**Abstract**

In species that advertise their toxicity to predators through visual signals, there is considerable variation among individuals in both signal appearance and levels of defence. Parental effects, a type of non-genetic inheritance, may play a key role in creating and maintaining this within-species diversity in aposematic signals, however a comprehensive test of this notion is lacking. Using the ladybird *Adalia bipunctata* we assess how egg coloration and defence level (concentration of the toxic alkaloid (-)-adaline) is influenced both by simulated predation risk in the egg laying environment, and by parental phenotype (coloration and toxin level). We found that egg toxin level and colour were predicted by parental phenotype, but were not altered in response to cues of egg predators. Egg luminance (lightness) was positively correlated with paternal elytral luminance, whilst maternal toxin level positively predicted egg toxin level. In response to egg predator cues, ladybird mothers altered the timing of laying and total egg number, but not egg toxin level or colour. It appears therefore that in *A. bipunctata* variation between individuals of the same morph in the colour and toxin level of the eggs they lay, i.e. egg aposematic phenotype, is more strongly influenced by individual variation in parental aposematic traits than environmental cues of egg predation risk. Furthermore, these results provide the first indication that, in a warningly coloured species, male coloration may play a dual role as predator deterrent and indicator of paternal quality, influencing maternal investment in offspring.

Key words: parental effects, aposematism, maternal effects, paternal effects, differential allocation, warning colour variation

**Introduction**

Prey can gain protection from predators through the association of a colourful warning signal with a toxic or distasteful defence (aposematism; Poulton, 1890). Individuals within a species may therefore benefit from sharing similar levels of defence and conspicuousness (Rowland et al., 2010). Despite this expectation, considerable variation in signal expression and associated toxin level is found amongst individuals of the same aposematic species (Merrill et al., 2015) and even within the same morph (e.g. Blount et al., 2012). Within morphs, individuals can vary in their conspicuousness and toxicity (Vidal-Cordero et al., 2012) and a number of hypotheses have recently been proposed that help to explain the existence of such apparently paradoxical variation, yet these focus almost exclusively on adult phenotypes (e.g. Summers et al., 2015). Natural selection, however, acts at every stage of an organism’s life cycle (Stearns, 1992), with both the strength and nature of selection pressures varying according to life stage (Moran, 1992). This is especially relevant for aposematic species, many of which have complex lifecycles (Joron, 2003) where each discrete phase (i.e. egg, larval, or adult) is likely to have different predators with very different sensory systems (e.g. Hemptinne et al., 2012). Furthermore, not only is offspring phenotype key in determining which individuals survive to contribute to the adult population, but many aspects of offspring phenotype carry over into adulthood (Burton & Metcalfe, 2014), including toxicity and warning coloration (Winters et al., 2014). It is clear therefore that a full understanding of warning signal variation requires consideration of how offspring aposematic phenotype is determined (Day & Bonduriansky, 2011; Marshall & Morgan, 2011).

In addition to genetic inheritance, parental effects are a powerful determinant of offspring phenotype (Mousseau & Fox, 1998), influencing key offspring traits in a way that reflects parentally detected environmental variation (Fox et al., 1997; Rollinson & Hutchings, 2013) and/or parental phenotype (Bonduriansky & Head, 2007). A large number of aposematic species are egg laying insects with no parental care (Joron, 2003) and egg provisioning is therefore the main conduit via which parental effects may occur (Newcombe et al., 2015). Maternal egg investment in a number of non-aposematic species has been shown to vary in response to reliable cues of environmental change (so called ‘Anticipatory Maternal Effects’ or AMEs; Marshall & Uller, 2007). This enables mothers to fine-tune their investment per reproductive event, maximising the total number of surviving offspring (reproductive success) and thereby maternal fitness (Bernardo, 1996). Maternally-controlled alteration of offspring toxin level in aposematic species is known to occur in response to environmental variation (Paul et al., 2015). However, aposematism is the direct relationship between a conspicuous signal *and* an associated defence. To date, no evaluation of the effects of offspring predation risk on maternal investment in both offspring colour and defence has been carried out in any aposematic species.

