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# Development of colour in an aposematic ladybird beetle: The role of environmental conditions

Christopher P. Grill*

*T.H. Morgan School of Biological Sciences, Center for Ecology, Evolution and Behavior, University of Kentucky, 101 Morgan Building, Lexington, KY 40546-0091, USA*

## ABSTRACT

I investigate the role of food quality and the use of a chemical defence in determining variability of warning colouration in an introduced aposematic beetle, *Harmonia axyridis*. I compare the development of colour in adults reared on different semi-natural diets and examine the effects of use of a chemical defence response, reflex bleeding, on adult colour. Adult colour differences are investigated using a combination of spectral inflection point data and principal component analysis. The two analysis techniques provide similar conclusions. Adult diet has very strong effects on the development of colour over time. Almost immediately, diet treatments diverge, with the high-quality aphid diet producing vibrantly coloured red adults, whereas a lower-quality pollen diet produces pale orange adults. Reflex bleeding has almost no effect on any aspect of adult beetle colour. The timing of reflex bleeding does not influence colour development. Finally, reflex bleeding and dietary stress do not result in reductions in the brightness, chroma or hue of adult *Harmonia* colour once colour development has taken place. The implications of these results for the evolution of colour are discussed.

*Keywords*: aposematism, chemical defence, colouration, food quality, *Harmonia axyridis*, reflex bleeding.

## INTRODUCTION

Animal colours and colour patterns have been of longstanding interest to biologists (e.g. Poulton, 1890; Punnett, 1915; Dobzhansky, 1933; Tan and Li, 1933; Endler, 1978). Colour is known to influence many different types of biological phenomena, including courtship, thermoregulation and predation events (Endler, 1978). The complexity of the phenomena that rely on colour, and of the colour patterns themselves, suggest a long evolutionary process leading to the appearance of contemporary phenotypes. The fundamental requirements of this process are selection and genetically based variability in the traits of interest.

The importance of colour as a selective agent has been demonstrated repeatedly. Colour may act directly as a deterrent to predators by suggesting the presence of some defensive strategy or substance (e.g. noxious chemicals) in the prey organism. Aposematism, or the

* Address all correspondence to Christopher P. Grill, Division of Natural Sciences, University of Hawaii at Hilo, 200 West Kawili Street, Hilo, HI 96720, USA. e-mail: grill@hawaii.edu

use of warning colouration in combination with chemical defence to ward off potential predators, has been frequently invoked as an explanation of the presence of conspicuous colours in defended species (e.g. Poulton, 1890; Cott, 1940; Edmunds, 1974; Guilford, 1988; Tullrot and Sundberg, 1991). A central theme in this field is that, in defended species, warning colour should communicate the presence and perhaps even the strength of the noxious defence (Cott, 1940; Edmunds, 1974; Guilford, 1988). In such aposematic systems, a strong genetic and phenotypic coupling is predicted between the indicator trait (i.e. colour) and the defensive trait, although little empirical evidence is currently available about this contention (but see Alatalo and Mappes, 1996; Vogler and Kelley, 1998).

Colour may also interact with other factors such as odour, taste and chemical defences 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). Colour can also influence the attractiveness of potential mates (e.g. Houde and Torio, 1992; Folstad *et al*., 1994).

Given that variability in colour patterns may affect the outcome of selective events and the course of evolution, I investigated how changes in environmental conditions influence variability in the expression of colour in an aposematic ladybird beetle, *Harmonia axyridis* Pallas.

Despite many previous studies of colour, quantifying variability in colour remains problematic. Many colour assessment techniques, such as human observation and comparison of samples to colour standards, are often unrepeatable and biased by observer effects. More accurate methods, such as spectrophotometry, have been prohibitively expensive. Recently, however, a new wave of inexpensive but very accurate spectrophotometers have become available. This advance in technology has made possible the accurate assessment of colour by even modestly funded researchers.

Colour changes over time in adult *Harmonia*; this change is usually most pronounced just after eclosion of adults when the exoskeleton becomes sclerotized, but colour continues to develop after eclosion. Under ideal conditions, colour changes from a pale yellow at eclosion to a rich crimson over a period of weeks or months (personal observation). Given that rates and magnitude of colour development are probably resource-limited (Grill *et al*., 1997), changes in environmental quality are likely to influence the expression of colour.

