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Christopher P. Grill · Allen J. Moore

Effects of a larval antipredator response and larval diet on adult phenotype in an aposematic ladybird beetle

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Abstract Many ladybird beetles respond to a potential predation event by 'reflex bleeding' or secreting a noxious defensive chemical that is similar to hemolymph. Both adults and larvae show this response. Reflex bleeding is known to reduce predator attack rates and increase prey survival after an attack, especially when reflex bleeding is employed in combination with other cues such as odor and warning coloration. In this experiment, we examined how variability in the number of reflex bleeding events and food quality in the larval stage of the aposematic ladybird beetle *Harmonia axyridis* affected elytral color, development time, and terminal size in adults. Effects of reflex bleeding were subtle and may have been influenced by diet treatments. Adult color did not differ between bleed treatment groups but beetles that reflex bled tended to take longer to develop and grow to smaller sizes than control group beetles. There were clear and strong effects of larval diet on adult phenotype: an ad libitum pollen diet resulted in paler adult coloration, shorter development time, and larger adult size relative to a limited-availability aphid diet. Our results suggest that the best environment for producing bright-red coloration may not be the best environment for favorable expression of life history characters, especially under stressful conditions. Interactions between different life history stages of *H. axyridis* are also discussed.

Key words Aposematic coloration · Chemical defense · Coccinellidae · *Harmonia axyridis* · Reflex bleeding

C.P. Grill (✉)[1]
Center for Ecology, Evolution, and Behavior,
T.H. Morgan School of Biological Sciences,
101 Morgan Building, University of Kentucky,
Lexington, KY 40506-0225, USA
Fax: (606) 257-1717; e-mail: cpg@ceeb.uky.edu

A.J. Moore
Department of Entomology, University of Kentucky,
Lexington, KY 40546-0091,USA

Introduction

Environmental conditions experienced during development can profoundly influence the expression of adult phenotypes (e.g., Werner and Gilliam 1984; Ebenman 1992). For example, juveniles that experience relatively low quality food are likely to have a decreased growth rate or adult size (Slansky and Rodriguez 1987). More complicated environmental effects may be seen in predator-prey systems where predators influence juveniles such that phenotypic expression in adults is affected directly (Lively 1986; Parejko and Dodson 1991) or indirectly (e.g., through mediation of behavior; Werner and Hall 1988; Werner 1991; Grill and Juliano 1996; Werner and Anholt 1996). Studies that simultaneously address multiple life history stages have also generally found strong interactive effects between stages (e.g., Werner and Hall 1988; Anholt 1991; Mittelbach and Osenberg 1993). Thus, the effects of environmental conditions in any one life history stage have the potential to cascade into subsequent stages.

Interactions between life history stages may be especially important for aposematic species. Aposematism is a complex antipredator strategy that depends on coordinated expression of morphological, biochemical, physiological, and behavioral traits. Environmentally induced change in one of the characters in an antipredator suite may undermine the effectiveness of the entire defensive strategy (Brodie 1992; Endler 1995). Color is the basis of an effective aposematic defense strategy although color may be associated with other important biological functions including communication, territorial defense, thermoregulation, and mate acquisition (Endler 1978). Animal color is certainly subject to change due to environmental variability. For example, changes in juvenile diet can result in changes in adult color, which may in turn influence risk of predation or mate acquisition (Kodric-Brown 1989). Such environmentally induced changes in color are expected to have especially serious consequences for aposematic

organisms if effectiveness of antipredator responses is dependent on color.

We examine the impact of different types of environmental variability in the larval stage on elytron (wing covering) color, development time, and terminal size in adults of the ladybird beetle, *Harmonia axyridis*. This beetle combines bright coloration with chemical defense, and occasional aggregation behaviors. Like many ladybird beetles, *Harmonia* can exude a noxious, alkaloid-based fluid when disturbed by potential predators (Pasteels et al. 1973). This response, known as reflex bleeding, occurs both in adults and larvae of *H. axyridis*; we examine effects of larval bleeding in this study. As in other ladybird species, reflex bleeding in *H. axyridis* is thought to be defensive (Komai 1956; Holloway et al. 1991, 1993) though the exact nature of the physiology and biochemistry underlying the response is poorly understood. Previous studies of other ladybird species have shown that reflex bleeding, in combination with aposematic coloration, is associated with reduced rates of attack, increased survival of prey animals after an attack (Wiklund and Jarvi 1982; Marples et al. 1994), and even death of potential predators (Marples et al. 1989). Reflex blood is very similar to hemolymph (Kay 1969; Bowers1992) and up to 20% of beetle weight can be expelled in a single bleeding event (Holloway et al. 1991). Thus, predator-rich environments, in which reflex bleeding is frequently employed, are expected to be costly to larvae, and such costs could affect adult performance.