Paternal quality can also influence maternal invesment in eggs (Burley, 1986, 1988), and this so called ‘Differential Allocation’ (DA) can be positive or negative depending on the species (Ratikainen & Kokko, 2010). Females can increase their per egg investment when mating with attractive males in order to maximise the survival of the resulting ‘good quality’ offspring (Positive DA; D'Alba et al., 2010; Sheldon, 2000). Alternatively, females may increase per egg investment when mating with less attractive males in order to compensate for their partner’s poor quality (Negative DA; Badas et al., 2017; Bolund et al., 2009). In some aposematic species, the conspicuousness of male warning coloration appears to act as a signal to females of the male’s quality (Summers et al., 1999), and influences mate choice (Finkbeiner et al., 2014; Maan & Cummings, 2008); whether it also influences maternal investment in offspring is unknown. Finally, maternal phenotype itself can dictate egg investment (Berkeley et al., 2004; Donelson et al., 2008). For example, mothers often provision their eggs and larvae with chemical defences in proportion to their own defence levels, leading to a positive correlation between maternal and offspring defence levels (Hanifin & Brodie, 2003; Hutchinson et al., 2008). Therefore, parental effects have the potential to create variation in aposematic phenotype, via maternal response to environmental and paternal cues, as well as maintaining it, perpetuating parental levels of conspicuousness and toxicity. Here we use the ladybird beetle *Adalia bipunctata,* to investigate whether maternal investment, specifically changes in egg toxin level and egg coloration, varies with egg predation risk and both paternal and maternal aposematic phenotype. *A. bipunctata* is an ideal study species as it is aposematic at all stages of its complex life cycle (egg, larval, pupal, and adult). We envisage three alternate pathways by which maternal investment in eggs may vary (Figure 1.):

*1) Influence of egg predator cues on egg phenotype via maternal investment (Anticipatory Maternal Effects)*

*A. bipunctata* eggs are aposematic and laid in environments with high levels of predation from the larvae of ladybird competitors (intraguild predation; Polis et al., 1989). Egg toxins deter heterospecific predators, but attract conspecific cannibals (Kajita et al., 2010) and females can alter egg laying behaviour in response to chemical cues of offspring predators (Seagraves, 2009). However, whether they also alter egg toxin level and conspicuousness in response to conspecific or native heterospecific predatory larvae is unknown; to date only changes in egg toxin level in response to invasive predators have been investigated (Paul et al., 2015). We predict that a) in the presence of conspecific larval tracks egg toxin level and conspicuousness will decrease in order to decrease the risk of egg cannibalism; and b) in the presence of heterospecific larval tracks egg toxin level and conspicuousness will increase, strengthening the egg aposematic signal.

*2) Influence of paternal phenotype on egg phenotype via maternal investment (Differential Allocation)*

In common with many other aposematic species, male conspicuousness is known to influence female mate choice in *A. bipunctata* (Majerus et al., 1982), however whether it also influences maternal investment in offspring is unknown. If positive differential allocation occurs then a positive correlation between paternal and egg conspicuousness and/or toxin level would be expected, whereas if negative differential allocation occurs the reverse of this scenario could reasonably be predicted.

3) *Influence of maternal phenotype on egg phenotype*

In aposematic species, including another species of ladybird (*Coccinella septempunctata*), both maternal and offspring conspicuousness and toxin level are known to correlate positively (e.g. Stynoski et al., 2014; Winters et al., 2014). We therefore predict that in *A. bipunctata*, maternal and egg toxin level and conspicuousness will positively correlate.

**Methods**

*Culture and experimental set up*

Stock culture of *A. bipunctata* (*typica*), obtained from Gardening Naturally (Love Lane Industrial Estate, Cirencester, UK), were maintained in culture on an *ad libitum* diet of pea aphids (*Acyrthosiphon pisum*) [*A. pisum* reared on dwarf bean (*Vicia faba*) Sutton variety] at 18oC with a 16L:8Dh photoperiod. Experimental individuals were 1st generation virgin adults of known age ( = 21 days post eclosion) obtained from stock culture: 104 females and 104 males from 20 families. Females and males were weighed to nearest 0.01 mg (analytical balance GR-200 A&D® Gemini™). Females were mated with a non-sib male, and 24 h after pairing males were removed, photographed, and stored at -80o C prior to toxin analysis (see below for colour and toxin analysis method details). Females were then placed into a clean Petri dish with *ad lib*. aphids (0.01g, ~ 40 aphids; Hodeket al., 2012)). After 24 h a cluster of eggs was randomly selected from those laid by the females and a subset of 3 eggs from the cluster were photographed and stored at -80o C. Females were then placed into an individual experimental arena, in one of three treatments (control (NN), conspecific risk (CP), or heterospecific risk (HP), with an *ad lib*. aphid supply. Females from different sibling clusters were distributed evenly between the treatment levels, so that family ID and mate ID were represented equally in all three treatments (NN: n= 41, CP: n=41, HP: n= 22). The simulated predation risk treatment levels were created using tracks of either 4th instar *A. bipunctata* larvae (CP) or *C. septempunctata* larvae (HP). For each replicate, tracks were created using 5 larvae, which were placed, without food, into individual sterile Petri dishes (9cm diam.), each containing a semicircle of corrugated filter paper (9 cm diam.) and left for 24 h (Doumbia et al., 1998; Magro et al., 2007). The control environment of no simulated predation risk (NN) consisted of a sterile Petri dish (9 cm diam.) and a clean semicircle of corrugated filter paper that had not been in contact with any ladybird larvae. Each female was left in its experimental arena for 2 d (48 h), with additional aphids being added after 24 h. Laying behaviour was monitored at 1, 3, 6, 9, and 24 h intervals over the 2 d, specifically the number of eggs laid, cluster size, and the number of clusters laid was recorded. A cluster was classified as a group of two or more eggs, with each egg being in physical contact with at least one other egg in that cluster. Once recorded eggs were removed to prevent filial cannibalism. The first and last clusters laid by a subset of females (NN: n= 19, CP: n=13, HP: n= 15) over the 2 d were chosen, and a further subset of 3 eggs from each cluster photographed and stored at -80oC prior to toxin analysis. After the full 3 days of the experiment had elapsed, females were removed, photographed and stored at -80oC prior to toxin analysis. All experiments were carried out in an incubator (Percival® model I-41LL, 505 Research Drive, Perry, IA 50220 USA) at 18oC with a 16L:8D h photoperiod.