In this paper, I examine how environmental conditions in both the larval and adult stages influence variability in adult *Harmonia* warning colouration. I examine the effects of adult diet quality on post-eclosion colour in adult beetles and on the subsequent ontogenetic trajectories of beetle colour as it develops over time. I also examine how use of reflex bleeding (a chemical defence), in both larvae and adults, affects the development of adult colour. Specifically, I address two questions: First, does change in adult colour over time depend on adult diet? Second, does change in adult colour over time depend on number or timing of reflex bleeding events?

## METHODS

### Study organism

*Harmonia axyridis* has repeatedly been introduced from Asia into different parts of the United States as a biological control agent (Tedders and Schaeffer, 1994). This brightly coloured beetle has spread quite effectively to non-agricultural habitats and is now found in

most parts of the United States. Native Asian populations of *Harmonia* are comprised of several different genetically distinct colour morphs, but US populations consist entirely of the *succinea* morph, which produces a predominantly red–orange, rather than black, elytral colour (Komai, 1956). This species has been used for a number of ecological studies; husbandry procedures are well-established (e.g. Ueno, 1994; Grill *et al*., 1997).

### Collection and maintenance of study animals

Beetles used in these experiments were either field-collected as adults and pupae near Lexington, Kentucky, or were laboratory-reared progeny of field-collected beetles. All experiments were run in incubators at 27°C and 15 : 9 light : dark. The availability of aphids fluctuated during the course of the field season. Thus the conditions varied from one experiment to the next. Feeding schedules for each experiment are described below.

### Experiment 1: Effects of adult diet

Pupae were collected, placed into petri dishes and left in an incubator until eclosion of adults. Upon eclosion, adults were placed immediately into one of two diet treatments. The pollen treatment consisted of *ad libitum* access to freeze-dried honeybee pollen; the aphid treatment consisted of three weekly feedings of 30–50 aphids (*Myzus nicotiana* Blackman) per feeding. Colour measurement (see below for details) was initiated on the third day following emergence of adults and continued every 2 days thereafter until a total of seven spectral measurements, spanning 17 days of colour development, was collected for each beetle.

### Experiment 2A: Effects of reflex bleeding in larvae

An F1 generation was derived from a field-collected population of adult beetles; F1 adults were randomly mated to produce the eggs used in this experiment. Newly hatched larvae were placed individually into petri dishes and given three weekly feedings of 20–30 aphids per feeding plus unlimited honeybee pollen. The larvae were treated in one of three ways. The no-bleed larvae were controls that were not induced to bleed. The one-bleed larvae were agitated once. If this single agitation did not induce a reflex bleeding event, the larvae were agitated every 2 days thereafter until a bleed was induced. The 2+ bleed larvae were agitated every 2 days and induced to bleed at least twice and a maximum of four times (mean = 2.3 bleeds per larva). I induced reflex bleeding in larvae by agitating sensory setae along the abdomen with a small paintbrush (see Grill and Moore, 1998, for additional details). Following a reflex bleeding event, the larvae were dabbed with a facial tissue to prevent resorption of any expelled fluids. After pupation and emergence, adults were maintained on the pollen/aphid combination diet (30–50 aphids per feeding). Starting on the second day after emergence, elytral colour was measured every 5 days until a total of five measurements, spanning 22 days of colour development, was collected for each beetle.

### Experiment 2B: Effects of reflex bleeding in newly eclosed adults

Larvae and pupae were collected in the field, isolated in petri dishes and placed in an incubator. Due to a shortage of aphids, the larvae were fed a combination of crushed field

corn and freeze-dried pollen. *H. axyridis* larvae and adults were observed consuming corn in the field; these beetles are not capable of penetrating corn kernels, but they can feed on field corn that has previously been damaged by Japanese beetles, *Popillia japonica* Newman (personal observation). *H. axyridis* larvae were reared to pupation and emergence and then placed into either the control or bleed treatment. For beetles in the bleed treatment, I attempted to induce reflex bleeding every 2–3 days by tapping on their elytra. This was generally, but not always, successful. Starting on the second day after emergence, elytral colour was measured (see below) every 5 days until a total of five measurements, spanning 27 days of colour development, was collected for each beetle.

