**Maternal effects and warning signal honesty in eggs and offspring of an aposematic ladybird beetle**

Anne E. Winters1,2, Martin Stevens3,1, Chris Mitchell1, Simon P. Blomberg2 & Jonathan D. Blount1\*

1*Centre for Ecology & Conservation, College of Life & Environmental Sciences, University of Exeter, Penryn Campus, Cornwall TR10 9EZ, UK*

2*School of Biological Sciences, University of Queensland, Brisbane QLD 4072, Australia*

3*Department of Zoology, University of Cambridge, Cambridge CB2 3EJ, UK*

Author for correspondence: j.d.blount@exeter.ac.uk

Running headline: Maternal effects and aposematism

**SUMMARY**

1. The eggs of oviparous species are often subject to intense predation pressure. One parental strategy to deter predators is to produce eggs that are laced with noxious chemicals and are conspicuously coloured (i.e. aposematism).
2. Ladybird eggs are conspicuously coloured and contain alkaloids; these traits are believed to function in concert as visual signal and chemical defence, respectively, to deter predators. However, it remains unclear whether such aposematic signals reveal the strength (rather than simply the existence) of chemical defences.
3. Furthermore, additional functions of egg pigments and toxins could apply; in particular mothers might deposit such resources into eggs to aid the development of offspring, or to provide resources that could contribute to aposematic traits in offspring.
4. We bred wild-caught seven-spot ladybird beetles (*Coccinella septempunctata*) in the laboratory, and then measured relationships between egg colouration and toxin concentrations (i.e. the alkaloids precoccinelline and coccinelline). We also measured relationships between egg carotenoids and egg colouration, and between egg colouration and toxin levels, and the elytra colouration and toxin concentrations of offspring at eclosion for a subset of eggs that were allowed to develop.
5. Egg carotenoids predicted egg colour saturation. In turn egg colour saturation and hue positively predicted egg concentrations of precoccinelline. However, there were no significant relationships between egg coccinelline concentration and any measure of egg colouration.
6. In recently eclosed adults of both sexes elytra saturation was significantly explained by variation in egg saturation and hue. Finally, body concentrations of coccinelline were significantly explained by variation in elytra hue.
7. These results suggest that the colouration of *C. septempunctata* eggs is a reliable signal of the strength of chemical defences contained therein, but in addition, maternal investment of pigments and toxins into eggs may serve to influence the reliability of aposematic signalling in resultant offspring.

Key-words: aposematism, carotenoids, coccinelline, maternal effects, precoccinelline.

**INTRODUCTION**

The eggs of oviparous species are highly nutritious, because they must contain all the resources required for embryonic development in a self-contained package. Consequently, such eggs are often subject to intense predation pressure. Parental strategies to mitigate the risk of predation include hiding or guarding eggs, or alternatively, producing eggs in such vast quantities that at least a few are likely to survive (Gundermann, Horel & Roland 1997; Machado & Oliveira 1998; Klug, Chin & Mary 2005; Huang & Wang 2009). Certain animal species, however, employ a different tactic: their eggs are laced with noxious chemicals and are conspicuously coloured, which is believed to function as a deterrent to predators (i.e. aposematism) (Twitty 1966; Heras et al. 2007; Dreon, Ituarte & Heras 2010). The best studied examples of aposematic eggs are those produced by freshwater apple snails (Ampullariidae). For example, *Pomacea* *canaliculata* produces eggs that contain a proteinase inhibitor, ovorubin, which limits predators’ ability to digest egg nutrients, and the eggs are coloured pinkish-red by the presence of a carotenoid pigment, astaxanthin (Heras et al. 2007; Dreon et al. 2010).

Until recently aposematic signals were considered to be qualitatively (although not quantitatively) ‘honest’, in the sense that they advertise the existence but not the strength of secondary defences (Ruxton, Sherrat & Speed 2004; Stevens & Ruxton 2012). Contrary to this view a positive correlation between aposematic signal expression and levels of chemical defence has been reported both within (Bezzerides et al. 2007; Blount et al. 2012; Maan & Cummings 2012; Vidal-Cordero et al. 2012) and across aposematic species (Summers & Clough 2001; Cortesi & Cheney 2010), suggesting that aposematic signals may be quantitatively honest indicators of the level of defence. Whether the colour of aposematic eggs is a reliable signal of their toxin content has not been studied before. However, eggs may be a particularly amenable context for studying signal honesty because of the relatively simple nature of the signal: no pattern elements, and no behavioural modifications (with the exception of effects of egg clustering on predator behaviour (Courtney 1984). Moreover, within eggs the molecules responsible for signal and defence are located in the same biochemical milieu. Indeed, in the freshwater apple snail *P.* *canaliculata*, ovorubin confers chemical defence whilst itself being the source of carotenoid pigmentation (Heras et al. 2007; Dreon et al. 2010).

It is conceivable that maternal investments of pigments and toxins into eggs may serve additional roles, unrelated to egg defence. Maternal effects occur where a mother's phenotype influences her offspring's phenotype, in addition to parents’ genetic contributions to their young, and can have important consequences for fitness (Wolf & Wade 2009). Maternally-derived carotenoid pigments in eggs could serve to provide antioxidant protection during embryogenesis or larval growth (Blount, Houston & Møller 2000). Alternatively, maternally-derived carotenoids and toxins in eggs could confer warning colouration and chemical defences to larvae and newly eclosed adults, at a vulnerable stage of life when the dietary sequestration of resources needed to produce aposematic traits has yet to reach its full potential. Additionally, maternal effect genes have been hypothesized as a potential mechanism for the initial evolution of aposematism. Producing an entire family of aposematic offspring could provide an immediate fitness advantage and help overcome the difficulty of initial rarity, which would be experienced by individuals with zygotically expressed genes (Brodie & Agrawal 2001).

