cause differences in food intake and consequently in mating success.

Fig. 1 The design of the predation experiment. Field-collected beetles were allowed to feed either on anemones or cat food for 7 days. In each test, three anemone-fed beetles (marked in grey) and three cat food-fed beetles (marked in black) were offered to chicks for a 10-min test session. In the anemone-red treatment, the anemone-fed beetles were presented in a red dish (solid circle), and the cat food-fed beetles were placed in a green dish (hatched circle). In the anemone-green treatment, the dish colors were reversed. Each bird was tested twice, 1 day, and 4 days after hatching



The body mass of both males was determined in 20 of the replicates at the end of the observation period using an AWS Gemini-20 portable milligram scale (12 anemone- vs. cat food-fed males, 8 cat food- vs. unfed males). This was done to ensure that incidental differences in body size did not affect the males' mating success.

Chemical analysis

Extraction of cuticle surface components We tested whether the secondary metabolite anemonin (a stable metabolite of the more toxic precursor protoanemonin) from the anemones accumulated on the beetles' body surface. Individuals that had fed for 1 week on freshly picked anemones or on cat food were frozen at -80°C until further analysis. For cuticle surface analysis, six beetles of each treatment were extracted individually, in 5 ml methanol (Merck, HPLC grade) for 5 min. The beetle was removed, and the liquid was centrifuged (5 min, $5,000\times g$) to remove any solid residue that might have detached from the beetle. As a reference sample, 100 mg red anemone pollen was extracted in 1 ml methanol, and the solid pollen was removed after 5 min by centrifugation. All supernatants were concentrated 50-fold under reduced pressure, and kept at 4°C prior to analysis by matrix-assisted laser desorption/ionization and time-of-flight mass spectrometry (MALDI-TOF MS). To avoid possible contamination by pollen from the digestive system of anemone-fed beetles, extracts of the beetles' soft tissues were not analyzed.

MALDI TOF analysis The methanol extracts were analyzed on a MALDI Micro MX machine, operating in the delayed extraction mode with a 3-ns pulse nitrogen laser (337 nm, 406 pulse/min, amplitude equal to 1,930) for desorption and ionization. Mass range of m/z 80–500 was scanned, ions were detected as positive on a time-of-flight mass detector

in the reflector mode, and external standards were used for mass calibration. The signal of interest (i.e., anemonin) appeared as a molecular ion, while no sodium adducts of the latter were detected. Application of a matrix was not necessary for the observation of molecular ions.

Data analysis

Predation experiments The following data were extracted from the videos (two tests/bird): (a) The order (ranked) of beetles picked up from the red or from the green dish. Beetles picked early were ranked highest. We summed the ranks for the green and the red dishes to produce pick-up scores for beetles from the red vs. green dishes, for each bird. (b) The ranked order of beetles consumed from the red or from the green dish. Two consumption scores (red and green) were calculated for each chick, as for pick-up scores. (c) The latencies since the start of the observation to the first picking up of beetles from a red dish and from a green dish. (d) The latencies since the start of the observation to first feeding on a beetle from each dish. (e) The total number of beetles picked up from each dish. (f) The total number of beetles consumed per dish. (g) The time spent handling each beetle, from first pick-up until it was consumed.

Parameters a–f resulted in two paired data points (for beetles from each of the dishes) per individual. After excluding individuals that did not pick up or consume a beetle from any of the dishes, sample size decreased to 25 (12 chicks in the anemone-red treatment, and 13 in the anemone-green treatment). We therefore analyzed these data using non-parametric statistics. We tested for the effects of background color and beetle diet on the above parameters. We used two-tailed Wilcoxon matched-pairs tests to examine whether latencies to pick-up differed between beetles from red and

from green backgrounds, and between anemone-fed and cat food-fed beetles. Similarly, we used Wilcoxon matched-pairs tests to determine the effects of background color and diet on the following variables: latency to consumption, pick-up scores, and consumption scores. This required the performing of two independent tests on the same dataset, looking both at the effect of background color and of beetle diet. The Šidák correction for multiple comparisons was used to adjust statistical significance levels. Therefore, P values lower than 0.0253 were considered significant.