### Experiment 2C: Effects of reflex bleeding in 'older' adults

Adult ladybird beetles of unknown age were collected in the field, isolated in petri dishes and placed immediately into one of two treatments: the bleed treatment, consisting of induced reflex bleeding every 2–3 days, and the no-bleed treatment, which served as a control. This differed from the previous experiment in several respects. Beetles were collected as adults and had undergone, to various degrees, development of elytral colour. The beetles were placed on a relatively poor diet of honeybee pollen (Pfannenstiel, 1995) with neither aphid nor corn supplements. Under these harsh conditions of poor food and repeated bleeding, I tested the hypothesis that colour, which was produced under presumably more favourable natural conditions, could be lost when environmental conditions degenerated. Beetle colour was measured (see below) on two different days, once 3 days after collection and once 28 days after collection. Thus, this experiment did not track the development of colour but focused on the end result of whether or not colour changed between treatments after 4 weeks of exposure to repeated reflex bleeding and dietary stress.

### Measurement and analysis of colour

I measured colour on each beetle using a Spectator™ portable spectroradiometer with a standardized halogen light source (World Precision Instruments; model F-O-LITEH) routed through a fibre optic cable fitted with a reflectance probe. Measurements were taken with the probe positioned approximately perpendicular to each beetle's elytron, or wing covering; spectra were taken only for the red portion of the elytra, not for the black spots. Data for this spectral analysis system are collected in approximately 0.3 nm increments. To reduce the size of the resultant data sets, I pooled data into 5 nm bins and averaged the data to produce a mean reflectance value within each bin. The reflectance spectra were analysed from 400 to 700 nm. The ultraviolet region of the spectrum (< 400 nm) may also be important in this species, but limitations of my equipment made it impossible to measure reflected light in that region. Two spectral measurements were taken for each beetle and then averaged to reduce measurement errors. I calculated reflectance as the ratio of transmitted (i.e. reflected) light from the beetle to transmitted light from a barium sulphate standard.

The reflectance spectra were analysed in two ways, using principal component analysis (PCA) (Bennett *et al*., 1997; Grill and Moore, 1998) and inflection point estimates (Grill and Moore, 1998). PCA treats reflectance at each wavelength as a separate dependent variable. PCA produces three principal components that account for > 95% of the overall variability in reflectance. This procedure has been shown to produce components that

correlate strongly with the three fundamental component parts that combine to produce colour: brightness, chroma and hue (Grill and Rush, in press). In these analyses, PC1 corresponds with brightness or the overall amount of light reflected off the sample. PC2 corresponds with chroma or the amount of neutral grey light present. As chroma increases so does the intensity or purity of the colour. For example, dark spray paint applied in a continuous stream to a white wall initially appears faded but attains a richness, or higher chroma, as more pigment is laid down. PC3 corresponds with hue, which many people think of as colour (e.g. red, yellow, blue, green, etc.). The principal components scores were then used as dependent variables in a repeated-measures analysis of variance to test for effects of diet, time (i.e. the repeated measures) and the diet × time interaction on the various components of colour. The second measure of colour exploits the sigmoidal shape of *Harmonia* reflectance spectra. The wavelength at which the inflection point occurs can be used as a repeatable ($t = 0.79$; $n = 275$ beetles) and easily identifiable estimate of colour; this inflection point wavelength is the point along the spectrum at which acceleration of reflectance is greatest (Endler, 1990; Grill, 1998). The higher the wavelength of the inflection point, the further the reflectance curve is shifted into the red region of the spectrum. Thus, higher values for the inflection point indicate redder individuals (Grill and Moore, 1998).

Although the differences between several principal components scores in different treatments were statistically robust, biological interpretation of the results is not entirely straightforward. Given the nature of PCA, orthogonal rotations of the data can result in reversal of signs for the principal components scores. Thus, it is not possible to state unequivocally that, for example, high values for PC1 are always associated with brighter ladybirds. The meaning of the principal components score depends on how the data are rotated. The important distinction is that of magnitude of difference in score between treatments, regardless of whether the value is positive or negative for any specific principal components score. One way to circumvent this problem is to include colour standards in the PCA. By comparing values of colour standards to real data points, colour can be estimated relative to the standards. In a hypothetical example, if all principal components for a red colour standard are positive, and negative for a pale orange colour standard, actual sample colours that have all, or even many, positive principal components scores may be considered more red than orange. Unfortunately, the addition of colour standards can affect the outcome of the PCA, sometimes drastically (Tabachnik and Fidell, 1989). In this paper, I suggest that some treatments produce adult beetles that are more red than others. These conclusions are consistent with three lines of evidence generated in this set of studies: principal components scores, inflection points and informal visual observation of the beetles. The conclusions regarding colour are not, however, based on PCA data sets that include colour standards.

