

Elytra color as a signal of chemical defense in the Asian ladybird beetle *Harmonia axyridis*

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Abstract Carotenoid- and melanin-based colors are valuable indicators of quality in many vertebrates, but their signaling role in invertebrates remains relatively unexplored. The Asian ladybird beetle *Harmonia axyridis* is an ideal organism for studies of this nature because males and females exhibit highly variable red and black colors on their elytra and are chemically defended with an alkaloid (harmonine). We used digital photography to quantify elytra coloration and absorbance spectrophotometry and gas chromatography mass spectrometry analyses to quantify pigment and alkaloid concentrations, respectively, in wild-caught male and female *H. axyridis*. We predicted that extensive or intense coloration would be an aposematic signal of high-alkaloid stores. We found that carotenoid pigments largely controlled variation in red elytra coloration. There was no relationship between alkaloid content and either elytra redness or carotenoid pigment concentration in either sex. However, we found a positive correlation between the extent of elytra coloration and alkaloid content.

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Animals with proportionally more red (or less black) on the elytra were more alkaloid-rich; this relationship was particularly strong in females. We also found that females with lighter black spots had greater amounts of harmonine than those with darker spots. These results suggest that elytra color patterns have the potential to reveal information about chemical defensiveness to mates or predators. Prior studies in this species show that nonmelanic forms are typically less active and yet more sexually attractive than melanic forms, and both results may be explained by the fact that nonmelanics are better chemically defended.

Keywords Alkaloids · Aposematism · Carotenoids ·
Coccinellidae · Melanins

Introduction

Of all the visual traits that animals use as signals, perhaps none is as well studied and understood both mechanistically and functionally as carotenoid-based pigmentation (reviewed in Hill 1999, 2002; McGraw 2006a). Several dozen studies have identified the presence of carotenoids in the integumentary tissues of animals from a variety of taxa (Fox 1976; McGraw et al. 2005a). In addition to serving as pigments, carotenoids have been shown to play a valuable role as antioxidants, immunostimulants, and photoprotectants (Vershinin 1999). Carotenoids are a class of compounds that animals must acquire via their diet (Goodwin 1984). As a consequence, many studies have shown that carotenoid levels are intimately linked to the overall health and nutritional state of animals (Grether et al. 1999; Hill and Montgomerie 1994; Hill et al. 2002; McGraw and Ardia 2003). As a result of this linkage and their inherent “honesty” as signals, carotenoid-based coloration is often

used as a sexually selected signal (reviewed in Hill 2006). Bright, colorful individuals signal to potential mates their strong foraging abilities, healthy immune system, and, in some cases, superior care of offspring (Olson and Owens 1998; Hill 1999; Møller et al. 2000).

To date, the sexual signaling role of carotenoid colors has been studied almost exclusively in birds and fish (Hill and McGraw 2006). Despite the incredible diversity of elaborate coloration present in the class Insecta, comparatively few researchers have focused on the signal content and value of conspicuous coloration in invertebrates of any kind. Work on insect coloration has largely been descriptive (e.g., Rutowski et al. 2005; Vukusic and Sambles 2003) or focused on patterns of mimicry in which intraspecific color variation is not central (e.g., Nijhout 1991). What little has been done on quality signaling using color in insects has been focused on a narrow range of taxa (e.g., lepidopterans, Talloen et al. 2004; odonates, e.g., Jennions 1998, Joop et al. 2006).

Here, we investigate causes and correlates of pigment-based color variation in the multicolored Asian ladybird beetle *Harmonia axyridis* (henceforth known as *Harmonia*). *Harmonia* is ideally suited for studies that investigate the signal content of coloration for several reasons. First, their elytra (forewing cases) are highly varied in a number of color parameters. Elytra have a base color that varies from pale orange to bright red and contain black spots that vary both in number (from 0 to 20) and darkness (Fig. 1). Second, *Harmonia* is chemically defended via a quantifiable alkaloid. As with other coccinellid beetles, adults reflex-bleed when attacked, and the secretion is dominated by the toxic alkaloid harmonine (Enders and Bartzén 1991). The origin of harmonine within the hemolymph of *Harmonia* is unknown, but its production is likely to be costly, as costs associated with the production of defensive chemicals have been demonstrated in other beetles (Rowell-Rahier and Pasteels 1986). There is also some evidence that predators can discriminate between individuals based on their chemical content (Skelhorn and Rowe 2006). Thus,

the concentration of harmonine within the body of individual beetles could serve as a tenable assay of chemical defense levels and likelihood of survival. Lastly, *Harmonia* are readily available, as they have become somewhat of a pest species in North America because of their ability to outcompete most native ladybird beetle species and their propensity to overwinter inside buildings (Koch 2003).