*Quantifying the colour of eggs and adult elytra*

Digital image analysis and visual modelling were both used to quantify egg and elytral luminance (perceived lightness) and saturation (colour richness) (Kelber, Vorobyev, & Osorio, 2003; Osorio & Vorobyev, 2005). Individual eggs and adults were photographed using a Nikon D7000 digital camera which had undergone a quartz conversion, enabling ultraviolet (UV) light sensitivity (Advanced Camera Services, Norfolk, UK), fitted with a Nikon 105-mm Nikkor lens. For photographs in the visible spectrum the camera was fitted with an ultraviolet (UV) and infrared (IR) blocking filter (Baader Planetarium, Mammendorf, Germany UV/IR Cut filter; transmitting between 400 and 700 nm). For photographs in the UV part of the spectrum the camera was fitted with a UV pass IR blocking filter (Baader U filter; transmitting between 300 and 400 nm). All photographs were taken in a dark room using standardized lighting provided by a UV daylight lamp (Iwasaki eyeColor arc lamp (6500k), with UV filter removed) with eggs or adults placed on a sheet of black ethylene-vinyl acetate (EVA), used for its low (<5%) UV reflectance, next to a Spectralon™ 40% diffuse grey reflectance standard (Labsphere, Congleton, UK) (Arenas, Troscianko, & Stevens, 2014; Stevens et al., 2007).

To correct for the non-linear response of the camera to light levels (radiance), and for any variation in light levels between photos, each image was linearized with respect to light intensity and equalized with respect to the grey standard (Stevens et al., 2007). This was carried out using the programme ImageJ 1.47t and the Multispectral Image Calibration and Analysis Toolbox plugin (Troscianko & Stevens, 2015). The entirety of each egg was selected for analysis, using a specialised egg selection tool plugin (Troscianko, 2014), and for adults an area of the elytra with no spectral reflectance and excluding the achromatic spots, was selected (Arenas, Walter, & Stevens, 2015). It is important when investigating any changes in animal coloration to do so in the context of the receiver, in this case either the potential predator or mate (Endler, 1978; Endler & Mielke, 2005; Stevens et al., 2007). Using an image transformation approach outlined in Troscianko & Stevens (2015), linearized egg images were mapped to the predicted responses of the ladybird larval egg predators used in this experiment. Ladybird vision is potentially trichromatic, containing three classes of retinular cells sensitive to medium wave (MW), short wave (SW) and ultraviolet (UV) light and here is modelled using the spectral sensitivity of the ladybird *C. septempunctata* (Linet al.*,* 1992). Adult predators are avian as opposed to insect (Hodek & Honek, 2012) and therefore images of adult elytra were mapped to blue tit (*Cyanistes caerulues*) cone sensitivities (Hart et al., 2000), to model avian predator perception. *C. caerulues*, like most passerines, are tetrachromats, and have five classes of cone receptors; longwave (LW), medium wave (MW), short wave (SW), ultraviolet (UV), and double cones (D). In order to model female perception of male coloration, adult male elytra were additionally mapped to the predicted responses of the ladybird visual system. This robust polynomial mapping technique is highly effective and yields higher R2 values (0.96-0.98) than similar cone catch values obtained via spectrometry (Stevens & Cuthill 2006; Stevens et al., 2007; Pike 2011; Troscianko & Stevens 2015).