## RESULTS

### Effects of adult diet

Beetle colour was influenced by the diet on which adults were reared. By the time measurements were initiated on the third day after eclosion, PC2 was already significantly different between diet treatments with higher values occurring in the aphid treatment (Table 1, Fig. 1). This difference was maintained throughout the measurement period. PC1 and PC3 were not affected by adult diet. The inflection point was significantly different between diet

**Table 1.** Results of Experiments 1 and 2 (PC1, PC2 and PC3 may be thought of as brightness, chroma and hue, respectively: Grill and Moore, 1998)[a]

| Experiment | Factor | d.f. | PC1 | | PC2 | | PC3 | | Inflect | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *F*-ratio | *P* | *F*-ratio | *P* | *F*-ratio | *P* | d.f. | *F*-ratio | *P* |
| 1 | Diet | 1,40 | 0.09 | 0.764 | 12.62 | 0.001 | 2.54 | 0.119 | 1,51 | 11.46 | 0.001 |
| | Time | 6,240 | 17.96 | < 0.001 | 4.96 | < 0.001 | 6.55 | < 0.001 | 6,206 | 6.91 | < 0.001 |
| | Diet × time | 6,240 | 3.55 | 0.002 | 2.08 | 0.061 | 9.77 | < 0.001 | 6,206 | 2.229 | 0.064 |
| 2A | Bleed | 2,117 | 0.53 | 0.590 | 0.13 | 0.882 | 0.35 | 0.708 | 2,110 | 0.61 | 0.547 |
| | Time | 4,468 | 30.41 | < 0.001 | 29.99 | < 0.001 | 17.36 | < 0.001 | 4,440 | 235.39 | < 0.001 |
| | Bleed × time | 8,468 | 0.91 | 0.511 | 1.17 | 0.317 | 0.30 | 0.965 | 8,440 | 0.61 | 0.746 |
| 2B | Bleed | 1,31 | 0.46 | 0.503 | 2.22 | 0.147 | 0.63 | 0.434 | | | |
| | Time | 4,124 | 4.66 | 0.002 | 7.62 | < 0.001 | 6.61 | < 0.001 | | | |
| | Bleed × time | 4,124 | 0.80 | 0.525 | 0.57 | 0.655 | 0.80 | 0.517 | | | |
| 2C | Bleed | 1,45 | 3.35 | 0.074 | 0.79 | 0.380 | 1.28 | 0.264 | | | |
| | Time | 1,45 | 9.79 | 0.003 | 3.89 | 0.055 | 5.46 | 0.024 | | | |
| | Bleed × time | 1,45 | 2.19 | 0.146 | 0.14 | 0.712 | 0.79 | 0.379 | | | |

[a] Inflection point data were not collected for Experiments 2B or 2C. The factor 'time' in each analysis of variance represents the repeated-measures element of each analysis.