Parameter g (handling time) yielded up to six data points per chick, the maximal number of beetles that could be consumed in a test. However, several individuals consumed fewer than six beetles, especially during the first test. Because of the many missing values, we were not able to analyze the handling time data using repeated-measures ANOVA. Two-way ANOVA was used to test the effects of background color and beetle diet on this parameter. All statistical tests were conducted with SPSS version 19.0.

Mate-choice experiments Binomial tests were used to test the null hypotheses that females mate equally frequently (a) with anemone-fed males and with cat food-fed males, (b) with cat food-fed males and with unfed males. Two-tailed t tests were employed to compare mating durations with anemone- vs. cat food-fed males. We used a paired t test to compare body masses of males that were housed with the same female.

Results

Predation experiment

Color reflectance of the background surfaces The green surface reflected maximally between 511 and 522 nm, while the reflectance of the red surface increased gradually beyond 560 nm. Both surfaces also reflected in the UV range (<400 nm, Fig. 2).

Chick body mass The chicks' body mass (mean \pm SE) was 42.96 \pm 0.83 g at the first observation, and 50.61 \pm 0.89 g at the second one. The increase in mass during the experiment was highly significant (paired t test, $t_{26}=13.29$, $P<0.001$).

Beetles from red vs. green dishes In the first test, some of the behavioral parameters indicated that the chicks preferred prey in the green dish: latencies to first pick-up and consumption of the first beetle were shorter for the green than for the red dish (Figs. 3 and 4, Wilcoxon paired-sample test, $P=0.025$ for latency to first pick-up, $P=0.012$ for latency to consumption). Pick up and consumption scores, the total

number of beetles picked up and consumed (Table 1) and prey handling times (Table 2), were not significantly affected by the beetles' background color.

In the second test, 3 days later, the chicks retained the preference for prey from the green dish, as reflected in the higher pick-up and consumption scores of beetles from green background compared with the red background (Figs. 5 and 6). The remaining behavioral parameters were not significantly affected by background color (Tables 1 and 2). The number of picked up and consumed beetles in the second test was higher than in the first test (Table 1), while handling times were shorter (Table 2). Handling times did not vary consistently with chick experience within each test.

Cat food-fed vs. anemone-fed beetles Pick up scores by 1- and by 4-day-old chicks were not significantly influenced by the beetles' diet. Nor were the consumption scores and latencies to consumption influenced by the beetles' diet at both ages. The total number of beetles picked up and consumed was not affected by diet either (Figs. 3, 4, 5, and 6, Table 1). Handling times of anemone-fed beetles were higher than those of cat food-fed beetles in 1-day-old chicks, but not in the 4-day-old (Table 2). The chicks showed no signs of discomfort while handling or after eating anemone-fed beetles.