Luminance is here used to refer to a visual system dependent measure of achromatic variation, or perceived lightness (Osorio & Vorobyev 2005; Stevens 2011). Ladybirds (*C. septempunctata*) and birds (*C. caeruleus*) differ in the receptor type used for luminance vision. The MW channel (~520nm) was used to model luminance for ladybird vision as this is the most abundant receptor (Linet al., 1992) and in insects the receptor type used for luminance vision is generally the most abundant of the retinular cell classes (Osorio & Vorobyev, 2005). In contrast, in birds it is the double cone receptor (D) that is thought to be responsible for achromatic vision (Osorio and Vorobyev, 2005) and therefore the double cone was used to model luminance for bird (*C. caeruleus*) vision.

Saturation or the perceived intensity of a colour (e.g. red vs. pink) in each visual system was calculated first by converting the single cone catch values (for both ladybird vision and bird vision respectively) into proportions to remove absolute variation in brightness (Endler & Mielke 2005). The proportional cone catch values for ladybird vision were then converted into two colour space coordinates (X, Y), giving each individual a location of colour in two dimensional colour space (Kelberet al., 2003; Endler & Mielke 2005). In contrast the proportional cone catch values for bird vision were converted into three colour space coordinates (X, Y, Z), giving each individual a location of colour in three dimensional colour space (Kelberet al., 2003; Endler & Mielke 2005). For each visual system, saturation was calculated as the shortest Euclidian distance from the achromatic origin, with saturation being greater the further a point was from the origin.

Eggs and adult elytra are all brightly coloured in the lw end of the visible spectrum (~570-750nm). As in previous studies (Winters et al., 2014) our a priori expectation was that there would be no difference in the *type* of pigment in eggs and adult elytra, and therefore the type of colour of eggs (i.e. hue), either between treatments or between female morphs, but that there would be differences in pigment quantity and therefore in luminance and saturation. Due to this and the strong correlation between hue and both luminance and saturation measures, hue was not included in the analysis (following Arenas et al., 2015).

*Quantifying levels of (-)-adaline*

*A. bipunctata* eggs and adults contain the toxic alkaloid (-)-adaline; this was assayed as follows. Each egg was weighed to the nearest 0.1 µg using an electronic microbalance (Cahn C33; Scientific and Medical Products Ltd, Manchester, UK.) and homogenized for 30 seconds in 200 µl of dichloromethane (DCM), using a handheld electronic pestle. Each sample was then centrifuged at 13RPM and 4o C for 10 minutes. 100 µl of solution was transferred into a screw top autosampler vial. Adults (male and females) were weighed to the nearest 0.1 mg (analytical balance GR-200 A&D® Gemini™), elytra removed (as it is the soft tissue that contains the (-)-adaline (Laurentet al.*,* 2002) and placed into a 2 ml centrifuge tube along with 1ml of DCM and 0.5 ml of glass beads (1 mm diameter). Samples were homogenised for 1 min at 5.5m/s in a tissue homogenizer (Precellys 24 Tissue Homogenizer; Bertin Technologies, France) and centrifuged at 13RPM and 4oC for 10 minutes. 100µl of supernatant from the resulting solution was transferred into a screw top autosampler vial along with 900µl of DCM. Samples (2µl) were analysed on a non-polar (HP-1, 50 m x 0.32 mm inner diameter x 0.5) Gas-Chromatograph (GC) (Agilent Technologies, UK) fitted with a cool-on-column injector, a deactivated HP-1 pre-column (1m x 0.53 mm inner diameter) and a flame ionisation detector (FID). The GC oven temperature was maintained at 30°C for 1 min after sample injection and then raised by 5°C min-1 to 150°C, then 10°C min-1 to 240°C. The carrier gas was hydrogen. Peak enhancement by co-injection with a pure (-)-adaline standard was used to confirm correct identification of the (-)-adaline peak. Absolute (-)-adaline concentration per egg (ng/mg) was quantified by transforming the peak area using a calibration curve created from an external standard of pure (-)-adaline in dichloromethane of the following concentrations; 100ng/µl, 50ng/µl, 10ng/µl, 5ng/µl, and 1ng/µl.