Our previous work has shown that color, development time and adult size are affected by diet quality in *H. axyridis* adults (Grill et al. 1997). In this study, we extend our examination of the effects of environmental variability to include defensive strategies, as well as a set of more natural diets. We predict that, in relatively stressful environments (i.e., those with more reflex bleeding events or lower-quality food), *H. axyridis* will be less brightly colored, take longer to develop, and grow to a smaller adult size than controls. We also investigate the possibility that different sources of environmental variability can interact, producing complex patterns of phenotypic expression.

Materials and methods

Study organism and husbandry

H. axyridis is a brightly colored ladybird beetle endemic to southeastern Asia. *H. axyridis* has long been the subject of ecological and evolutionary research (Dobzhansky 1993; Komai 1956) and has become important in biological control programs in the United States (De Quattro 1995). This beetle is a very effective colonizer and has spread over most of the United States following agricultural releases in the southeastern and western United States (Tedders and Schaeffer 1994). Husbandary procedures of *H. axyridis* are well-established, easily executed, and inexpensive, making this beetle an excellent candidate for experimental studies of ecological questions.

H. axyridis adults were field-collected from a tobacco farm in Scott County, Kentucky, in early June 1996. These adults and all subsequent generations were maintained in incubators (27°C,15:9 light:dark), and supplied with aphids (*Myzus nicotiana*) three times per week (see below for details). Field-collected beetles were housed individually in petri dishes during a 3-day acclimation period and were then sexed and isolated into pairs for mating. Upon reaching maturity, this first laboratory generation was mated to produce a second laboratory generation, which was subsequently used for this experiment. We used beetles one generation removed from the field to control for maternal and other environmental effects (Falconer and Mackay 1996). Hatchlings were separated from their siblings within 24 h of hatching to minimize common family environmental effects and cannibalism. Larvae were reared individually for the remainder of each experiment.

Experiment 1 – effects of larval reflex bleeding on color and life history

All larvae in experiment 1 were reared identically on three feedings per week of approximately 50 aphids per feeding. At the fourth instar, simulated predation trials, or 'bleed treatments,' were initiated resulting in reflex bleeding. These treatments consisted of agitating beetle larvae with a fine-tipped paint brush until reflex blood was exuded. Ladybird larvae possess a large number of sensory setae that, when stimulated, can trigger the bleeding response. Repeated agitation of setae, especially those along the dorsal surface of the abdomen, was usually sufficient to initiate a bleed.

Fifteen full-sib larvae from each of ten families were split evenly into three bleed treatments. Larvae in the control (0) treatment were raised to adulthood without any additional manipulation. Larvae in the single-bleed (1) treatment were subjected to one bleed; the initial bleed attempt was performed after the molt to the fourth instar. Larvae in the multiple-bleed (2+) treatment were bled daily, again starting after the molt to the fourth instar, until four bleeds had been elicited or pupation occurred (mean number of bleeds per larva in 2+ treatment = 2.3). Reflex bleeding could not always be initiated, especially with older larvae in the 2+ treatment. If reflex bleeding could not be induced on a given day, additional attempts were made on subsequent days until the requisite number of bleeds had been achieved.

Experiment 2 – effects of larval reflex bleeding and diet on color and life history

In the second experiment, effects of reflex bleeding (0 vs. 2+ bleeds) and two different diets, as well as the interaction effect, were evaluated simultaneously in a 2×2 factorial design. The protocol for bleeds was similar to that used in the first bleed experiment with two exceptions: the single-bleed treatment was eliminated and, after bleeding, any visible exudate was dabbed with a tissue to prevent resorption of expelled fluids by the larvae. Both diets were considered food limited relative to experiment 1; the first diet consisted of three feedings per week of approximately 35 aphids (*M. nicotiana*) (the 'few-aphid' treatment) while the second consisted of one aphid feeding per week plus unlimited access to dried honeybee pollen (the 'pollen+' diet). Previous work has shown that an aphid diet is superior to a pollen diet in *H. axyridis*, but this study was performed under ad libitum food conditions (Pfannenstiel 1995). During the course of this experiment, aphid colony densities rapidly declined and food for the beetles became scarce. The feeding schedule was maintained but the number of aphids available (approx. 15 aphids per feeding) became relatively small. Thus, the food treatment in experiment 2 represented a contrast between two relatively low quality habitats, one in which food was of relatively high quality but scarce (few-aphid treatment), and the other, in which food was abundant but of lower overall nutritive value (pollen + treatment).