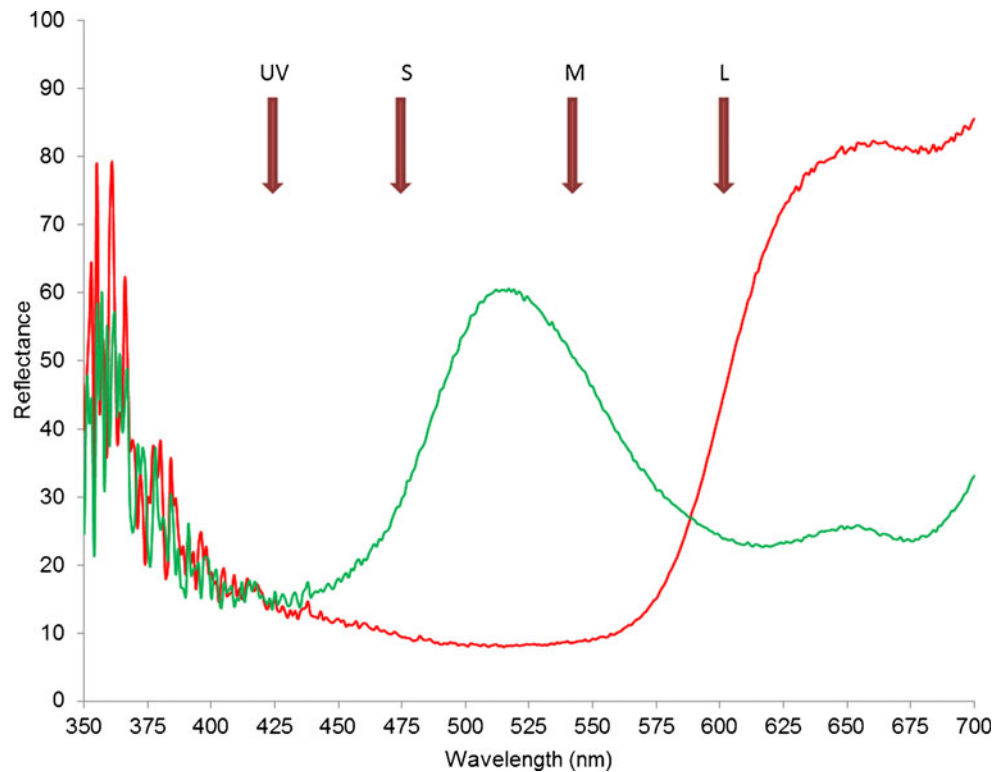
Preliminary mating observations

The beetles fed readily on the cat food and banana. All individuals survived from collection until the end of the second observation, 5–6 days later. Of the 36 pairs, 32 mated during the first observation session, and 19 of these pairs re-mated during the second observation session. Mating duration was 118 \pm 95.60 min (mean \pm SD). We concluded that cat food and banana sustain the beetles and can be used for the main mate-choice experiment. We also concluded that females are capable of re-mating, allowing the use of field-collected beetles of unknown mating status in the mate-choice experiment.

Mate-choice experiment

Males that had fed on cat food mated sooner than males that had fed on anemones in 22 replicates, while the anemone-fed males were the first to mate in eight cases. No mating occurred in the remaining 12 replicates. This result allows rejection of the null hypothesis of equal mating chances to both males (binomial test, $P=0.008$), and suggests that cat food-fed males have a mating advantage over anemone-fed males. Mating durations were 131.25 \pm 20.94 min (mean \pm SE) for anemone-fed males and 115.00 \pm 4.05 min for cat food-fed males. These means did not differ significantly (two-tailed t test, $t_8=0.26$, $P=0.80$).

Fig. 2 Spectral reflectance functions of the red and green backgrounds used in the experiment. The *arrows* denote the peak sensitivity wavelengths of the four receptors (*UV*, *S*, *M*, and *L*) in the chicks' color-vision system, as reported by Osorio et al. (1999)



Seven females mated with a cat food-fed male rather than with a male that received only water (unfed), while one female mated with the unfed male. The remaining eight females did not mate with either male. Thus, fed males mated significantly more often than unfed ones (binomial test, $P=0.035$). Mating and non-mating males did not significantly differ in body mass (mean±SDs, 0.059 ± 0.013 and 0.054 ± 0.010 g for mating and non-mating males, respectively; paired, one-tailed t test, $t_{19}=1.44$, $P=0.08$).

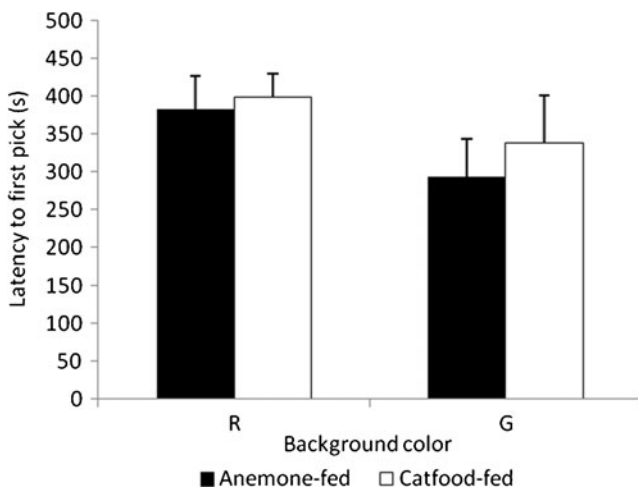


Fig. 3 Latencies to first pick-up of a prey item (Test 1)

Chemical analysis of the cuticle surface

Anemones are known for their high content of glucosides, mainly ranunculin. When the plant tissue is subjected to a physical damage, alkaline hydrolysis or steam distillation, the ranunculin is converted to protoanemonin, an unstable noxious compound that later dimerizes to the more stable compound, anemonin (Hill and van Heyningen 1951; Bonora et al. 1985). Cuticle surface chemical analysis demonstrated the presence of m/z 192 (attributed to anemonin) in four out of six anemone-fed beetles (Fig. 7), whereas none of six cat food-fed beetles contained this peak. The anemonin peak was also detected in the sample of anemone pollen.