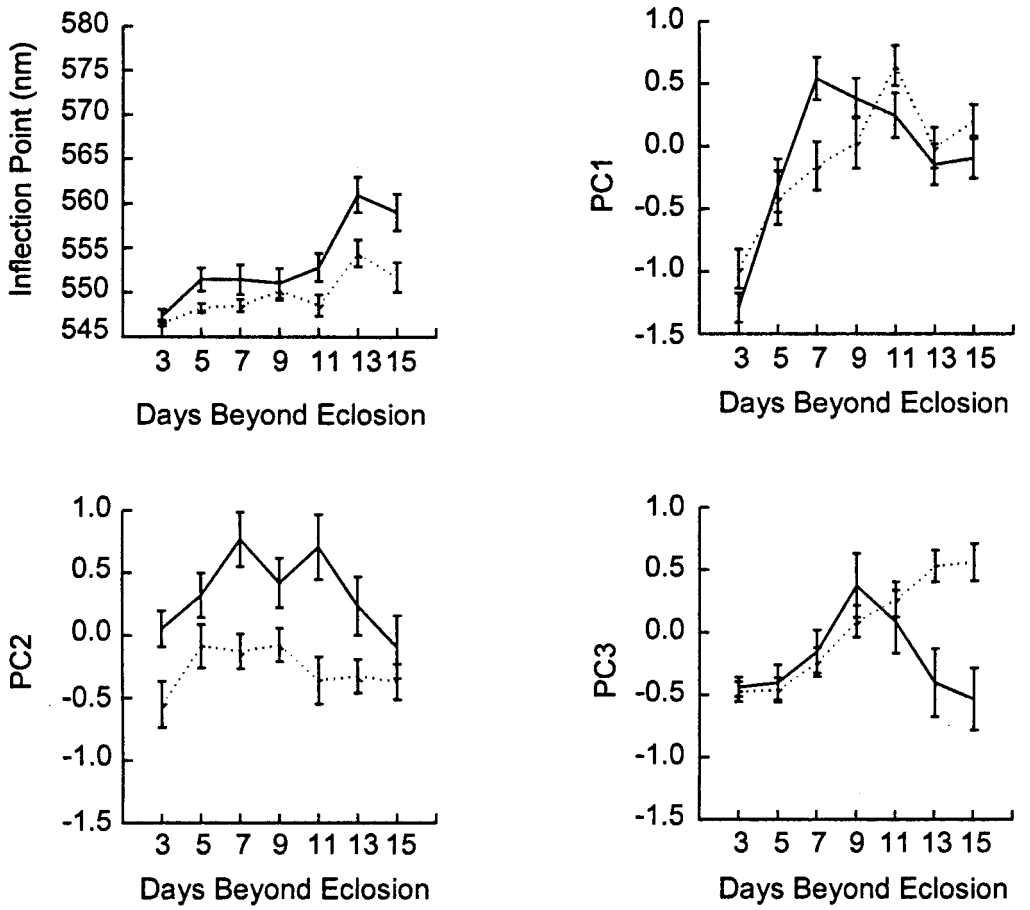


**Fig. 1.** Results of Experiment 1. PC1, PC2 and PC3 may be thought of as brightness, chroma and hue respectively. For all graphs, the axes have been rotated such that higher values indicate brighter and/or redder individuals. Diet treatments: dotted lines, pollen; solid lines, aphids.

treatments; aphid treatment beetles had consistently higher inflection point values after the first set of measurements. All characters were subject to strong time effects, indicating that all aspects of colour change over time. All colour characters also showed weak to moderate diet × time interactions, indicating that the rates at which the different colour components change over time varied between diets for all characters, regardless of whether or not a significant main effect of diet was observed. All beetles initially became brighter (PC1), but then declined in brightness, presumably as darker red pigments accumulated. Beetles also became more red over time based on inflection point, but this result was not corroborated by the results for PC2 (Table 1, Fig. 1). The results for PC3 were inconsistent with the results for the other measures of colour in all experiments. PC3 initially increased as expected, but then tailed off in the latter half of Experiment 1 for the aphid treatment only. This was unexpected and may have been the result of some unknown change in the quality of the field-collected aphid food source.

### Effects of reflex bleeding

Neither larval nor adult reflex bleeding in laboratory-reared *Harmonia* had any significant effects on any component of adult colour. Time had strong effects on all components of colour as expected, with the exception of PC2 in Experiment 2C, which was marginally non-significant (Table 1, Fig. 1). The absence of bleed × time interactions indicated that the developmental trajectories of colour in *Harmonia* did not differ between bleed treatments in any of the experiments (Table 1, Fig. 2).