Based on prior work, it seems very likely that orange-to-red coloration of the elytra in *Harmonia* is at least partially driven by the deposition of carotenoids. *Harmonia* are predominately aphidophagous and have been shown to develop redder elytra when fed on an aphid-exclusive diet (Grill and Moore 1998). Carotenoids are known to be present in aphids and have also been described from the elytra of other ladybird beetles (Britton et al. 1977a,b). It is also likely that such coloration in *Harmonia* is driven by the presence of more than just a single pigment. The bright coloration of the feathers and beaks of several species of birds has been shown to be attributable to a suite of carotenoids (McGraw et al. 2005a). In addition, there are several other types of compounds that can lead to the presence of bright orange-red tissues. Several studies in insects, amphibians, reptiles, and birds, for example, have revealed that red, orange, and yellow colors are often due to the presence of pterin pigments (Oliphant and Hudon 1993), which are particularly abundant in butterfly wings and insect eyes (Pfleiderer 1992, 1994).

Melanin pigments are the likely chemical colorants of black spots on *Harmonia* elytra, given that they are the common black pigments in insect cuticles and wings (Stoehr 2006) and in other animals (Fox and Vevers 1960; McGraw and Wakamatsu 2004, McGraw 2006b). Levels of melanization can also be linked to certain attributes of individual quality in animals (McGraw 2006b), including in insects (reviewed in Stoehr 2006). Based on works in other taxa (mostly birds), however, some have argued that melanin and carotenoid colors should have different patterns of signal information and use (e.g. McGraw and

Fig. 1 Color differences in *H. axyridis* elytra ($n=49$) before extraction of carotenoids. Female elytra ($n=27$, left side of figure) tended to be larger and have a greater proportion of area covered in black than male elytra ($n=22$, right side of figure). A color version of the figure is available in the electronic form of the manuscript



Hill 2000, Senar et al. 2003, but see Griffith et al. 2006), because they incur different costs of production and maintenance. *Harmonia* provides a unique opportunity to study the interaction of carotenoid and melanin-based coloration in a nontraditional insect taxon.

Therefore, we aimed to determine: (1) if the base coloration of *Harmonia* elytra is due to the presence of carotenoid or pterin pigments and (2) if there is a relationship between red or black coloration and levels of defense compounds in ladybird beetles.

Materials and methods

Forty-nine ladybird beetles (27 females, 22 males) were collected from the wild in the winter of 2002–2003 in Newfield, NY, USA and frozen at -20°C upon collection. The left elytron of all animals was photographed with a Fuji Finepix 200 digital camera under standardized indoor lighting and background conditions. We chose to analyze only one elytron in this study because pilot analyses showed that all color and pigment variables were highly significantly intercorrelated between the left and right elytra of individual animals (all $r > 0.92$, $n = 10$, all $p < 0.0001$). We also only quantified elytra color variation within the visible range of wavelengths, and not also in the ultraviolet (UV), because *Harmonia* elytra do not reflect UV light (A.L. Bezzerides and T. Eisner, personal observation). Photos were imported into Adobe® Photoshop® (Adobe Systems, San Jose, CA, USA) at a resolution of $1,600 \times 1,200$ pixels, and several parameters of elytra coloration were quantified: (1) orange hue (a reliable tristimulus score when animals vary in true color; Hill 2002), (2) black spot brightness (a measure of the intensity and reflectance of the dark spots on the elytra), and (3) area of red and black coloration. The first two measures were obtained by using the “lasso” marquee to select relevant pigmented areas and then using RGB values from the Histogram palette to calculate hue and brightness in the Color Picker function (Dale 2000). Extent of coloration was determined by separately outlining the entire elytra and the black spots using the “lasso” marquee and then using the Histogram function to determine the number of pixels occupied by each (McGraw et al. 2002). Areas of black and red coloration were then determined as percentages of the total elytra covered (summed for each animal to equal 100%).

Pigment analyses

The left elytra was then weighed to the nearest 0.0001 g with an electronic balance and added to 1 ml of chloroform to remove carotenoids. Elytra mass is a reliable cue of adult body mass in *Harmonia* ($n = 25$, $r^2 = 0.78$, $p < 0.0001$). Elytra

still remained a light orange color after carotenoid extraction, so we investigated the presence of pterins by adding elytra from a few test samples to 0.1 M NaOH. The remaining orange color leached into NaOH and exhibited UV fluorescent characteristics typical of red pterin pigments, probably drosoppterins (Needham 1974; Grether et al. 2001). To get a quantitative impression of pterin contributions to color, we rephotographed the carotenoid-extracted elytra and again measured the orange hue (as above).