Data collection

One of the most noticeable characteristics of *Harmonia* is extreme variation in elytron (i.e., wing covering) color and spot patterns even within small populations. Several genetically distinct color morphs exist in native Asian populations but in Kentucky, *H. axyridis* populations consist entirely of the *succinea* color morph, which is homozygous recessive for predominantly red-orange, rather than black, elytral color (Komai 1956). Previous studies that examined 'color' in ladybird beetles have typically focused on distribution of spot patterns (Komai 1956; Holloway et al. 1995) or comparisons between discrete color morphs (e.g., melanic vs. nonmelanic elytra; Tan and Li 1934; Kearns et al. 1990,1992; O'Donald and Majerus 1992; Osawa and Nishida 1992). Elytral color is highly and continuously variable within the *succinea* morph, ranging from a deep crimson to a pale yellow-orange. Only recently have quantitative data been collected on the spectral properties of *H. axyridis* elytron color within a single color morph and how that color is influenced by environmental effects (Grill et al. 1997).

We quantified adult color by measuring spectral reflectance using a Spectator portable spectroradiometer with a standardized halogen light source (Word Precision Instruments, model F-O-LITEH). Measurements were taken on live animals at right angles to the surface of the elytra using a fiber-optic probe. Approximately 48 h after adult emergence, measurements were made with a fiber-optic probe placed approximately 2 mm from the posterior end of the body and 2 mm from the elytral surface. Data were collected in approximately 0.3-nm increments and pooled into 5-nm 'bins.' Reflectance readings for each bin were calculated as the average of all reflectance readings within the bin. Reflectance was calculated as the ratio of transmitted (i.e., reflected) light from the beetle relative to transmitted light from a barium sulfate standard. Two measurements were taken for each beetle and the average of these measurements was used in subsequent analyses.

We measured development time and adult size to examine the effects of larval environment on life history characters. Development time was recorded as the number of days between oviposition and emergence of mature adults for experiment 1. For experiment 2, oviposition date was not recorded. Instead, we used number of days between egg hatch and adult emergence as our measure of development time. The period between oviposition and hatching of eggs is relatively invariant at a given photoperiod and temperature. We therefore calculated the mean number of days between oviposition and hatch in a previous experiment which was run under similar conditions and added this value (2.31 days) to the development time of each beetle in experiment 2 in order to put development times for both experiments on the same scale. Adult size was estimated by measuring pronotum width to the nearest 0.01 mm for each adult beetle. Pronotum widths were measured no sooner than 24 h after adult emergence using a PowerMac 8500 and the public domain NIH Image video analysis package (download at **http://rsb.info.nih.gov/nih - image**/or **ftp://zippy.nimh.nih.gov/pub**/). Image measurements are highly repeatable (Becker 1992) within individuals ($t = 0.98$; $n = 15$ beetles with two measurements per beetle; Grill et al. 1997).

Statistical analyses

Simulated predation did not always result in reflex bleeding. While this phenomenon was unanticipated, it did allow us to test for confounding effects of simulated predation events, independent of the reflex bleeding events. Characters were analyzed, using ANOVA, for effects of simulated predation that did *not* result in reflex bleeding. Only individuals from the multiple-bleed treatments were included in this analysis. We have no evidence of any effects of simulated predation, independent of reflex bleeding events, on *H. axyridis* character expression or fitness in either experiment, nor does the simulated predation regime interact with diet (Table 1).

To examine the effects of treatment on color, we quantified reflectance using inflection point of reflectance curves (see Fig. 1) and principal components analysis (PCA) of color spectra (Endler 1990). Reflectance spectra for adult color were generally sigmoidal. The inflection point of such a curve indicates, by definition, the point on the curve at which the increase in reflectance switches from accelerating to decelerating. Thus, the inflection point can be used as a crude, but reliably identifiable indicator of beetle elytron color (Fig. 1). We estimated inflection point wavelength for each curve by sight. Higher values of 'infection point' (i.e., inflection points occurring at higher wavelengths) tend to be associated with redder individuals (see Fig. 1). The repeatability (Becker 1992) of any single estimate of inflection point is high ($t = 0.79$; $n = 275$ beetles with two measurements each; Grill et al. 1997). We used the mean value of two measurements for each beetle to reduce the error associated with individual measurements.