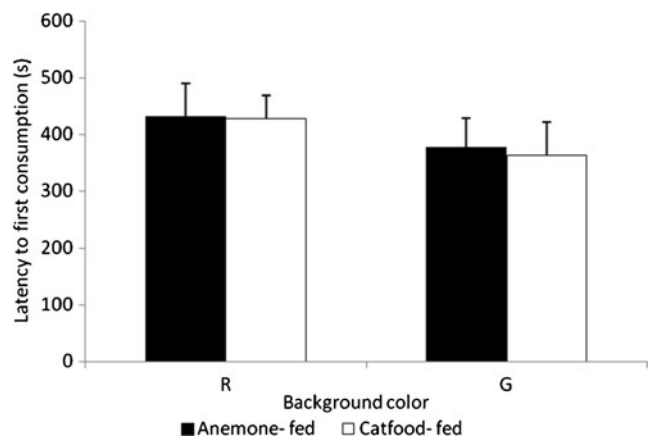


Fig. 4 Latencies to first consumption of a prey item (Test 1)

Table 1 Summary of feeding parameters that were not significantly affected by background color and diet

Test no.	Parameter	Mean±SE anemone-red treatment Beetle diet		Mean±SE anemone-green treatment Beetle diet	
		Anemone	Cat food	Anemone	Cat food
1	Consumption score	4.50±0.85	6.15±1.13	4.95±0.99	4.72±0.98
1	No. beetles picked up	1.87±0.26	2.29±0.21	2.25±0.22	1.83±0.26
1	No. beetles consumed	1.58±0.27	1.67±0.24	1.67±0.24	1.58±0.26
2	Latency to pick-up (s)	92.58±27.38	70.08±15.94	94.77±27.44	77.61±25.53
2	Latency to consume (s)	113.08±31.44	137.42±47.29	110.92±40.55	119.15±42.83
2	No. beetles picked up	3.00±0.00	3.00±0.00	2.77±0.11	2.92±0.07
2	No. beetles consumed	2.83±0.10	2.75±0.16	2.50±0.21	2.69±0.16

Discussion

The results of the present study are compatible with our "color hypothesis," which posits that the red color of *A. coronaria* reduces avian predation on *P. israelitus* beetles while they feed on the flowers. Accordingly, naïve chicks picked up beetles from a green background sooner than from a red background, and required a shorter duration to the first pick-up and consumption of beetles from a green background. Interestingly, the chicks preferentially picked up and consumed the green-background beetles also during re-testing, indicating that the chicks' color preferences persisted in individuals with previous foraging experience. The results do not clearly support the "diet hypothesis": although anemone-fed beetles required longer handling time than cat food-fed beetles, the difference was significant on the first test only. In addition, none of the remaining behavior parameters pointed to an avoidance of anemone-fed beetles. We also found no evidence for interaction between diet and background color, i.e., no indication for increased avoidance of anemone-fed beetles when presented on a red background.

Our results add to the increasing body of evidence of recent years for an aposematic function of many visual signals of plants. Such signals include coloration of leaves, flowers, and

fruits that is commonly yellow, orange, red, brown, black, white, or a combination of these colors. Warning coloration is expressed by thorny, spiny, prickly, and poisonous plants and by plants that are unpalatable for various other reasons (Lev-Yadun 2009). Here, we extend this notion by proposing that plant warning coloration can also protect herbivores and pollinators, as they feed on their host plants. This suggestion is compatible with previous studies, demonstrating that predators can learn color cues associated with their prey's background: chicks learned to identify palatable food items by the color of the background on which they were presented (Gamberale-Stille and Guilford 2003). Furthermore, great tits learned to prefer one type of food when presented with a red background cue, and to switch their preference to a second food type when coupled with a blue background signal (Hansen et al. 2010).