Carotenoids are known to be responsible for the pigmentation of aposematic eggs in a range of species (e.g. polar leaf beetle, *Melasoma populi* (Fox 1976); large white butterfly, *Pieris brassicae* (Feltwell 1981); apple snail, *P.* *canaliculata* (Heras et al. 2007)). Carotenoids are also often responsible for aposematic signal colouration in larvae and adults (e.g. the yellow-orange spots of larvae and the ground pigmentation of elytra in ladybirds (Britton et al. 1977; Bezzerides et al. 2007; Blount et al. 2012); red wing markings in burnet moths *Zygaena filipendulae* (Bornefeld & Czygan 1975)). Similarly, toxins might be transferred from eggs to offspring. For example, ladybirds are chemically defended at all life stages (Daloze, Braekman & Pasteels 1994), and at least some of the toxins in larvae and young adults could derive from initial maternal investments in eggs. However, whether the development and survival of offspring is influenced by levels of carotenoids in eggs is not known. Similarly, whether there are significant relationships between levels of carotenoids and toxins in eggs and resultant offspring has not been studied before.

Here, using the aposematic 7-spot ladybird (*Coccinella septempunctata*), we tested the following hypotheses: (1) rates of offspring developmental rate and survival would be positively correlated with levels of maternally-derived carotenoids in eggs; (2) components of egg colour would be positively correlated with egg levels of carotenoids; and (3) egg toxin levels would be positively correlated with components of egg colour. Furthermore, we hypothesised that (4) components of elytra colouration of newly eclosed adults would be positively correlated with the same colouration measures in eggs derived from the same mothers; and (5) egg toxin levels would be positively correlated with levels of the same compounds in newly eclosed adults. Finally, we hypothesised that elytra colouration of newly eclosed adults would be positively correlated with their body toxin levels. We assessed these relationships using objective measures of colouration derived from digital images obtained using a human-UV visible camera, and biochemical analyses of alkaloids obtained using LC-MS and GC-MS. We used generalized linear mixed models (GLMM) to test these hypotheses in order to account for random factors such as mother and father ID and to include multiple terms. For most models, we expect a priori causal pathway between the x and y variables. For instance, in analyses comparing egg phenotypes to adult phenotypes, egg measurements were used as the predictor terms, as we expect egg phenotypes to predict offspring phenotypes. Maternally-derived carotenoids were also used as the predictor term in analyses investigating egg colouration, as we would expect egg colouration to depend on the concentration of pigment contained within. However, for analyses testing signal honesty in eggs and offspring, where we do not expect a causal path, we have used colour measurements as our predictor terms, as our measurements of colour had a smaller error rate than those of toxins. Ladybirds are sexually size dimorphic, females being the larger sex, and therefore females may be more susceptible to resource limitation than males (Blount et al. 2012). Thus, we controlled for the potential effect of sex in the statistical analyses.

**MATERIALS AND METHODS**

***(a) Ladybird rearing***

A total of 78 Adult *C. septempunctata* (n=52 females, n=26 males) were collected in April-May 2011 from field sites within a 5 km radius of the University of Exeter’s Penryn Campus, Cornwall, UK (GPS co-ordinates: 50°10’15” N, 05°07’30” W). Individuals were sexed under a dissecting microscope (Leica MZ125) using diagnostic features of the cuticular plates on the underside of the abdomen (Majerus 1987). Individuals were then photographed under standardised conditions for calculation of pronotum width (± 0.001mm) using imageJ, before being housed singly in Petri dishes (9cm diameter) under standardised conditions in the laboratory (25ºC, 16:8 L:D, 60% RH). Ladybirds were provided with an *ad lib.* supply of live pea aphids (*Acyrthosiphon pisum*), which were cultured on broad bean plants (*Vicia faba*; Sutton Dwarf variety), and were transferred to a clean Petri dish (9cm diameter) daily. Females may have mated in the wild prior to this experiment, which could increase paternally derived genetic variance amongst offspring. However, studies of other ladybird species (*Harmonia axyridis* and *Adalia bipunctata*)have found a last male precedence (Hodek & Ceryngier 2000). To reduce the possible effects of prior breeding history, females were housed for 39 ± 19 (mean ± s.e.) days under standardised conditions in the laboratory and any eggs laid during this period were discarded. Since maternal investment in eggs may be expected to vary based on body size, females that had been field collected on the same day were matched based on pronotum width (no significant difference between matched females: paired *t*-test, *t* = 0.21, d.f. = 18, *P* = 0.83). Females of each matched pair were then mated consecutively on the same day to a randomly selected individual male. The same male was mated with both females to reduce paternally derived genetic variance amongst offspring, and to avoid any differential allocation amongst females in response to variation in male phenotype. Pairs were observed until they had mated, which typically occurred within the first 5 minutes, and were then immediately separated.