**Ethical note**

This research adheres to ASAB Guidelines for the use of animals in research and was approved by the Exeter University Ethics Board. No national licences were required. Predator cues were used instead of live predatory larvae in order to prevent potentially stressful inter-specific interactions. The predator cues used to simulate offspring predation risk are abundant in the ladybird’s natural environment and the minimum number of larvae cited to have elicited a detection response in female ladybirds was used to create this cue. All individuals were terminated at the end of the experiment as analysis of toxin level in *A.bipunctata* is lethal and the *C. septempunctata* used to create the experimental cues were from non-local populations. Individuals were terminated via gradual cooling (5oC, -20oC and then -80oC), a more humane technique than direct transfer to -80oC, as it decreases invertebrate activity and the risk of physiological stress due to cell rupture.

*Data analyses*

Data were analysed using R version 3.2.2 (R Core Team, 2016). Where appropriate, data were examined for normality, homoscedasticity and outliers. Alpha level was set at 0.05 for all tests and stepwise backwards deletion was employed to reach the minimum adequate model (Crawley, 2014). General or generalised linear models (package=MASS) were used to assess the effect of treatment, female mass (mg) and female age (days) on time first eggs were laid (family= negative binomial), the number and average size of clusters (family= negative binomial), the total number of eggs (family = Gaussian), whether or not females laid single eggs (family= binomial) and how many single eggs they laid (family= negative binomial). Egg volume (mm3), (-)-adaline levels (ng/mg), luminance and saturation were repeatable (Egg volume: R = 0.695, SE = 0.038, CI = [0.613, 0.763], P<0.001; Egg (-)-adaline: R = 0.867, SE = 0.02, CI = [0.82, 0.898], P<0.001; Luminance: R = 0.808, SE = 0.027, CI = [0.746, 0.855], P<0.001; Saturation: R = 0.701,SE = 0.038, CI = [0.621, 0.769], P<0.001 ('rptr' package Nakagawa & Schielzeth 2010, 2013). The effect of treatment, day, treatment by day interaction, female and male (-)-adaline level (ng/mg) or mass (mg), and female age on the square root of egg (-)-adaline level (transformed to normalise) or egg volume were assessed using a general linear mixed effects model (GLMER; package=lme4 (Bateset al.*,* 2015)), where female identity was the random effect. Variation in egg luminance and egg saturation attributable to the effect of egg (-)-adaline level (ng/mg), treatment, day, egg (-)-adaline level (ng/mg) by treatment by day interaction, female and male luminance/saturation was also assessed using a LMER, where female identity was the random effect. Based on these results the relationship between maternal toxin level and elytral saturation and luminance, calculated using bird visual system (*C. caeruleus*) as outlined in the visual analysis methods above, was also investigated.

**Results**

Predator treatment had a significant effect on the latency of females to lay eggs, and both the total number of clusters and the number eggs laid (Table 1.). In the presence of conspecific tracks females took the longest to lay eggs (control [NN] – conspecific [CP]: mean difference +SE = 0.33+0.15hrs, Z2,101 = -2.17, *P* = 0.03; heterospecific [HP] – conspecific [CP]: mean difference +SE = 0.48+0.19hrs, Z2,101 = -2.53, *P* = 0.01), and also laid fewer egg clusters and had a smaller total number of eggs than the control treatment (control [NN] – conspecific [CP]; Cluster number: mean difference +SE = 0.25+0.0.12, Z2,103 = -2.07, *P* = 0.04; total egg number: mean difference +SE =-10.03+2.80, t2,103 = -3.56, *P* <0.001). The effect of treatment on both average and maximum cluster size was non-significant (Table 1). Egg luminance and saturation varied significantly between treatment groups, but there was no interactive effect of treatment and day on egg (-)-adaline levels, volume, saturation, or luminance (Figure 2; Table 2). Egg (-)-adaline concentration was also positively correlated with egg luminance and egg saturation (Table 2). Egg (-)-adaline concentration, volume, and luminance increased with maternal (-)-adaline concentration, maternal mass, and paternal luminance (bird and ladybird vision) respectively (Figure 3; Table 2). Maternal toxin level was also negatively correlated with maternal elytral saturation (bird vision; F 1,44= 6.83, *P* = 0.012, R2 = 0.13) but not elytral luminance (F1,44=0.42, *P* =0.52, R2 = 0.01).