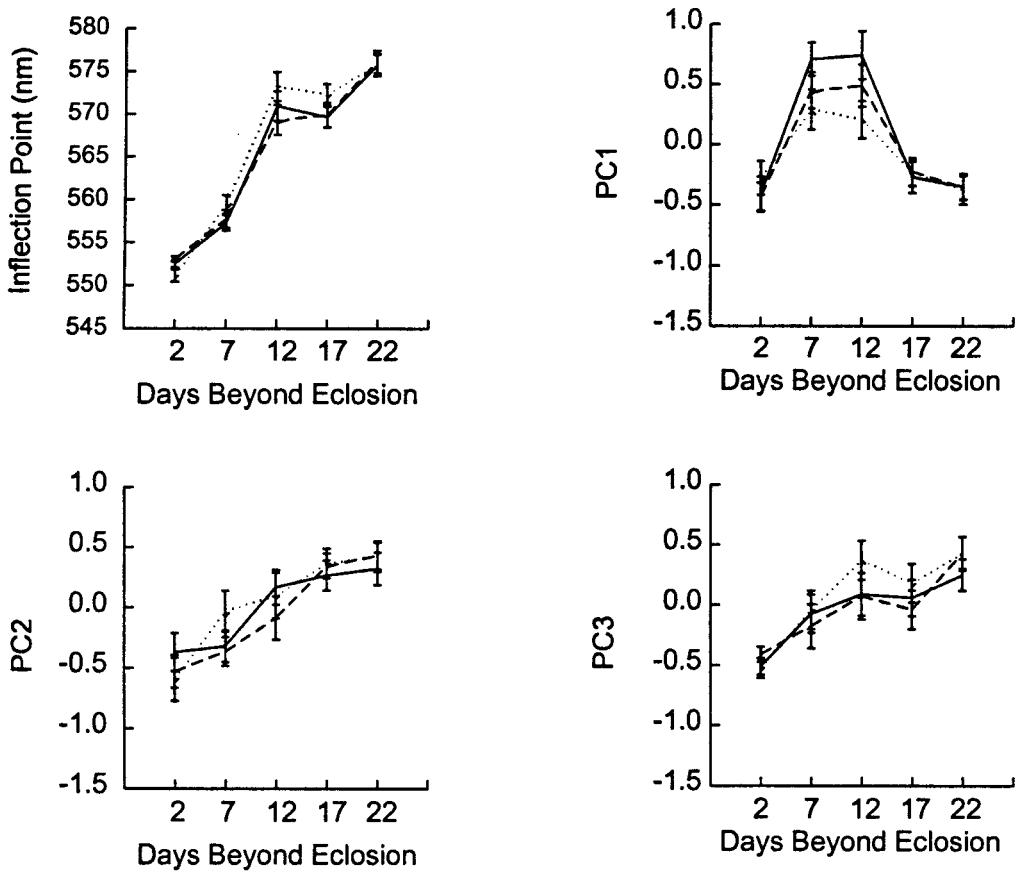


**Fig. 2.** Results of Experiment 2A. PC1, PC2 and PC3 may be thought of as brightness, chroma and hue respectively. For all graphs, the axes have been rotated such that higher values indicate brighter and/or redder individuals. This figure is also representative of the results of Experiments 2B and 2C, with the exception of the inflection point, which was not measured in those experiments. No effects of bleed were observed in any of the experiments, but effects of time were nearly universal for all dependent variables measured. Bleed treatments: dashed lines, 2+ bleeds; dotted lines, 1 bleed; solid lines, no bleed.

## DISCUSSION

### Effects of diet quality

Diet quality is known to affect the expression and variability of ladybird beetle life-history and morphological characters (Grill *et al*., 1997; Grill and Moore, 1998; Wagner *et al*., 1999). *H. axyridis* larvae reared on pollen are at a substantial disadvantage relative to those reared on a higher-quality aphid diet; they take longer to develop, grow to smaller adult sizes, and produce relatively pale adult colouration (Pfannenstiel, 1995; Grill and Moore, 1998). This study extends those findings to include information on the influence of diet on developmental trajectories for colour (Table 1, Fig. 1). Adult beetles reared on pollen showed a reduced rate of colour development and a paler, less conspicuous terminal colouration than their aphid-reared counterparts. These results suggest that beetle colouration may be influenced by some dietary component that, when limited, results in less conspicuously coloured individuals. Alternatively, the production of beetle colouration may be energetically expensive and lower-quality diets may not provide sufficient energy to generate large amounts of pigment.