Several previous studies have suggested that red coloration is spontaneously avoided by vertebrate predators (particularly birds) and could facilitate avoidance learning. Naïve bobwhite quail innately discriminated against red-colored food items in choice tests (Mastrota and Mench 1995). Naïve chicks innately avoided red-painted insects (Gamberale-Stille and Tullberg 2001), and preferred dishes with green/black food items to dishes with red items (Roper and Marples 1997). After

Table 2 The effects of background color and diet on handling times: means with associated standard errors and results of two-way ANOVAs for the two tests

		Test	
		1 (1-day-old chicks)	2 (4-day-old chicks)
Background color	Red	0.55±0.17	0.19±0.04
	Green	0.58±0.12	0.36±0.10
ANOVA results		$F_{1, 73}=0.11, P=0.92$	$F_{1, 132}=2.37, P=0.13$
Beetle diet	Anemone	0.79±0.18	0.33±0.10
	Cat food	0.30±0.06	0.22±0.05
ANOVA results		$F_{1, 73}=6.09, P=0.016$	$F_{1, 132}=0.98, P=0.32$

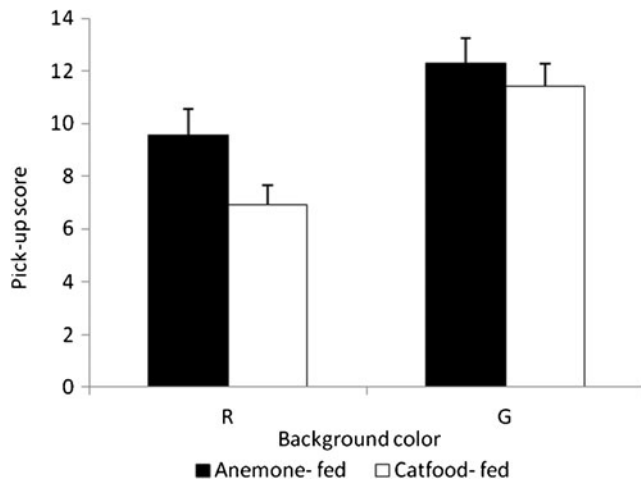


Fig. 5 Pick-up scores in Test 2. Beetles that were picked up earlier received higher scores

experiencing noxious prey items, chicks reduced their consumption of palatable items colored red and yellow, whereas their intake of green palatable items was unaffected (Rowe and Skelhorn 2005). In a survey of 51 Australian *Hakea* species, a positive correlation was established between red flower color, floral cyanide, and bird pollination. Possibly, cyanides reduce the chances of the flowers being eaten by the bird pollinators of the plants, so that the red color advertises both the flower's nectar reward and its distasteful floral tissue (Hanley et al. 2008). Great tits, *Parus major*, learned to avoid red distasteful bug larvae faster than a mutant grey phenotype of equal toxicity (Sillén-Tullberg 1985). Tits that learned to avoid red aposematic firebugs did not generalize their experience to yellow or white morphs of the same species, whereas birds that had learned to avoid the yellow morph generalized their experience to red firebugs (Svádová et al. 2009). Other studies, however, found no color biases by birds, or even demonstrated