**Discussion**

Parental effects have the potential to influence within-morph variation in coloration and toxicity in aposematic species in three main ways. Firstly, mothers may create variation by changing offspring phenotype in response to signals of predation risk (‘Anticipatory Maternal Effects’; AMEs), for example by increasing egg conspicuousness and toxicity when egg predation risk is high. Secondly, through the alteration of offspring phenotype in response to paternal quality (‘Differential Allocation’; DA), mothers may have the capacity to perpetuate the paternal aposematic phenotype (positive DA) or create offspring with levels of coloration and toxicity that differ from that of their father (negative DA). Finally, patterns of variation in aposematic traits present in the adult population may be maintained through the perpetuation of maternal phenotype in their offspring; mothers could, for example, lay eggs that have an aposematic phenotype that reflects maternal phenotype. In *A. bipuncata* mothers did not alter either egg colour or toxin content in the presence of egg predator tracks (i.e. no treatment by day interaction). Whereas, paternal luminance (as viewed by both predators and conspecific females) positively predicted egg luminance and maternal toxin level and mass positively predicted egg toxin level and volume, respectively. Our results indicate that maternal and paternal phenotype play a greater role in determining the colour and toxin content of eggs than egg predation risk in this aposematic ladybird.

***Response to tracks of offspring predators (‘Anticipatory Maternal Effects’)***

Previous work has shown that female *A. bipunctata* can detect tracks of heterospecific and conspecific egg predators (Frechette et al., 2004) and alter their reproductive investment in response (Paul et al., 2015). Therefore, we predicted that in the presence of conspecific larval tracks egg toxin level and measures of egg coloration related to egg conspicuousness would decrease, in order to reduce egg cannibalism risk. We further predicted that the reverse would occur in the presence of heterospecific larval tracks, as mothers strengthened egg aposematic signal and thereby egg predator deterrence.

Conspecific ladybird larvae benefit from the consumption of conspecific eggs, i.e. cannibalism, as they possess the biochemical pathways to prevent the toxic effects of egg chemical defence and the eggs themselves represent an energy and nutrient rich resource (Sloggett & Lorenz, 2008). It has also been demonstrated that cannibalistic larvae preferentially consume eggs with a higher toxin content (Paul et al.*, unpublished data*) and that they may be able to sequester toxins from consumed eggs (Kajita et al., 2010; Laurent et al., 2002). It is therefore in the interests of mothers to reduce any signals of egg toxin content, such as aspects of coloration, which may increase the likelihood of egg detection and consumption in the presence of such conspecific cannibals. As in previous studies (Martini et al., 2009), females were initially reluctant to lay in the presence of conspecific tracks and, perhaps as a consequence, laid both fewer eggs and a smaller number of egg clusters. However, contrary to predictions there was no alteration in egg toxin content, luminance, or saturation in the presence of conspecific predator tracks.

There are two possible and non-mutually exclusive explanations for the lack of change in egg aposematic phenotype observed in response to the presence of cannibalistic egg predator cues. Firstly, the costs of changing egg phenotype in the presence of this predator may not be outweighed by the benefits, i.e. a decrease in egg toxin level and egg conspicuousness may not significantly reduce egg predation risk in the face of conspecific predators. Consequently a strategy of offspring predator avoidance may more reliably maximise reproductive success, as seen in many egg laying species (Fontaine & Martin, 2006; Otsuki & Yano, 2017; Rieger et al., 2004). Although females did eventually lay eggs in the presence of conspecific tracks this may have been because they were constrained to one specific laying environment. In the wild females are highly mobile and able to move away from an area where egg/offspring predators are present to find a new and less risky locality in which to lay eggs (Jeffries et al., 2013; Seagraves, 2009). Secondly, the early life and adult toxin levels of individuals are tightly correlated in ladybirds (Winterset al., 2014), and toxicity as well as colour is important in deterring predation on adults (Marples et al., 1989). Decreasing egg toxin level in early life may therefore negatively impact adult survival. Females may consequently have to balance reducing predation on offspring during early and later life. Such antagonistic selection pressures between life stages are not uncommon (Aguirre et al., 2014; Schluter et al., 1991), though they remain largely unexplored in aposematic species.

There was also no significant difference between the colour and toxin content of eggs laid in the presence or absence of the tracks of heterospecific predators (*C. septempunctata* larvae). Predators are known to respond negatively to even small increases in prey toxicity and unpalatability (Skelhorn & Rowe, 2006, 2009), and make strategic state-based decisions about prey consumption based on visual signals of prey toxicity (Barnett et al., 2012). For example, *C. septempunctata* larvae are more likely to consume *A. bipunctata* eggs if resource constrained (Hemptinne et al., 2000), the nutritional benefit of egg consumption outweighing the costs of toxin load under these circumstances (Barnett et al., 2007). It is therefore surprising that mothers do not increase egg toxin level in the presence of these egg predators. One explanation could be that in contrast to experimental systems using birds as predators, the toxicity of the *A. bipunctata* toxin ((-)-adaline) to *C. septempunctata* larvae is high and in some cases lethal (Hemptinne et al., 2000). The effect of modulating toxin level on predation risk, at this end of the toxicity spectrum, may therefore be negligible, and may not outweigh the physiological costs of investment. The toxicity of (-)-adaline might also explain the differential maternal response to *C.septempunctata* tracks compared to tracks of invasive *H.axyridis* larvae (Paul et al., 2015). The negative effects of the consumption of *A.bipunctata* eggs (and thereby (-)-adaline) being far greater on *C. septempunctata* larvae than on invasive *H. axyridis* larvae who are able to tolerate large quantities of (-)-adaline in their diet (Hemptinne et al., 2000; Katsanis et al., 2013; Ware et al., 2009).