A more detailed analysis of color spectra was performed using PCA. Depending on the angle of the reflectance probe to the insect, a spectral curve can be transposed up or down while retaining the same basic shape; deviations from absolute perpendicularity of the

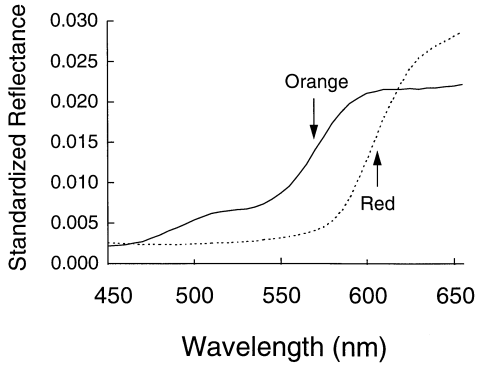

Fig. 1 Illustration of 'pure'-red vs. orange curves, based on reflectance spectra of maximum chroma red and orange Munsell color chips. Reflectance is measured relative to a barium sulfate standard. The *arrows* indicate the approximate location of the inflection point on each curve

Table 1 Tests for confounding effects of bleed protocol, independent of the effects of the reflex bleeding event. One of our characters, development time, is confounded with number of bleeds because bleeds were attempted every day until eclosion; we did not analyze development time for confounding effects of the simulated predation regime (*PC* principal component)

Dependent variable	*df*	Mean square	*F*-ratio	*P*
Effects of bleeding protocol in experiment 1				
Pronotum	9,99	0.022	1.236	0.282
Inflection	9,99	2.735	0.319	0.967
PC1	9,99	0.716	0.744	0.668
PC2	9,99	1.334	1.308	0.242
Effects of bleeding protocol in experiment 2				
Pronotum	8,317	0.031	1.295	0.245
Inflection	8,317	19.404	0.516	0.844
PC1	8,317	0.664	0.645	0.740
PC2	8,317	0.641	0.804	0.600

probe to the beetle result in lower reflectance at all measured wavelengths. Therefore, all color spectra were standardized to a common area under the curve (= 1) before PCA to correct for differences between curves due to measurement error (Endler 1990). We then analyzed for differences in PC scores, treating each score as a dependent variable in our analyses (see Results for interpretation of principal components).

Treatment and family effects for both experiments were assessed using mixed-model ANOVA (Fry 1992). Family effects were tested using family-by-bleed interaction sums of squares as outlined in Fry (1992). Results were similar to those produced using the family-by-diet interaction sums of squares. Not all measurements were available on all beetles, so depending on the characters, different numbers of individuals were available for analysis. Pairwise comparisons for experiment 1 were performed using Fisher's least-significant-difference tests. Normality and variance assumptions of ANOVA were tested using Lillifoers tests and graphical inspection of data; no variables required transformation. All analyses of treatment effects were performed using the GLM procedure from the SYSTAT analysis package (Wilkinson et al. 1996). PCA and the Lillifoers test were also performed using SYSTAT.

Results

Principal components of color

PCA revealed very similar overall component loading patterns for both experiments, though some differences existed between treatments within experiment 2 (Table 2, Fig. 2). After standardization most of the variance (≥98%) in different color spectra fell out in the first two principal components (Table 2).

As discussed by Endler (1990), color is composed of three primary components: value, chroma, and hue. *Value* is the overall brightness or total intensity of light at all visible wavelengths reaching the eye. *Chroma* or 'saturation' of beetle color, is a measure of the amount of neutral gray tones present in a color; colors with maximum chroma contain no neutral gray tones. *Hue* is what most people refer to as color (e.g., red, orange, yellow). In our raw samples, differences in value or brightness were due primarily, though not entirely, to measurement error; our measurement protocol, combined with the standardization of spectra to a common brightness level, minimizes measurement error.