Petri dishes housing females were monitored daily for the presence of eggs. Upon the discovery of eggs, females were moved to a new Petri dish to prevent filial cannibalism. We collected eggs from n=37 females (i.e. n=18 matched-pairs of females and one additional female whose matched-pair died before laying) for colour measurement and biochemical assays of alkaloids and carotenoids. For each female, eggs laid on the same day were weighed to the nearest 0.1µg using an electronic microbalance (UMX2 ultra-microbalance; Mettler-Toledo Ltd.), before being collected using a fine tipped paintbrush and stored in Eppendorf tubes at -80°C until analyses were carried out within 6 months. Of these females, n=31 individuals laid additional eggs on a different day (either the day before, or the day after the production of eggs which were collected and frozen as described above). These eggs were allowed to develop *in situ* under standardised conditions (25ºC, 16:8 L:D, 60% RH) to yield larvae for rearing (see below). Eggs produced on the same day by paired females were always used for the same purpose (i.e. both used for biochemical analyses, or both allowed to develop).

Female ladybirds typically lay on multiple occasions over the course of a breeding season, often producing more than one discrete cluster of eggs on a single day, and sometimes depositing eggs singly or in numerous discrete clusters. To ascertain whether maternal allocation of resources varied amongst eggs produced on a single day, or across consecutive days, we collected an additional 15 females in the wild, allocated them to matched-pairs based on body size, and then mated them with a single male per matched pair of females (as described above). We compared the phenotypes of n=2 randomly collected eggs laid within a single day, and n=2 randomly collected eggs laid on successive days; repeatabilities were calculated according to (Lessells & Boag 1987). There was significant repeatability (R) both within- and between-days for egg mass (within day: *F*14,15 = 4.60, *P* = 0.002, R = 0.64; between day: *F*14,15 = 4.02, *P* = 0.005, R = 0.60), and egg concentrations of carotenoids (within day: *F*14,15 = 3.78, *P* = 0.008, R = 0.58; between day: *F*14,15 = 3.09, *P* = 0.019, R = 0.51), precoccinelline (within day: *F*14,15 = 5.59, *P* = 0.001, R = 0.70; between day: *F*14,15 = 2.75, *P* = 0.031, R = 0.48), and coccinelline (within day: *F*14,15 = 10.80, *P* < 0.0001, R = 0.83; between day: *F*14,15 = 6.22, *P* = 0.0006, R = 0.72). Therefore, eggs laid on the same day or on different days by the same female had comparable phenotypes. We can therefore examine the relationships between levels of carotenoids and toxins in eggs produced on one particular day, and the phenotypes of adults derived from eggs laid either the day before or after. Concentrations of carotenoids and alkaloids were determined as described below.

Upon hatching, larvae were counted along with un-hatched eggs. Five randomly selected larvae per family were used for the rearing experiment. Individuals were carefully picked up using a fine-tipped paintbrush, weighed to the nearest 0.1µg using an electronic microbalance (UMX2 ultra-microbalance; Mettler-Toledo Ltd.), and then transferred to individual 3.5 cm Petri dishes and allowed to develop at 21°C, 16:8 L:D and 60% RH. Larvae were fed once daily using a stock of frozen pea aphids that had been stored at -80°C for less than three weeks. Aphids were thawed at room temperature and offered to larvae irrespective of instar stage, except 1st instar larvae were not given late instar aphids, as they are too large for consumption. Larvae were reared on a diet of 1.37mg ±10% aphids day-1 (1st instar), 3.49mg ±10% aphids day-1 (2nd instar), 6.98mg ±10% aphids day-1 (3rd instar), 44.74mg ±10% aphids day-1 (4th instar) as in previous studies of *C. septempunctata* (Murdoch & Marks 1973). Petri dishes were cleaned daily to remove waste. We recorded larval weight on the first day of each instar. On the day of adult eclosion, ladybirds were weighed to the nearest 0.1µg, killed by freezing at -80°C, and sexed under a dissecting microscope (Leica MZ125) as described above. The elytra were carefully removed using dissecting scissors and stored at -80oC until colour measurement. Similarly, the remaining body was stored at -80oC until analyses of alkaloids (see below). A small number of larvae escaped or changed instars undetected and were therefore excluded from the analyses, giving a final sample size of n=151 larvae derived from n=31 mothers.

***(b) Measurements of egg and elytra colouration***

Ladybirds are prey to a variety of predators (e.g. ants, spiders, other ladybird larvae, birds and small mammals), which may vary over the ladybird life cycle, and consequently the colour signal of ladybird eggs and elytra may be aimed at several possible receivers (Wiklund & Jarvi 1982; Sloggett 2010), each with different visual systems (Stevens 2007). Therefore, colour was analysed objectively to obtain measurements not linked to any particular visual system. Digital photography was used instead of spectrometry due to the small size of ladybird eggs, and to ensure comparability of results the same methodology was used for elytra. Elytra or eggs were placed on white paper and allowed to thaw at room temperature, before being photographed with a Fujifilm IS Pro ultraviolet (UV) sensitive digital camera with a quartzCoastalOpt UV lens (Coastal Optical Systems). For photographs in human visible wavebands a UV and infrared (IR) blocking filter (Baader UV/IR Cut filter; transmitting between 400 and 700 nm) was used. For UV images, a UV pass IR blocking filter (Baader U filter; transmitting between 300 and 400 nm) was used. Each image contained a SpectralonTM diffuse grey reflectance standard (Labsphere, Congleton, UK), reflecting light equally at 40% between 300 and 750 nm and beyond. All eggs were photographed using standardized lighting provided by two UV daylight lamps (Kaiser RB260 Digital Lighting Unit). Each image was then linearized with respect to light intensity to accommodate the nonlinear response in image value with changes in radiance, and equalized with respect to the grey standard using custom written programmes and the Image Processing Toolbox of MATLAB (The MathWorks, Inc., MA, USA). This produced four images per object, each representing broadband reflectance information in the long wave (LW), medium wave (MW), short wave (SW), and ultraviolet (UV) parts of the spectrum. Reflectance values were extracted from each of the image channels using the Histogram Tool in Image J (National Institute of Health, USA). We aimed to collect colouration data for n=3 eggs produced by each female, although some eggs were damaged during handling after having been thawed and therefore we were only able to measure 1 or 2 eggs for n=25 females, and we could not measure any eggs for n=5 families. Each egg or elytron was selected and measured using the Freehand Selection Tool. Where multiple eggs were measured per family means were used for subsequent analyses. For elytra, means were calculated per individual.