***Paternal phenotype (‘Differential Allocation’)***

There was a positive correlation between paternal elytral luminance (as viewed by both predators and conspecific females) and egg luminance. Furthermore, eggs with higher luminance were also more saturated and had a greater toxin content. Therefore as predicted under a scenario of positive differential allocation (DA) brighter males fathered brighter and more toxic eggs (Horvathova et al., 2012; Ratikainen & Kokko, 2010). Positive DA is predicted to be a more common strategy than negative DA (Harris & Uller, 2009) and is a phenomenon well recorded in relation to visual signals of male quality in non-aposematic species. Female mallards (*Anas platyrhynchos*), for example, lay larger eggs with higher albumen lysozyme concentration, and have greater reproductive success after mating with more attractive males (Cunningham & Russell, 2000; Giraudeau et al., 2011; Sheppard et al., 2013).

Studies have shown that more conspicuous males are favoured by females in aposematic species of frogs and butterflies (Cummings & Crothers, 2013; Finkbeiner et al., 2014), while high levels of conspicuousness and toxicity leads to lower predation risk, and therefore potentially a better ‘quality’ of mate (Arenas et al., 2015). Therefore in some aposematic species male coloration in may be multifunctional, acting both as a warning signal to deter predators and a signal of male quality to females (Summers et al., 1999). Though correlative, our data suggest that such a scenario may be occurring in *A.bipunctata*, providing the first potential example of differential allocation in an aposematic species in response to male coloration. It is, however, important to point out that further work, for example the manipulation of male attractiveness, is needed to confirm whether the signal used by females to infer male quality is in fact visual and not another correlated factor, such as chemical signals (Kingma et al., 2009). The direct transfer of compounds from fathers that contribute to egg coloration, in either seminal fluid or via nuptial gifts, though unlikely in *A.bipunctata*, also cannot be ruled out, without the use of labelled chemicals for verification (Camarano et al., 2009).

***Maternal phenotype***

A positive relationship between maternal size and offspring size is pervasive across taxa (Lim et al., 2014; Roff, 2002), and similarly examples of positive correlations between maternal and offspring coloration and toxicity abound (e.g. Stynoski et al., 2014; Williams et al., 2011; Winters et al., 2014). Accordingly, large mothers and those with a high toxin concentration laid larger and more toxic eggs, respectively. A recent review of work on the relationship between maternal size and offspring size indicate that it is most likely maternal nutritional status or condition, as opposed to maternal size *per se*, that dictates per offspring investment and therefore offspring size (Rollinson & Rowe, 2016). The females used in this experiment were reared and maintained on *ad lib*. aphids, i.e. high resource availability, and therefore likely to have a generally good nutritional status. However, variation between individuals in size and toxin level, indicates that despite abundant resource availability there was some variation between mothers in condition, perhaps driven by phenotypic differences in resource acquisition or assimilation (Biro et al., 2014; Han et al., 2016).

The lack of a relationship between maternal colour and egg colour was surprising considering the association between size and toxin level of mothers and their eggs. A possible explanation lies in the link between maternal resource availability and signalling honesty. Theory predicts that when resources are abundant the conspicuousness and toxicity of aposematic species will cease to be positively correlated (Blount et al., 2009). Under high resource conditions individuals are able to increase their toxicity to a point where they can ward off attackers and therefore benefit from a decrease in their conspicuousness to predators (Leimar et al., 1986). In line with these theoretical predictions the females in this experiment, which were reared under high resource conditions, showed a negative relationship between toxicity and conspicuousness. Accordingly there may have been a much weaker link in these females between the availability of compounds responsible for egg warning coloration (carotenoids; Blount et al., 2012) and their ‘condition’, enabling investment in egg coloration independent of levels of maternal coloration.