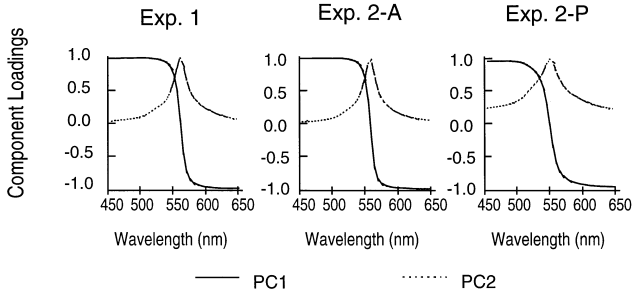

Fig. 2 Component loadings for each measured wavelength from principal component (*PC*) analyses of color spectra (*2-A* few-aphid, *2-P* pollen+)

Our PCA provides scores that can be interpreted as components of color. We interpret PC1 as chroma, or a measure of the amount of 'neutral colors' present. After standardization, PC1 accounted for the majority of variance (74–88%) between spectra within each of the food treatments (Table 2), suggesting that chroma is an important determinant of color differences between *H. axyridis* adults. Component loadings indicate that PC1 conveys information about the ratio between reflectances at high and low wavelengths in each spectrum (Fig. 2). Changes in chroma result in changes in the ratio of high to low wavelengths (e.g., via change in the slope of the reflectance curve; Endler 1990). Thus, PC1 values appear to be very tightly linked with chroma. Beetles with high PC1 scores generally appear pale or 'faded' while those with low PC1 scores possess richer, more vibrant coloration.

We interpret PC2 as a generalized measure of hue (orangeness vs. redness). The wavelengths with the highest component loadings for PC2 coincided roughly with the location of inflection points (approx. 560 nm). This suggests that inflection point and PC2 are conveying similar information. A moderate correlation between PC2 and inflection point ($r = 0.618$, $n = 141$ individuals, $P = 0.0005$) supports this contention but also suggests that both characters may contain sources of variation that are not shared by the other character. Beetles with low values of PC2 generally appear more red and beetles with high values of PC2 appear more orange. PC2 accounted for most of the remaining variation (11–24%) after accounting for PC1 in each of the food treatments (Table 2).

Experiment 1 – effects of larval reflex bleeding on color and life history

The effects of reflex bleeding on color depended on how color was measured. Individuals that experienced a single bleed treatment had lower inflection points (i.e., were less red) than controls or 2+ bleed individuals. In contrast, bleed treatments in experiment 1 did not have a significant effect on either of the PC scores (Figs. 3, 4;

Table 2 Principal component (*PC*) analysis of elytral reflectance data from beetles reared under three environmental (food) conditions. Component loadings are presented graphically in Fig. 2

	Experiment 1 (many aphids)		Experiment 2 (few aphids)		Experiment 2 (pollen+)	
	PC1	PC2	PC1	PC2	PC1	PC2
Eigenvalue	35.221	5.075	35.907	4.467	30.348	9.682
Variance explained (%)	85.9	12.4	87.6	10.9	74.0	23.6

Table 3). Bleed treatments did affect expression of life history characters. In experiment 1, adult pronotum width was significantly larger when no bleeding occurred than in either bleed treatment. There was no difference in pronotum width between the 1 bleed and 2+ bleed treatments (Fig. 4; Table 3). Development time increased with number of bleeds taken, though this effect was not significant.

A significant effect of family indicates that, on average, expression of characters was different between families. Inflection point was the only measured character significantly affected by family. Marginally significant family-by-treatment interactions existed for development time and for PC1 (Table 3).

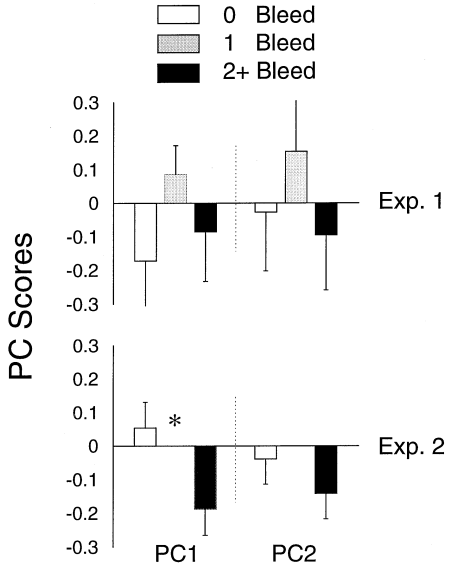

Fig. 3 Effects of reflex bleeding on chroma and hue as measured using principal components (*PC*) analysis in experiments 1 and 2. *Asterisks* indicate significant differences between treatment means for each component score. Experiments 1 and 2 were not compared statistically

Experiment 2 – effects of larval reflex bleeding and diet on color and life history

Experiment 2 – revealed little influence of reflex bleeding on any of the measured characters (Figs. 3, 4). The single exception was a significant effect of reflex bleeding on PC1, which is the factor we interpret as a chroma (Fig. 3; Table 4). Individuals in the 0 bleed treatment had significantly higher values of PC1 (i.e., these beetles were more pale), though PC2, or hue (orangeness vs. redness), did not differ between treatments.