Reflectance values were then used to calculate three measures of colour: hue, saturation, and brightness (HSB). Brightness corresponds to overall reflectance across the spectrum, and calculated independently of colour by applying the formula: (LW+MW+SW+UV)/4. To calculate saturation and hue, each set of reflectance values was first converted into a proportion (i.e. pLW=LW/(LW+MW+SW+UV) to remove absolute brightness variation. These proportional reflectance data were then converted into colour space coordinates following (Kelber, Vorobyev & Osorio 2003; Endler & Mielke 2005). This method calculated three (X,Y,Z) coordinates for each set of reflectance values, giving each clutch or individual offspring a location of colour in tetrahedral space. Saturation (*r*) was calculated as the shortest (Euclidian) distance from the achromatic origin, where saturation increases the further a point is from the origin. To calculate hue, individual averages of LW, MW, SW and UV values were analysed using PCA. Results from the PCA were then used to make an informed decision based on the type of colour variation that was present and therefore the type of colour channels (ratios) that would be logical (Stevens 2011; Spottiswoode & Stevens 2011). In eggs, PC1 (75%) and PC2 (17%) explained over 92% of the variation. In elytra, PC1 (69%) and PC2 (28%) explained about 97% of the variation.. PC1 is effectively the combination of LW plus MW to SW plus UV (i.e. longer wavelengths to shorter wavelengths). Thus, the colour channel (hue 1) is simply (LW+MW) / (SW+UV). Hue 2 is (LW+UV) / (SW+MW). We have used ratio values in our analysis rather than using real PC values to allow for repeatability analysis and because when visual systems analyse colour using opponent channels, there is little evidence that they weigh the output of one colour channel more than another.

Egg colour metrics were repeatable for females (saturation: *F*32,42 =4.16, *P* <.001, R = 0.58; brightness: *F*32,42 =10.11, *P* <.001, R = 0.80; hue1: *F*32,42 =4.75, *P* <.001, R = 0.62; hue2: *F*32,42 =5.09, *P* <.001, R = 0.64), and similarly offspring’s elytra measurements were repeatable (saturation: *F*120,121 =10.42, *P* <.001, R = 0.82 brightness: *F*120,121 =5.16, *P* <.001, R = 0.68; hue1: *F*120,121 =11.77, *P* <.001, R = 0.84; hue2: *F*120,121 =8.53, *P* <.001, R = 0.79).

***(c) Measurement of egg carotenoids***

For measurement of total carotenoids, all chemicals were HPLC grade, and chemical solutions were prepared using ultra pure water (Milli-Q Synthesis; Millipore, Watford, UK). Ten eggs were weighed to the nearest 0.1µg using an electronic microbalance (UMX2 ultra-microbalance; Mettler-Toledo Ltd.) and then homogenized for 1 min in 3ml chloroform and 1ml 70% methanol. Samples were then centrifuged at 12 x g and 4oC for 4 min. The chloroform phase containing the carotenoids was removed and evaporated to dryness under a vacuum. The remaining substrate appeared colourless, although we did not confirm the absence of any other pigment types by further analyses. The extract was redissolved in 100µl ethanol, and 50µl was injected into a Dionex HPLC system (Dionex Corporation, California, USA) fitted with a Waters Spherisorb NH2 column (5µm particle size, 4.6 x 250mm) maintained at 21oC. The mobile phase was 97% methanol, and the flow rate was 1.5ml/min. Absorbance was maximal at 445nm and was recorded using a PDA-100 photodiode array detector (Dionex). Total carotenoids were quantified using a calibration curve of lutein in ethanol (Kemin Europa, Herentals, Belgium). Results are expressed as µg g-1 egg.

***(d) Measurement of alkaloids in eggs and newly eclosed adults***

The whole body (minus the elytra) was cut down the midline using a scalpel; one half was used for coccinelline analysis, and the other half was used for precoccinelline analysis. Alkaloids were measured using LC-MS for coccinelline and GC-MS for precoccinelline, as described previously (Sloggett, Obrychi & Haynes 2009; Blount et al. 2012) with some modifications (see electronic supplementary material).