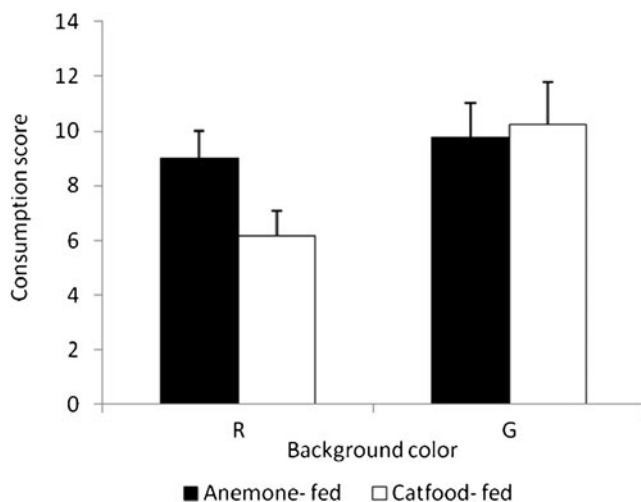


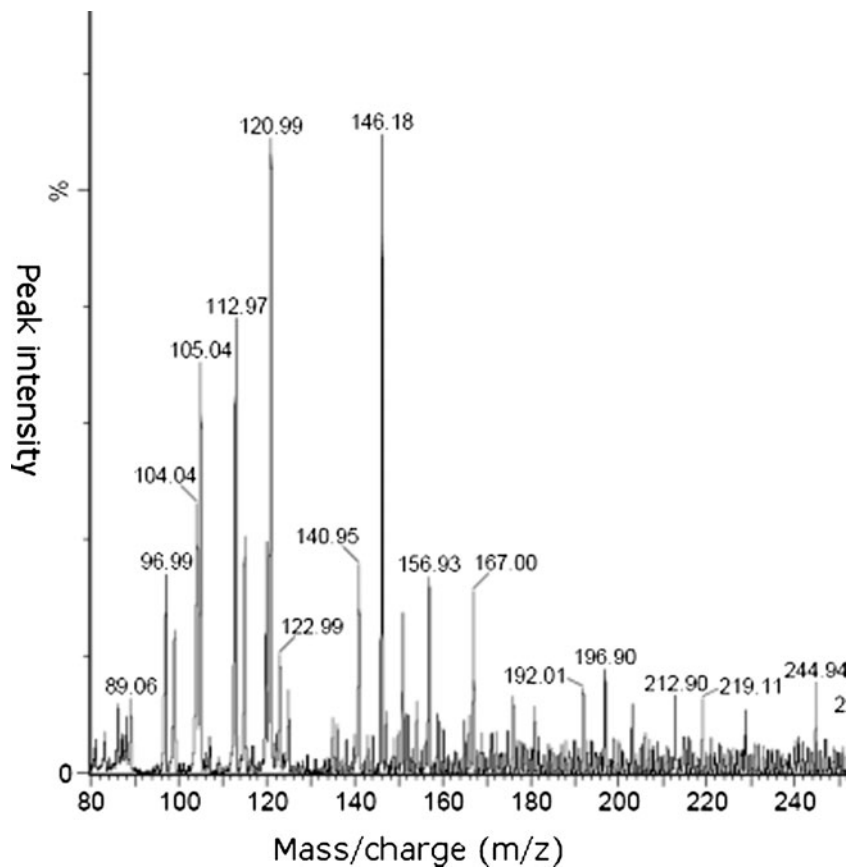
Fig. 6 Consumption scores in Test 2. Beetles that were consumed earlier received higher scores

a preference for red: Great tits learned equally well to associate red, yellow, and grey color with unpalatable prey (Ham et al. 2006). Naïve blackcaps (*Sylvia atricapilla*) preferred red artificial fruit to other colors, while wild-caught adults showed no color preference (Schmidt and Schaefer 2004). These mixed results suggest that red coloration of prey can deter avian predators under some, but not all, circumstances. It has been suggested that red coloration is an ambiguous signal in nature because it is associated with both attractive (e.g., female sexual receptivity) and repulsive (e.g., toxicity) messages (Humphrey 1976). Gamberale-Stille and Tullberg (2001) suggested that red has context-dependent behavioral effects on birds and, in particular, elicits avoidance when associated with insect prey. Our results support this view, as the chicks discriminated against beetles on red background, regardless of the beetles' diet.

Aposematism involves both the warning coloration and unprofitability of the prey, and often requires predators to learn to associate these two traits. In the present experiment, we predicted that learning by the chicks would reduce the number of anemone-fed beetles handled and consumed in the second test, compared with the first. Contrary to this prediction, the chicks' avoidance of picking up and consuming anemone-fed beetles did not increase between tests (Table 1). Handling times of anemone-fed beetles actually decreased by more than 50 % (Table 2), suggesting that manipulation of this prey type improved with predator age or experience. Possibly, the chicks' exposure to the anemone-fed prey was too brief to allow avoidance learning in our experiment. Learning of background cues was demonstrated after ten training trials in chicks and after six training trials in tits (Gamberale-Stille and Guilford 2003; Hansen et al. 2010), while our study included only two sessions, 3 days apart. A second possible interpretation for the lack of avoidance learning is that the anemone-fed beetles were profitable prey for the birds, even though they had fed on a chemically defended plant. According to this interpretation, beetles on anemones would indeed gain protection from avian predators by exploiting the birds' possible innate aversion to red color, rather than by being distasteful.