Several researchers have suggested that conspicuous colouration can provide an advantage in the form of reduced predation risk (e.g. Sillen-Tullberg, 1985; Guilford, 1986; Endler, 1991). In a population of aposematic individuals, however, a pale individual may be perceived as poorly defended and desirable relative to its local conspecifics. *H. axyridis* is known to form large aggregations in the field, especially during early spring and late fall. Against a field of uniformly bright red adults in such an aggregation, a pale individual may be seen as rare and desirable to predators, especially if predators have learned to associate conspicuous colour with some negative stimulus such as noxious chemicals (Marples *et al*., 1994; Roper, 1994). Thus, in addition to the negative effects of a reduction in diet quality on size, growth rate and initial adult colour, food stress may result in a higher risk of predation.

### Effects of reflex bleeding

The conditions under which these experiments were conducted were somewhat stressful. This was by design to prevent the beetles from compensating for any costs of larval reflex bleeding with increased uptake of a high-quality resource. Nonetheless, larval reflex bleeding had virtually no effect on the development of colour in recently eclosed adults (Table 1). There are two possible explanations. First, previous work on *Harmonia* has suggested that if food quality is not greater than some minimum threshold, the stress of the poor diet may effectively swamp out any effects of reflex bleeding (Grill and Moore, 1998). The second possible conclusion is simply that there is no relationship between colour and reflex bleeding. This appears unlikely but little is known about correlations between colour and defensive chemicals in *H. axyridis*. More work is also needed on how the environment influences the chemical composition of reflex blood.

I also investigated whether or not reflex bleeding in adult beetles could influence the spectral characteristics of existing colours within the beetle elytra. I found that reflex bleeding was not in any way associated with changes in adult colour (Table 1). Once beetle colour was produced or deposited, no amount of reflex bleeding affected any of the components of adult colour (i.e. brightness, chroma or hue). Even on the poorest diet, adult colour continued to progress over time from pale orange towards a more saturated

red–orange, regardless of which reflex bleeding regime was applied. Thus, colour in adult *H. axyridis* appears to be largely, if not completely, independent of the number of reflex bleeding events experienced.

### The role of colour in *Harmonia axyridis*

Adult ladybird colour can influence the outcome of a predation sequence (Sillen-Tullberg, 1985; Marples *et al.*, 1994) and is also associated with mating preferences (Grill *et al.*, in prep.). These experiments are focused on the 3–4 weeks that follow eclosion of adults. During this time, adults continue to develop and mature, prior to any matings. Colour development is critical during this period because it may influence both the likelihood of survival through the post-eclosion maturation period and the probability of mating successfully once that maturation period has ended. Clearly, diet influences many aspects of the *Harmonia* phenotype, including development of colour, during this important phase of the life-cycle. This set of studies does not, however, provide evidence of an energetic cost to chemical defence, nor of any environmentally induced limitations to chemical defence. It remains unlikely that beetles have unlimited access to the resources needed to generate reflex blood. Rather, as the number of reflex bleeding events increases, the deterrent quality of a beetle's reflex blood probably diminishes, perhaps drastically after an initial bleed or two. This issue of reflex blood quality has been addressed in other ladybird species (e.g. Holloway *et al.*, 1993) but, to the best of my knowledge, has not been attempted in *H. axyridis*, or across different sets of ecologically relevant environments.

### Evolution of warning colouration

I was surprised not to find any relationship between beetle colour and the number of reflex bleeding events. This has implications for the evolution of colour and the evolution of the defensive 'suite' of colour-coupled-with-chemical-defence. Conventional wisdom suggests that you should see a tight linkage between these traits. If so, evolution of the defensive suite may be constrained. But I observed no such coupling.

Other work has also suggested that the linkage between colour and defence is not always as tight as might be expected (e.g. Vogler and Kelley, 1998). There are several possible explanations that need further investigation (e.g. quality of chemical defence changes over time). Thus, while some of the expected environmental effects were observed (i.e. food quality influences colour development), the expected coupling of colour and chemical defence was not evident.

## CONCLUSIONS

This set of studies brings to light some important issues and raises some additional questions. Development of colour is heavily influenced by diet, but not by the number of reflex bleeding events. These experiments provide little evidence of any relationship between colour and chemical defence. It remains possible, however, that existing relationships were masked by the harsh conditions of the treatments. Little is known about how environmental factors influence quality and effectiveness of chemical defence, or correlations between chemical defence and other traits. Stringent quantitative investigation of these issues will help to further uncover the mechanisms underlying successful use of aposematic defence strategies.

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