***(e) Data analyses***

All analyses were conducted using R version 3.0.2 (R Core Team 2010). Data were examined for normality, homoscedasticity, and outliers. In non-normal models, a Box-Cox power transformation was used to improve the approximation of normality. Generalized linear mixed models (GLMM) were fitted with Gaussian errors and an identity link function using the lme4 package; survival was fitted with a binomial error structure (Bates & Maechler 2009). First, we examined whether egg carotenoid concentration predicted hatching success, development time, growth rate, and survival to eclosion. Development time was deemed to be the total number of days between hatching and eclosion as adults. Growth rate was calculated as the regression coefficient of an exponential curve fitted to the x(age in days at the start of each instar stage) and y(body mass) for each larva. Second, we tested whether egg carotenoid concentration predicted egg colouration (saturation, hue 1, hue 2, and brightness, respectively). Third, we tested whether each of the egg colouration metrics in turn predicted egg concentrations of precoccinelline or coccinelline, respectively. Fourth, we tested whether egg colouration metrics predicted elytra colouration metrics, and whether egg concentrations of toxins predicted levels of toxins in the bodies of newly eclosed adults. Fifth, we tested whether elytra colouration metrics predicted body concentrations of alkaloids in newly eclosed adults (i.e. honest signalling). In all analyses father ID was included as a random factor to account for non-independence of eggs produced by matched-pairs of mothers and which shared the same father. As may be expected, females that spent more days in the lab on an *ad lib* diet prior to breeding had higher levels of diet derived carotenoids in their eggs (Pearson r=0.508, t=3.00, P=0.007). Therefore, we included this term as a covariate in all analyses of clutches. However, the amount of time females were kept in the lab prior to breeding did not influence any of our response variables: saturation (t=0.78, d.f. = 22.85, P ≥ 0.44), hue1 (t=0.35, d.f. = 22.99, P = 0.73), hue2 (t=0.26, d.f. = 21.52 , P = 0.80), brightness (t=-0.12, d.f. = 21.33, P =0.91), precoccinelline (t = 1.79, d.f. = 20.03, P = 0.09), coccinelline (t =0.51, d.f. = 22.16 , P =0.61). Results are reported from models that have been simplified to remove lab time as a covariate. In analyses of offspring phenotypes, sex and its two-way interaction with the relevant predictor was added as a fixed factor, and mother ID was included as a nested random factor to account for the non-independence of offspring produced by the same mother. Models were simplified by examining Wald t-tests and dropping terms starting with the interactions (where applicable) from the model until only those terms whose removal would significantly reduce the explanatory power of the model remained. Degrees of freedom were calculated using the Satterthwaite approximation (Schaalje, McBride & Fellingham 2002). Results are reported as predicted means ± s.e.

**RESULTS**

***Do egg carotenoids predict offspring development and viability?***

There were no significant relationships between the concentration of egg carotenoids and hatching success, development time, growth rate, survival to eclosion, or body mass at adult eclosion. Males grew slightly but significantly faster than females during larval development (slope ± s.e: female, 1.435 ± 0.008; male, 1.442 ± 0.005; Figure S1), while as expected, females had a significantly higher body mass at adult ecolosion (mean ± s.e: female, 32.10 ± 0.53; male, 29.32 ± 0.36; Figure S2) (see Table S1 in electronic supplementary material).

***Do egg carotenoids predict egg colouration?***

Egg carotenoids significantly predicted egg saturation (GLMM: t=2.10, d.f.= 29.84, *P*=0.044; 0.49 ± 0.23 (estimated slope ± s.e.). In contrast, there were no significant relationships between egg levels of carotenoids and egg colour metrics: hue1 (t=1.75, d.f.= 29.99, *P*=0.091), hue2 (t=1.51, d.f. = 27.02, *P*=0.141), brightness (t=-0.50, d.f. = 30.75, *P*=0.618).

***Does egg colouration predict egg toxin levels?***

Egg precoccinelline concentration was significantly explained by variation in egg saturation and egg hue1 (Figure 1 and Table 1), but there were no significant relationships between egg precoccinelline concentration and either egg brightness or hue2 (Table 1). There were no significant relationships between egg coccinelline concentration and any measure of egg colouration (Table 1).

***Does egg phenotype predict offspring phenotype?***

In recently eclosed adults of both sexes elytra saturation was significantly explained by variation in egg saturation, and similarly, elytra hue1 was explained by variation in egg hue1 (Table 2 and Figure 2). Neither elytra brightness nor hue2 were significantly explained by variation in egg brightness or hue2, respectively. However, there were no significant relationships between concentrations of either precoccinelline or coccinelline in eggs and newly eclosed adults of either sex (Table 2).

***Does elytra colouration predict body toxin concentrations of newly eclosed adults?***

At adult eclosion, body concentrations of coccinelline were significantly explained by variation in elytra hue2 (positive correlation) and elytra brightness (negative correlation) (Figure 3 and Table 3). In addition, females had higher body concentrations of coccinelline than males (male, 25.13 ± 0.91µg mg-1; female, 28.73 ± 1.28 µg mg-1) (Table 3). There were no significant relationships between body concentrations of coccinelline and elytra saturation or hue 1, or body concentrations of precoccinelline and any colouration metric (Table 3).

**DISCUSSION**

This study of 7-spot ladybirds suggests that maternal deposition of carotenoids into eggs contributes to aposematic colouration in eggs and offspring. Furthermore, warning signalling appears to be quantitatively honest, though the significant toxin and predominant colour metrics changed from eggs to adulthood: the yellow/orange colouration of eggs was correlated with maternal investment in precoccinelline, while the red colouration of offspring was correlated with levels of coccinelline.