Elytra carotenoids were analyzed via high-performance liquid chromatography (HPLC) to determine the diversity of types present (see McGraw et al. 2003 for methods). A suite of ten carotenoids was detected; although we were not able to identify all of them by comparison to authentic external standards, we determined that three different carotenoids— α -carotene, β -carotene, and lycopene—comprised the majority ($72.2 \pm 2.2\%$) of carotenoids. We used total carotenoid concentration (microgram pigment per gram elytra) in statistical analyses. Total concentration was determined, before HPLC analysis, by dissolving carotenoid extracts in 1 ml of hexane and measuring absorbance at $\lambda_{\text{max}} = 458$ nm with a spectrophotometer (see McGraw et al. 2002 for additional details).

We also verified that the black color of elytra spots is due to the presence of melanin pigments. Following the analytical methods of McGraw and Wakamatsu (2004), we found that black spots contained an average (\pm SE) melanin concentration of 1.64 ± 0.42 mg pigment/g tissue ($n = 4$ animals).

Alkaloid analyses

We ground 42 individual elytra-less *Harmonia* (25 females, 17 males) with a glass rod in 1 dram vials in 500 μl 9:1 methanol/water and 500 μl hexanes. We added a small stir bar to each sample and stirred them for 2 h at room temperature. The contents were then transferred to a 1.5 ml Eppendorf tube and centrifuged at 13,000 RPM for 5 min. We pulled 480 μl off of the bottom layer (methanol/water) and evaporated it under a stream of nitrogen. We brought the sample up in 300 μl ethanol and let it sit overnight in a -20°C freezer.

For analysis of harmonine, the ethanol was first evaporated under a stream of nitrogen. We then added 50 μl of pyridine to the sample, vortexed it, added 50 μl of bis-(Trimethylsilyl)-trifluoroacetamide (BSTFA), and put it in a 70° oven for 30 min. We let the sample cool for 5 min, added an additional 300 μl of acetonitrile, and 1 μl of the solution was injected onto an HP1 column connected to an HP 6890 gas chromatograph. The oven was held at 200° for 2 min, ramped up $5^{\circ}/\text{min}$ to 260° , and then ramped up $25^{\circ}/\text{min}$ to 280° . Each run ended with a hold of the oven at 280° for

3 min. The retention time of harmonine was 12.1 min. An HP 572 mass selection detector was used for the detection of harmonine, using a mass to charge ratio of 426 (the molecular weight of harmonine). For calibration purposes, known quantities (0.25, 0.5, and 1.0 mg/ml) of a pure sample of harmonine (generously provided by Frank Schroeder, Cornell University) were also analyzed. Regressions of the concentrations of the known samples and their corresponding areas (integrated from the gas chromatography-mass spectrometry traces) were linear ($R^2 > 0.999$).

Statistical analyses

Several of the color- and alkaloid-related variables in this study were not normally distributed and could not be transformed to a normal distribution using traditional transformations, so nonparametric Spearman rank correlations were used to evaluate relationships between pigments, color, and alkaloid content. The Wilcoxon rank sum test was used to test for differences between the two sexes. Note that in all analyses, lower hue scores signify redder beetles, because hue is assigned along a 360° color wheel, with red arbitrarily set at 0° (see McGraw et al. 2003 for details). Additionally, lower spot brightness values indicate beetles with darker black spots. Sample sizes for all color analyses were 27 females and 22 males, with the exception of spot brightness, where one male was omitted because his elytra lacked black spots. Sample sizes for all analyses of alkaloid data were 25 females and 17 males.