Domestic chicks, selected as predators for our experiment, have often been used in prey-choice studies (e.g., Marples and Roper 1996; Roper and Marples 1997; Gamberale-Stille and Tullberg 2001; Rowe and Skelhorn 2005). Their advantages include high availability and ease of maintenance, which allow for large sample sizes, and control over previous foraging experience. Moreover, there is ample psychophysical and physiological evidence of their capacity for color discrimination, which is based on their tetrachromatic photoreceptor composition (Osorio et al. 1999). The reflectance spectra of the stimuli in our predation experiment are well within the capacity of chicks' color discrimination. Specifically, the birds' medium-wavelength receptor is maximally sensitive at 540 nm (green), while the peak sensitivity of the long-wavelength receptor is at 600 nm (red, Fig. 2). Osorio et al.

Fig. 7 A representative MALDI-TOF MS spectrum of one of the methanol surface extracts of anemone-fed beetles. A peak of m/z 192 is attributed to anemonin—a dimer of protonemonin that is responsible for the poisonous character of several Ranunculaceae



(1999) showed that chicks can be trained to discriminate effectively between long- and medium-cone signals. This suggests that they could differentiate between the red and green backgrounds in the present study. The experiment's red background resembles *A. coronaria*'s red signal in strongly reflecting light above 600 nm, but differs from the anemone display in reflecting in the UV range as well (see Martinez-Harms et al. 2012 for reflectance spectra of *A. coronaria* and other glaphyrid-pollinated flowers). As the green background reflected UV as well (Fig. 2), we hypothesize that the chicks' color discrimination in the experiment involved mainly differential excitation of medium- and long-wavelength cones.

The main drawback for using chicks in the present study is that they did not coevolve with glaphyrid beetles, which may limit the ecological relevance of the results. It has been argued that domestic chicks are suitable for insect-feeding experiments because their wild progenitor is the red jungle fowl, a generalist forager (Gamberale-Stille and Tullberg 2001). Nevertheless, we suggest that the experiment be repeated using a local insectivore avian predator, to validate the generality of our findings.

The mate-choice experiment aimed to test whether anemone metabolites affect sexual behavior in male beetles. The "diet hypothesis" predicted a positive effect, either by making anemone-fed males more attractive to females or by conferring an advantage in male–male competition. Contrary to this prediction, we found a mating advantage

to cat food-fed over anemone-fed males. We also observed a mating advantage of cat food-fed males over unfed males. This raises the possibility that anemone-fed males were at a disadvantage because of poorer nutrition condition compared with the cat food-fed males. Regardless of the reason, feeding on anemone seems to incur a cost for the beetles in terms of mating success. This cost may be balanced by the mate-attracting benefits of *A. coronaria*'s visual signals, or by the benefits of predator deterrence.

In the field, glaphyrid beetles feed and mate on large, red, bowl-shaped flowers, such as *A. coronaria*. Field observations, flower-choice experiments, and field experiments with flower models suggest that both males and females are attracted to large red floral displays, which may increase their prospects of encountering mates (Kearse et al. 2010). The current mate-choice experiment suggests that feeding on anemones reduces male mating success rather than enhancing it. This further supports the importance of the anemones as a visual signal for beetle mating.

To conclude, our study suggests that glaphyrid beetles feeding on *A. coronaria*'s pollen gain some visual protection from avian predators that are repelled by the red coloration of the beetles' toxic food plant. The avoidance of beetles by predators because of ingested anemone-produced secondary metabolites was not clearly supported, and warrants additional testing. On the other hand, feeding on anemone

reduces the mating success of glaphyrid males, a cost that may be offset by the attraction of potential mates to the plant's red display. The combined results suggest an important role for the visual signals of the host plant in mediating the beetles' interactions across trophic levels.

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Ethical standards The reported experiments comply with the Israeli law.

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