Many oviparous species invest carotenoids in eggs (Blount et al. 2000). Such carotenoids must be obtained in the diet, and consequently we found a positive correlation between the amount of time females spent in the laboratory on an *ad lib*. diet of pea aphids and the concentrations of carotenoids which they subsequently deposited into their eggs. One possible function of such carotenoids is to facilitate rapid growth (Lakeh et al. 2010) while protecting developing embryos and neonates against oxidative damage (Blount et al. 2002; McGraw, Adkins-Regan & Parker 2005; Constantini & Møller 2008). Ladybirds are sexually size dimorphic with adult females being larger than males, while the total time taken to complete development does not differ between the sexes (Majerus 1987). The growth rate is similar between the sexes until the fourth instar, when females have a higher relative growth rate than males (Yasuda & Dixon 2002). However, we did not find any significant relationships between levels of maternally-derived carotenoids in eggs and offspring growth or survival to adult eclosion. Rather, our results suggest that egg carotenoids could play a role in aposematic signalling in ladybird beetles. Indeed, carotenoid levels in eggs influenced egg colour in terms of saturation.

The mechanisms which underpin the evolution and information content of aposematic displays have been a focus of much attention. Recent theoretical work has predicted that signal expression and the strength of defences may correlate positively, i.e. honest signalling (Blount et al. 2009; Lee, Speed & Stephens 2009; Speed et al. 2010; Holen & Svennungsen 2012). Indeed, positive signal-defence correlations have recently been reported across dendrobatid species of poison frogs (Summers & Clough 2001) and marine opisthobranch species (Cortesi & Cheney 2010), across populations of the strawberry poison frog *Dendrobates pumilio* (Maan & Cummings 2012), and within single populations of harlequin ladybirds *Harmonia axyridis* (Bezzerides et al. 2007), 7-spot ladybirds at adult eclosion (Blount et al. 2012), and paper wasps *Polistes dominula* (Vidal-Cordero et al. 2012). Empirical studies have shown that diet can affect coloration and toxins levels in adult ladybirds (Grill et al. 1997; Blount et al. 2012), and it has been hypothesised that the honesty of aposematic signals will depend on nutritional resource availability (Blount et al. 2009). However, the diet of larvae used in this study was standardized, and all individuals received equal amounts of food. Therefore, we expect that the variation in aposematic traits observed in this study resulted from other factors such as maternal effects. To the best of our knowledge, our study is the first to have considered whether honest signalling is apparent in aposematic eggs, and how this is related to aposematic phenotypes at adulthood.

Chemical defence in 7-spot ladybirds comprises the alkaloids coccinelline and its free base, precoccinelline, which are synthesised in the fat body, and are distributed throughout the body and deposited into eggs (Pasteels et al. 1973; Holloway et al. 1991; Daloze et al. 1994; Kajita et al. 2010). Empirical evidence shows that 7-spot adults are aversive and harmful to bird predators (Marples, Brakefield & Cowie 1989; Dolenska et al. 2009), while consumption of 7-spot eggs by larval harlequin ladybirds has been shown to retard growth and developmental time (Kajita et al. 2010). In the present study, the mean precoccinelline : coccinelline ratio in eggs was 1 : 2.19 (± 0.12 s.e.), whereas in adults at eclosion it was 1 : 18.49 (± 0.61 s.e.). It therefore seems likely that during ontogeny, maternally-derived precoccinelline in eggs is transformed into coccinelline. Whether precoccinelline functions solely as the precursor of coccinelline, or whether it confers chemical defence in its own right is uncertain. However, we note that precoccinelline is found in the defensive secretions of a variety of taxa which do not contain coccinelline, including the ladybird beetles *Tytthispis sedecimpunctata, Micrapis 16-punctata* and *Coleomegilla maculata* (Daloze et al. 1994), Australian soldier beetle (*Chauliognathus pulchellus*) (Moore & Brown 1978), bufonid toads (Garraffo et al. 1993), and dendrobatid frogs (Daly et al. 1994). Further studies will be necessary to evaluate whether precoccinelline functions to deter predators of 7-spot ladybird eggs and adults.

Ladybirds are prey to a variety of predators, the identity of which being likely to change over the ladybird life cycle. For example, it is likely that eggs are more susceptible to predation by ants, spiders, and other ladybird larvae, whereas adult beetles may more commonly fall prey to birds, reptiles, amphibians, or small mammals. Aposematic colouration may be most beneficial to adults, which seem more likely to be targeted by visually hunting predators. Though amphibians, reptiles and certain visually hunting insects and spiders may perceive the yellow/orange colouration of eggs (Kelber et al. 2003), further research is needed to determine whether they may discriminate the colour variation seen here. In addition, it is possible that toxicity caused by ingestion of precoccinelline and coccinelline differs among these predator types; this will also require further study. We found the positive correlation between aspects of colouration and chemical defence switched from precoccinelline in eggs (where the ratio of precoccinelline:cocinelline was high), to coccinelline in adults (where the ratio was low), which could indicate that egg and elytra colouration honestly signal the level of chemical defence that is functionally most relevant at that life stage. The significant colouration metrics (saturation and hue1 in eggs; and hue2 and brightness in adults) are logical because the eggs appear to human eyes relatively yellow/orange, which is essentially what hue 1 (LW + MW) measures, and elytra appear dark red, which hue 2 (LW colours) measures.