Results

Relationships between red color intensity, carotenoid content, and body mass

In both sexes, carotenoid content was significantly correlated with the preextraction elytra hue; that is, redder beetles deposited greater amounts of carotenoids in their elytra than paler individuals ($r_s = -0.8$ for females, -0.69 for males, $p < 0.001$ for both sexes, Fig. 2). Males did have a slightly higher concentration of carotenoids than females, but this was not statistically significant (Wilcoxon 2-sample test, $z = 1.65$, $p = 0.10$). There were no significant sex differences in preextraction ($z = -0.7$, $p = 0.48$) or postextraction hue ($z = -0.55$, $p = 0.58$). In females, there was no significant correlation between pre- and postextraction hue ($r_s = 0.24$, $p > 0.2$), but in males, naturally redder individuals were still redder after the extraction of carotenoids ($r_s = 0.68$, $p < 0.001$). A comparison of the variances in preextraction ($s^2 = 7.02$) and postextraction ($s^2 = 1.40$) hue in males indicated that the natural color variability in the elytra was driven more by the presence of carotenoids than

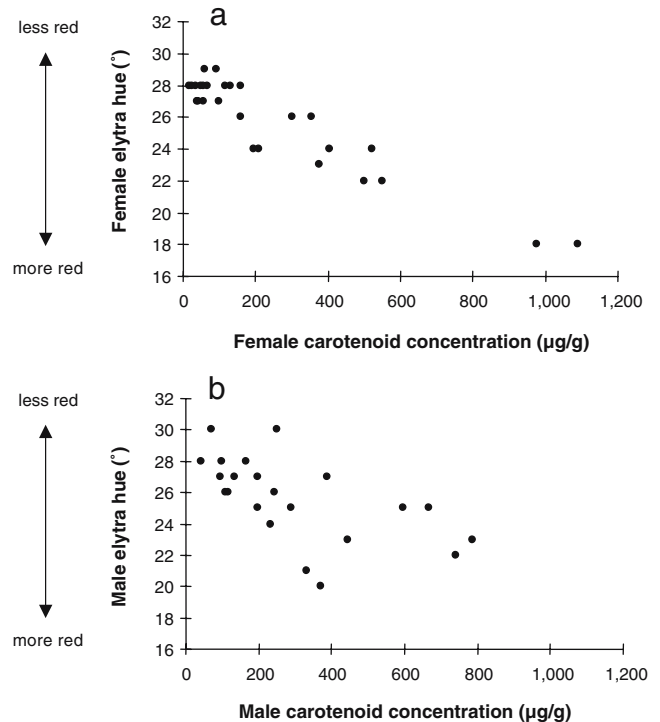


Fig. 2 Correlations between carotenoid concentration and elytra hue in **a** female and **b** male *Harmonia*. In both sexes, individuals had redder elytra when their elytra contained greater concentrations of carotenoids

by pterins (Levene's test for unequal variances, $p < 0.01$). For this reason, only carotenoids were extracted and analyzed via spectrophotometry. Females were significantly larger than males ($z = -3.03$, $p < 0.01$), but elytra mass was not significantly correlated with preextraction hue or carotenoid concentration in either sex ($p > 0.48$ for all comparisons).

Spot darkness, and extent of red/black coloration

Males, on average, had a greater percentage of their elytra covered by red coloration (or, conversely, less of a percentage covered by black) than did females ($z = 3.33$, $p < 0.001$, Fig. 3a). In addition, black spots on the elytra of females were significantly darker (less bright) than spots on males ($z = 2.86$, $p < 0.01$, Fig. 3b). In both sexes, the fraction of the elytra that was red was positively correlated with the mass of the beetles ($r_s = 0.44$ for females, 0.52 for males, $p < 0.03$ for both sexes, Table 1). In both sexes, there was no statistically significant relationship between the percent of the elytra area covered by red and any of the color variables ($p > 0.08$ for all comparisons, Table 1). In both sexes, however, individuals with darker black spots tended to also have a redder hue. This relationship was statistically significant in males ($r_s = 0.52$, $p < 0.02$), but not in females ($r_s = 0.36$, $p = 0.06$). There was no statistically significant