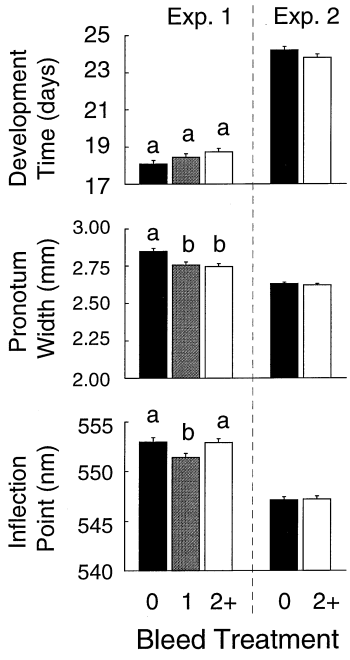

Fig. 4 Effects of reflex bleeding in size, development time, and inflection point position in experiments 1 and 2. In experiment 1, treatment means with different letters are significantly different from each other. There were no significant effects of reflex bleeding in experiment 2. Experiments 1 and 2 were not compared statistically

Table 3 Effects of reflex bleeding (*B*), family (*F*), and $B \times F$ interactions in experiment 1. Significant results are reported in *italics* (*PC* principal component)

Variable	Source	*df*	Mean square	*F*-ratio	*P*
Inflection	Bleed	2	46.1553	7.083	*0.0014*
	Family	8	23.7518	3.024	*0.0284*
	B × F	16	7.8536	1.205	0.2817
	Error	83	6.5162		
PC1	Bleed	2	0.6315	0.740	0.4801
	Family	8	1.2510	0.837	0.5841
	B × F	16	1.4945	1.751	*0.0512*
	Error	90	0.8537		
PC2	Bleed	2	0.6375	0.609	0.5460
	Family	8	1.3953	2.048	0.1058
	B × F	16	0.6812	0.651	0.8333
	Error	90	1.0464		
Development time	Bleed	2	3.3368	2.278	0.1084
	Family	8	3.4939	1.315	0.3041
	B × F	16	2.6564	1.813	*0.0411*
	Error	90	1.4648		
Pronotum	Bleed	2	0.0873	5.184	*0.0074*
	Family	8	0.0141	0.570	0.7876
	B × F	16	0.0247	1.466	0.1306
	Error	89	0.0168		

Table 4 Effects of family, diet, reflex bleeding, and all possible two-way interactions in experiment 2. The three-way interaction term for all variables tested was non-significant and was dropped from the analysis. Significant results are reported in *italics*

Variable	Source	*df*	Mean square	*F*-ratio	*P*
Inflection	Bleed	1	0.4330	0.030	0.8620
	Diet	1	6707.7069	469.217	*< 0.0001*
	Family	27	21.5043	1.451	0.1698
	B × D	1	1.2937	0.091	0.7638
	B × F	27	9.1664	0.641	0.9162
	D × F	27	14.8232	1.037	0.4191
	Error	250	14.2937		
PC1	Bleed	1	4.1727	5.022	*0.0259*
	Diet	1	51.8560	62.415	*< 0.0001*
	Family	27	0.7731	0.843	0.6698
	B × D	1	1.2387	1.491	0.2232
	B × F	27	1.1827	1.423	0.0862
	D × F	27	0.9171	1.104	0.3355
	Error	248	0.8308		
PC2	Bleed	1	0.7547	0.952	0.3302
	Diet	1	77.5386	97.802	*< 0.0001*
	Family	27	0.9062	1.194	0.3239
	B × D	1	0.0879	0.111	0.7394
	B × F	27	0.7957	1.004	0.4643
	D × F	27	0.7588	0.957	0.5298
	Error	248	0.7928		
Dev. Time	Bleed	1	10.284	1.799	0.1810
	Diet	1	317.466	55.523	*< 0.0001*
	Family	28	14.754	1.559	0.1230
	B × D	1	8.710	1.523	0.2182
	B × F	28	4.943	0.864	0.6668
	D × F	28	9.462	1.655	*0.0230*
	Error	280	5.718		
Pronotum	Bleed	1	0.0113	0.640	0.4245
	Diet	1	1.8178	103.157	*< 0.0001*
	Family	28	0.0389	2.028	*0.0332*
	B × D	1	0.0119	0.676	0.4115
	B × F	28	0.0154	0.876	0.6508
	D × F	28	0.0192	1.088	0.3513
	Error	280	0.0176		