These findings are at odds with the results of an earlier diet manipulation study, where 7-spot larvae reared on a relatively high food supply had increased body levels of precoccinelline, although not coccinelline, and elytra carotenoid pigmentation was positively associated with body levels of precoccinelline at adult eclosion (Blount et al. 2012). The discrepancy amongst studies could indicate a difference in the rate of transformation of precoccinelline to coccinelline, although why this should be the case is unclear. One possibility is that there were differences between the two studies in the nutritional condition of laying mothers and hence their offspring. Whether maternal nutritional condition influences the capacity of offspring to transform alkaloids requires further study.

Egg colour also predicted the elytral colouration of adults at eclosion in terms of saturation and hue1. This association could be non-adaptive, in the sense that mothers’ eggs and offspring may have aspects of their phenotypes in common simply because mothers and offspring share genes and environments. For example, there could be a genetic component to the ability to sequester carotenoids from the common rearing diet of pea aphids. As in 2-spot ladybirds (Holloway et al. 1995), aposematic colouration is likely heritable to some extent in 7-spot ladybirds. However, there are other potential adaptive explanations, i.e. maternal effects. Aposematic traits controlled by maternally expressed genes will cause all offspring to share the same phenotype regardless of their individual genotypes (Brodie & Agrawal 2001). This could provide a possible explanation for the lack of variation amongst clutches despite the potential for multiple mating in the wild-caught females used for this study. It seems unlikely that the association between egg and offspring phenotypes can be explained entirely by transfer of maternally-derived carotenoid molecules from eggs to larval integument; the average concentration of carotenoids in eggs in this study (0.044 ± 0.005 µg g-1, mean ± s.e) is considerably less than that previously reported in the elytra of a sample (n=31) of 7-spot ladybirds at adult eclosion (155.47 ± 8.48 µg g-1; (Blount et al. 2012). However, an intriguing possibility is that carotenoids in ladybird eggs have programming effects on developing larvae, altering their capacity to sequester dietary carotenoids as previously reported in birds (Koutsos et al. 2003). By depositing more carotenoids into eggs, mothers might ensure that their offspring will develop greater levels of elytral carotenoid pigmentation.

In conclusion, aposematic signal expression is dependent on a variety of factors including environmental conditions, heritability, and as this study suggests, maternal investment. We have reported positive associations between aspects of egg colouration and egg levels of precoccinelline, suggesting that maternally-derived pigments in eggs could confer honest aposematic signalling. Furthermore, aspects of egg colouration predicted the elytra colouration of recently eclosed adults; such an association could arise through various mechanisms, but one possibility is that maternally-derived carotenoids in eggs directly influence the development of colouration in offspring. It would therefore be interesting to determine whether carotenoids in eggs have programming effects on larvae, which influence their adult aposematic phenotype. Offspring were themselves found to have honest aposematic signals at adult eclosion, colour being positively associated with body levels of coccinelline. Our study therefore highlights the possibility that the functional importance of precoccinelline and coccinelline as agents of chemical defence differs across life stages, a possibility which awaits investigation.

**ACKNOWLEDGEMENS**

We thank Louise Selwood, Abi Brown and Georgia France for their assistance in the lab, and the anonymous reviewers for many helpful comments on an earlier draft. This study was supported by a Royal Society University Research Fellowship (to JDB) and by the Natural Environment Research Council (NERC). MS was supported by a Biotechnology and Biological Sciences Research Council (BBSRC) David Phillips Research Fellowship (BB/G022887/1).

**APPENDICES**

The following files are provided as electronic supplementary material:

Detailed methodology for alkaloid analyses.

Table S1: Relationships between egg carotenoids and sex, and the development and survival of seven-spot ladybird offspring.

Figure S1: Sex differences in larval growth rate.

Figure S2: Sex differences in body mass at adult eclosion.

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**Figure legends**

**Fig. 1.** Relationships between aposematic colouration and toxin levels in eggs of seven-spot ladybirds. (a) Saturation and precoccinelline (b) Hue1 and precoccinelline. Fitted lines are predictions arising from GLMM analyses controlling for Father ID (see text of Results).

**Fig. 2.** Relationships between aposematic colouration of eggs and offspring at adult eclosion in seven-spot ladybirds. (a) Egg saturation and elytra saturation. (b) Egg hue1 and elytra hue1. Males are denoted by filled circles and females by open circles. Fitted lines are predictions arising from GLMM analyses controlling for Father ID and Mother ID; a single line is fitted in each panel because the effect of sex was non-significant (see text of Results).

**Fig. 3.** Relationships between aposematic colouration and body toxin levels at adult eclosion in seven-spot ladybirds. (a) Adult elytra hue2 and body coccinelline. (b) Adult elytra brightness and body coccinelline. Males are denoted by filled circles and solid lines, whereas females are denoted by open circles and dashed lines. Fitted lines are predictions arising from GLMM analyses controlling for Father ID and Mother ID (see text of Results).