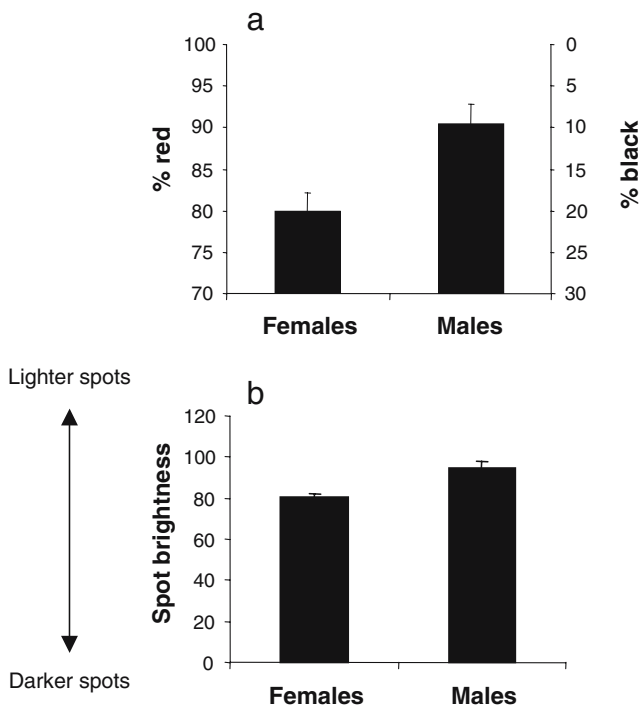


Fig. 3 Sex differences in **a** elytra area covered in red/black and **b** spot brightness in *Harmonia* (note that lower brightness values signify darker spots)

relationship between spot darkness and body mass in males ($r_s=0.01$, $p=0.96$) or females ($r_s=0.2$, $p=0.2$).

Relationships between alkaloid content and color/pigmentation in both sexes

There was no significant correlation between total alkaloid content and preextraction hue, postextraction hue, carotenoid concentration, or mass in either sex ($p>0.07$ for all comparisons, Table 1). In females, there was, however, a strong positive correlation between the percentage of the elytra that was covered by red and total alkaloid content ($r_s=0.61$, $p<0.01$, Fig. 4a). This same effect was seen to a degree in males, although the result was not statistically significant ($r_s=0.41$, $p=0.10$). There was also no statisti-

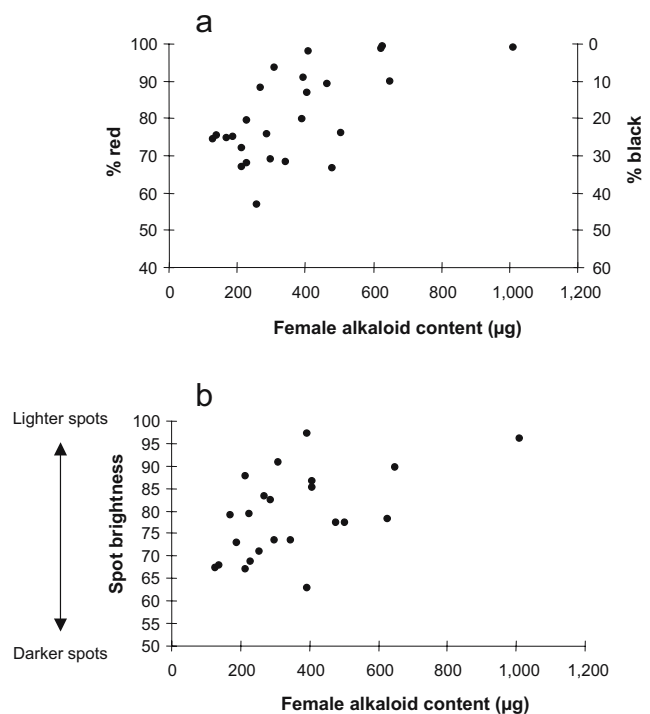


Fig. 4 Correlations between **a** total alkaloid content of females and the percentage of their elytra covered by red/black, and **b** total alkaloid content of females and black spot brightness

cally significant difference ($z=-1.79$, $p=0.07$) in the average (\pm SE) total alkaloid content of males ($268.43 \pm 37.28 \mu\text{g}$) versus females ($369.68 \pm 40.25 \mu\text{g}$). Focusing on just the black spots, females with fainter black spots tended to have a higher amount of alkaloid than those with darker spots ($r_s=0.56$, $p<0.01$, Fig. 4b). This relationship between spot brightness and alkaloid content was not seen in males ($r_s=0.16$, $p=0.56$).

Discussion

We have shown that carotenoid pigments are largely responsible for the vibrant orange-red coloration of *H.*

Table 1 Spearman rank correlations for measures of color, pigmentation, mass, and alkaloid content in male and female *Harmonia*

	Pre-hue (°)		Post-hue (°)		Carotenoid concentration (μg/g)		Mass (g)	
	r_s	p	r_s	p	r_s	p	r_s	p
Percent red, females ($n=27$)	0.01	0.96	-0.07	0.73	0.05	0.8	0.44	0.02
Percent red, males ($n=22$)	0.37	0.09	0.06	0.78	-0.37	0.09	0.52	0.01
Alkaloid content (μg), females ($n=25$)	-0.03	0.9	0.3	0.14	-0.02	0.93	0.14	0.5
Alkaloid content (μg), males ($n=17$)	-0.19	0.49	-0.28	0.28	0.37	0.14	0.44	0.08