All characters measured showed a strong, significant response to diet in this experiment (Figs. 5, 6; Table 4). In the few-aphid treatment, the inflection point was higher (more red), development time was longer, and pronotum width was smaller relative to the pollen+ treatment (Fig. 6). Results for both PCA factors are consistent with results for inflection point with few-aphid treatments producing relatively bright and red ladybird adults and the pollen+ food treatment producing relatively pale and orange adults (Figs. 5, 6; Table 4). Results indicating that colors differ between treatments are further supported by comparing plots of mean standardized reflectances for each of the two diet treatments (Fig. 7).

Pronotum width was the only measured character significantly affected by family (Table 4). Of all possible interactions between family and the two treatments, only one, the family-by-food interaction for development time, was significant (Table 4).

No significant reflex bleed-by-diet interaction effects were detected for any of the measured characters in experiment 2 (Table 4). There were, however, differences in how simulated predation affected trait expression in the two different experiments. Given that the primary difference between experiments 1 and 2 was diet related, the possibility remains that effects of reflex bleeding interact with diet (see Discussion).

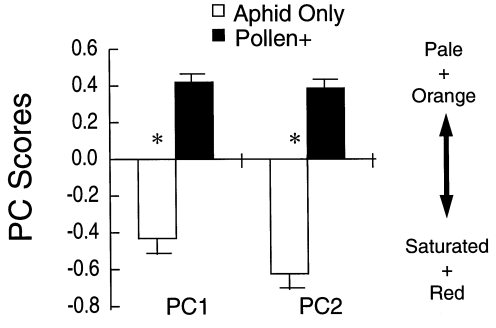

Fig. 5 Effects of diet on chroma and hue as measured using principal components (*PC*) in experiment 2. *Asterisks* indicate significant differences between treatment means for each component score

Discussion

Effects of larval reflex bleeding on adult phenotype

Overall, there were few effects of reflex bleeding on the color of *H. axyridis* (Figs. 3, 4). The possibility remains,

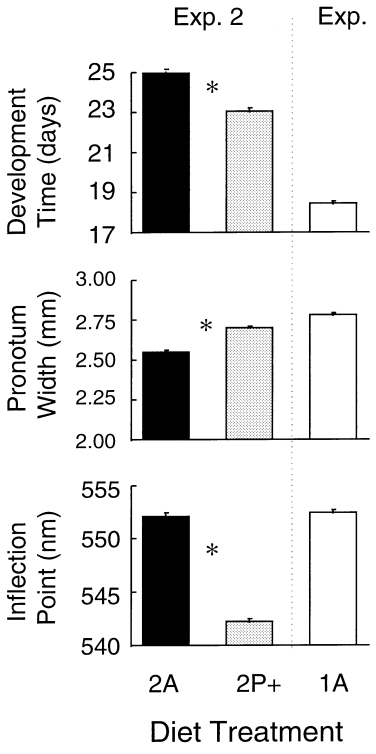

Fig. 6 Effects of diet on size, development time, and inflection point in experiments 1 and 2. *Asterisks* indicate significant differences between the few-aphid (2A) and pollen+ (2P+) treatments in experiment 2. Experiments 1 and 2 were not compared statistically

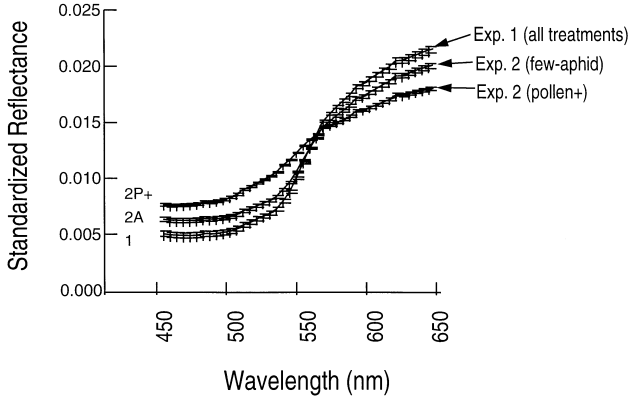

Fig. 7 Mean reflectance at each measured wavelength for beetles under different food regimes. Sharply sloped curves indicate more-saturated beetles while more gently sloping curves are associated with less-saturated (i.e., more pale), beetles (Endler 1990). Color spectra of the three different bleed treatments of experiment 1 were virtually identical and are condensed into a single pooled mean curve for clarity of presentation

of course, that reflex bleeding has major effects on other aspects of the aposematic defense strategy employed by *Harmonia*, particularly if the quality of reflex blood declines with repeated use and if that decline is associated with a reduction in the effectiveness of the reflex blood as a deterrent to predators. Additional work is needed on how environmental variability influences toxicity and quantity of reflex blood, as well as regeneration of expelled fluid.