Table 1: Relationships between aposematic colouration and toxin concentrations in seven-spot ladybird eggs. Results are from General Linear Mixed Models including Father ID as a random factor. See Methods for details. Significant *P*-values are shown in bold.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Response | Explanatory | Estimate ± s.e. | t-value | d.f. | *P*-value |
| Precoccinelline | Saturation | 2.99 ± 0.71 | 4.21 | **29.98** | **<0.001** |
| Precoccinelline | Hue1 | 0.06 ± 0.01 | 3.84 | **29.98** | **<0.001** |
| Precoccinelline | Hue2 | 0.21 ± 0.26 | 0.81 | 25.38 | 0.43 |
| Precoccinelline | Brightness | -0.00 ± 0.00 | -0.09 | 29.87 | 0.93 |
|  |  |  |  |  |  |
| Coccinelline  Coccinelline  Coccinelline | Saturation  Hue1  Hue2 | 26.10 ± 15.72  0.48 ± 0.34  3.43 ±4.399 | 1.66  1.41  0.78 | 29.67  25.65  29.69 | 0.11  0.17  0.44 |
| Coccinelline | Brightness | 0.06 ± 0.05 | 1.21 | 23.20 | 0.24 |
|  |  |  |  |  |  |

Table 2: Relationships between the phenotypes of seven-spot ladybird eggs and offspring at adult eclosion in terms of aposematic colouration and toxins. Results are from General Linear Mixed Models including Father ID and Mother ID as random factors. See Methods for details. Significant *P*-values are shown in bold. All 2-way interactions were tested and were non-significant (t≤ 1.53, *P* ≥ 0.13).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Response | Explanatory | Estimate ± s.e. | t-value | d.f. | *P*-value |
| Adult elytra saturation | Egg saturation | 0.02 ± 0.01 | 2.88 | **91.36** | **0.005** |
|  | Sex | 0.00 ± 0.00 | 0.10 | 91.08 | 0.94 |
|  |  |  |  |  |  |
| Adult elytra hue1 | Egg hue 1 | 0.08 ± 0.04 | 2.15 | **94.99** | **0.034** |
|  | Sex | 0.03 ± 0.15 | 0.21 | 94.37 | 0.83 |
|  |  |  |  |  |  |
| Adult elytra hue2 | Egg hue 2 | -0.00 ± 0.00 | -0.00 | 25.23 | 1.00 |
|  | Sex | 0.00 ± 0.01 | 0.01 | 94.75 | 0.95 |
|  |  |  |  |  |  |
| Adult elytra brightness | Egg brightness | -0.03 ± 0.02 | -1.15 | 94.997 | 0.25 |
|  | Sex | 0.46 ± 0.67 | 0.68 | 94.997 | 0.50 |
|  |  |  |  |  |  |
| Adult body precoccinelline | Egg precoccinelline | -0.03 ± 0.03 | -0.81 | 25.20 | 0.42 |
|  | Sex | -0.05 ± 0.08 | -0.61 | 86.98 | 0.55­­­­­­ |
|  |  |  |  |  |  |
| Adult body coccinelline | Egg coccinelline | -0.02 ± 0.02 | -1.20 | 21.32 | 0.25 |
|  | Sex | -0.17 ± 0.11 | -1.52 | 77.85 | 0.13 |
|  |  |  |  |  |  |

Table 3: Relationships between aposematic colouration and toxin levels at adult eclosion in seven-spot ladybirds. Results are from General Linear Mixed Models including Father ID and Mother ID as random factors. See Methods for details. Significant *P*-values are shown in bold. All 2-way interactions were tested and were non-significant (t≤ 1.18, *P* ≥ 0.24).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Response | Explanatory | Estimate ± s.e. | t-value | d.f. | *P*-value |
| Adult body precoccinelline | Adult elytra saturation | 1.28 ± 1.91 | 0.67 | 109.26 | 0.50 |
|  | Sex | -0.03 ± 0.07 | -0.46 | 105.52 | 0.65 |
|  |  |  |  |  |  |
| Adult body precoccinelline | Adult elytra hue 1 | 0.04 ± 0.10 | 0.41 | 110.69 | 0.68 |
|  | Sex | -0.03 ± 0.07 | -0.46 | 105.52 | 0.65 |
|  |  |  |  |  |  |
| Adult body precoccinelline | Adult elytra hue 2 | 0.19 ± 0.26 | 0.74 | 110.65 | 0.46 |
|  | Sex | -0.03 ± 0.07 | -0.46 | 105.52 | 0.65 |
|  |  |  |  |  |  |
| Adult body precoccinelline | Adult elytra brightness | 0.003 ± 0.01 | 0.33 | 104.14 | 0.75 |
|  | Sex | -0.03 ± 0.07 | -0.46 | 105.52 | 0.65 |
|  |  |  |  |  |  |
| Adult body coccinelline | Adult elytra saturation | -1.74 ± 3.23 | -0.54 | 108.18 | 0.59 |
|  | Sex | -0.25 ± 0.12 | -2.13 | **106.06** | **0.036** |
|  |  |  |  |  |  |
| Adult body coccinelline | Adult elytra hue1 | -0.26 ± 0.18 | -1.47 | 108.67 | 0.14 |
|  | Sex | -0.25 ± 0.12 | -2.13 | **106.06** | **0.036** |
|  |  |  |  |  |  |
| Adult body coccinelline | Adult elytra hue2 | 1.07 ± 0.35 | 3.01 | **109.45** | **0.003** |
|  | Sex | -0.25 ± 0.09 | -2.13 | **106.12** | **0.037** |
|  |  |  |  |  |  |
| Adult body coccinelline | Adult elytra brightness | -0.05 ± 0.02 | -2.62 | **110.02** | **0.01** |
|  | Sex | -0.25 ± 0.12 | -2.13 | **106.06** | **0.036** |
|  |  |  |  |  |  |