Pre-hue and post-hue refer to the hue of the elytra before and after extraction of carotenoids, respectively. Percent red refers to the fraction of the total elytra covered in red (as opposed to black).

axyridis elytra. In both males and females, a strong positive correlation exists between the concentration of carotenoids in the outer wings and the intensity of red coloration. In males, there is also a positive relationship between the initial hue of the elytra and the postcarotenoid extraction hue, indicating that individuals with high levels of carotenoids also have relatively high levels of pterins contributing to their overall reddish color. Correlated levels of carotenoids and pterins have been found previously in the orange skin spots of guppies (*Poecilia reticulata*; Grether et al. 2001), and mechanistically, this may be explained by similar metabolic costs of producing both color types (Grether et al. 2005).

Carotenoid content and red color of male and female elytra were not correlated with body harmonine content. This suggests that the color intensity of red elytra does not function as an intraspecifically variable signal of chemical defense in these beetles. This, of course, does not preclude red coloration from functioning as a potentially valuable signal to either conspecifics or predators. Bright individuals could be advertising their ability to effectively forage for carotenoids, process them, and transport them for deposition in the elytra; all are potentially costly steps (reviewed in McGraw et al. 2005b; McGraw 2006a). In addition, although the hue of the elytra may be unrelated to defensive levels, in females, the fraction of the elytra taken up by red areas is strongly positively correlated with their total harmonine content. This effect was also seen to a degree in males, but the correlation was not statistically significant, likely driven by the fact that fewer males were included in the study. In both sexes, this result is important because color patterns (as opposed to intensity) have been shown to play a role in mate selection in *Harmonia*, and at least one study has shown nonmelanic forms to be preferred over melanic ones (Osawa and Nishida 1992). Early work speculated that the red elytra of ladybird beetles acts as an aposematic signal to potential predators (Edmunds 1974; Frazer and Rothschild 1962), and our results indicate that it may be the total area covered by red (rather than its hue) that indicates unpalatability.

Harmonia offers a unique opportunity to investigate the signaling roles of carotenoids and melanins simultaneously. The situation is made even more interesting by the fact that the pigments are being displayed on the size-limited surface of the elytra. To present a greater area of melanins, by nature, a smaller area of carotenoids has to be displayed. There are potential benefits to having both black and red elytra that may place *Harmonia* under selective pressure from multiple directions. Melanic morphs have been experimentally shown to absorb more thermal energy (Brakefield and Wilmer 1985; Stewart and Dixon 1989), and as a consequence, have increased activity rates compared to nonmelanic morphs. On the other hand, we

have shown that beetles that have mostly red wings (and consequently little melanin) may be signaling high levels of alkaloids, which may help to explain the female mating preference for nonmelanic males witnessed by Osawa and Nishida (1992). With regards specifically to the melanic spots of animals in the current study, we found that females with fainter spots were more chemically defended than females with bold black spots. Again, a potential trade-off exists in which individuals are either active but not well defended (more melanic beetles) or less active but possessing greater amounts of defensive compounds. Sex differences in spot brightness (and corresponding correlations with alkaloid levels) are likely driven by the fact that females are larger and under different energetic constraints (e.g., costs associated with egg laying) than males.

Further studies are necessary to fully elucidate the relationship between coloration (both carotenoid- and melanin-based) and fitness in *Harmonia*. First, much more needs to be known about how harmonine is generated to understand potential trade-offs between color and defense. In addition, mate choice trials and predation assays will be necessary to determine exactly what role the red and black colors are playing in communication. Because coccinellids themselves are unable to see red wavelengths (Lin and Wu 1992), it is likely that, in *Harmonia*, it is the contrast between the two colors (and not their specific hues) that plays a role in interactions between conspecifics. Lastly, although it is well established that diet and the environment play a strong role in shaping the red color of adult *Harmonia*, there is also a genetic component to the melanic coloration that should not be overlooked (Grill et al. 1997). It would be interesting to perform studies similar to the ones described here with different color morphs of *Harmonia*. Our studies focused solely on the *succinea* color morph, in which the red/orange coloration tends to dominate the area of the elytra. This is the predominant morph in the area where the beetles for this study were collected, but several other genetically distinct morphs occur in native Asian populations (Grill and Moore 1998). Studies similar to the one described here, focusing on these other morphs of *Harmonia*, would greatly enhance our understanding of how color and fitness are related in this highly polymorphic ladybird beetle.

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