In contrast to the color characters, use of the reflex bleeding response does adversely affect *H. axyridis* life history (Fig. 4). Decreased performance of life history characters in bleed vs. control treatments suggests that energetic constraints are imposed with use of reflex bleeding. Beetles that expel copious amounts of fluid are at greater risk of dehydration, as well as nutrient loss, and possibly increased predation risk due to reduced effectiveness of the bleeding response with repeated use. Our results show that life history traits are influenced by use of reflex bleeding, but only if food quality is above some minimum threshold. Detailed quantification of the characters underlying reflex bleeding (e.g., Holloway et al. 1991, 1993) under different environmental conditions would be valuable.

Effects of larval diet on adult phenotype

As in previous work (Grill et al. 1997), diet had strong significant effects on all characters we measured in *H. axyridis* (Figs. 5, 6; Table 4). Results from the different diet treatments indicate that the best diet in terms of *H. axyridis* life history may not be the best diet for producing conspicuous coloration under food stress conditions (Figs. 5, 6). Previous studies provide evidence that ladybird coloration can affect the likelihood of predation in several ways. Beetle color can interact with smell, taste, and toxicity to produce a stronger repellent effect than that produced individually by any of these characters (Rothschild 1961; Sillen-Tullberg 1985; Marples et al. 1994; Marples and Roper 1996). Our results suggest that, if beetles are dependent on warning coloration as a primary antipredator strategy, requirements of this strategy may be in direct conflict with the life history needs of *H. axyridis*, at least under food stress conditions. How *H. axyridis* mediates this potential trade-off remains to be investigated.

Interactive effects between larval diet and reflex bleeding?

Although we recognize the need for caution in comparing the results of our two experiments, we suggest that differences in food quality, rather than differences in protocol between our experiments, resulted in the observed differences in character expression between experiments. In experiment 1, beetles consistently performed better than in either food treatment of experiment 2. The effects of bleed treatments, however, were not consistent between experiments. Phenotypes for the experiment 1 control groups were similar to those found in nature and in previous experiments (Ueno 1994; Grill et al. 1997) while those for experiment 2 reflected severe stress relative to natural populations (Fig. 6). Beetles in experiment 2 took longer to develop,

were smaller and relatively less red than those from experiment 1. This combination of results suggests that, while no diet-by-reflex bleeding interactions were evident in experiment 2, diet quality can mediate the effects of reflex bleeding on phenotype expression. Additional experiments are needed to determine if interactions between diet and other environmental factors are subject to threshold effects.

Do different life history stages interact?

These experiments demonstrate that adult phenotypes, adult life history and to a lesser degree, defensive strategies, can be contingent on the larval environment. Not surprisingly, different types of environmental conditions produce different effects on phenotype expression. Responses to larval environment are often complex and not easily generalized. Our results underscore the need for more comprehensive studies of complex life cycles. Effects of environmentally induced changes in one life history stage are likely to cascade into other stages; the overall impact of such change is unpredictable.

Contrary to our expectations, we found little evidence for a strong link between beetle color and frequency of reflex bleeding in larval *H. axyridis*. Frequency of bleeding may well be linked with other predator cues, such as toxicity, odor, or quality of reflex blood. Effects of reflex bleeding on these cues is largely unstudied. There was, however, a moderately strong effect of reflex bleeding on *H. axyridis* life history. More work is needed on the role of food quality in mediating the effects of reflex bleeding with an emphasis placed on reflex bleeding as a trait, rather than an imposed treatment (e.g., Holloway et al. 1991, 1993).

The results of these experiments are not simply examples of treatments affecting phenotypic expression. Environmental variability in the *H. axyridis* larval stage has the potential to influence the entire suite of traits associated with aposematic defense in the adult stage as well as important life history characters. This work suggests that favorable expression of antipredator characters can be in direct conflict with performance of life history characters, depending on the quality of diet. Clearly more studies are needed, not only on the direct and interactive effects of environmental variability, but on how that variability influences the interactions between the different developmental stages of organisms with complex life cycles.

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