***Summary***

Parental effects play a key role in determining the colour and toxin content of *A. bipunctata* eggs and therefore in contributing to aposematic signal variation in early life. Of the different subsets of parental effects investigated, it was maternal and paternal aposematic phenotype that had the biggest effect on egg traits, rather than maternal responses to offspring predators (AMEs). We found no evidence that mothers altered their investment in egg defences, either toxin level or warning coloration, in response to egg predation risk. Reproductive adjustment in response to egg predation risk occurred with respect to when and where eggs were laid rather than due to alterations in egg content. The positive correlation between aspects of paternal and egg luminance provides the first tentative evidence that differential allocation (DA) may occur in an aposematic species. These results open up the possibility that warning signals can impact male fitness, not just by influencing male survival and mating opportunities, but by their effects on the survival of the offspring a male sires. Maternal toxin concentration positively predicted the concentration of toxins in eggs, supporting previous work on the role of maternal effects in chemical defence and adding weight to the theory that maternal condition may mediate many of the maternal effects recorded in field and laboratory systems. Despite having different predators, with different sensory systems, in *A. bipunctata* there is a perpetuation of aspects of adult aposematic phenotype in their offspring.

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Figure 1. Potential routes via which offspring aposematic phenotype could be influenced by parental effects in an egg laying aposematic species: 1) female response to reliable cues of the offspring environment (‘Anticipatory Maternal Effects’ e.g. predator presence), 2) alteration of female investment in response to mate ‘quality’ (‘Differential Allocation’), and 3) a direct result of female aposematic phenotype. For illustration purposes the two spot ladybird *Adalia bipunctata* is used and orange eggs are more conspicuous and have a higher toxin levels than yellow eggs.

Figure 2. Egg toxin level ((-)-adaline concentration - ng/mg), luminance and saturation across days (day 0= pre treatment internal control and days 1-2 = treatments applied) and between the control (NN), conspecific (*A. bipunctata* larval tracks; CP), and heterospecific (*C. septempunctata* ; HP) treatment levels.

Figure 3. Relationships between a) egg volume and maternal mass, b) egg (-)-adaline concentration and maternal (-)-adaline concentration, c) egg luminance and paternal elytral luminance as viewed by a mate (ladybird ●) or predator (bird ●). Trend lines are GLMM model predictions with associated CIs (see Methods for model details).

Table 1. Effect of predation risk (conspecific and heterospecific) and female age and mass on female laying behaviour.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  |  | **Explanatory variables** | | |
|  |  | **Predation risk** | **Female mass (mg)** | **Female age (days)** |
| **Response variables** | **Latency to lay eggs** | **X2 2,101 = 8.22** | X2 1,99 = 0.01 | X2 1, 100 = 0.01 |
| ***P* = 0.016** | *P* = 0.95 | *P* = 0.91 |
| **Number of clusters** | **X2 2,100 = 6.78** | **X2 1,100 = 13.63** | X2 1,99 = 0.69 |
| ***P* = 0.03** | ***P* <0.01** | *P* = 0.41 |
| **Average cluster size** | X2 2, 101= 5.51 | X2 1, 99= 0.038 | X2 1, 100= 0.31 |
| *P* = 0.06 | *P* = 0.85 | *P* = 0.58 |
| **Total Eggs** | **F2, 99 = 6.49** | **F1, 99 = 35.93** | **F1, 99 = 6.71** |
| ***P* <0.01** | ***P* <0.001** | ***P* = 0.01** |
| **Single eggs laid** | X2 2, 101 = 4.59 | X2 1, 100 = 1.89 | X2 1, 99 = 1.26 |
| *P* = 0.13 | *P* = 0.17 | *P* = 0.26 |
| **Total number of single eggs** | X2 2, 101 = 2.84 | X2 1, 99 = 0.1 | X2 1, 100 = 0.25 |
| *P* = 0.24 | *P* = 0.75 | *P* = 0.62 |

Results are given as, test statistic with associated d.f. and *P*-value for model details see methods.

Table 2. Effect of predation risk (conspecific and heterospecific), experimental day, their interaction, and the effects of parental values of (-)-adaline, mass, luminance, and saturation on egg (-)-adaline, volume, luminance, and saturation respectively .

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  |  | **Explanatory variables** | | | | | | | | | | | | | |
|  |  |  | **Predation risk** | **Day** | **Egg (-)-adaline (ng/mg)** | **Predation risk \*Day** | **Maternal** | | | | **Paternal** | | | | | |
|  |  |  | **(-)-adaline (ng/mg)** | **Mass (mg)** | **Elytral Luminance (Bird vision)** | **Elytral Saturation (Bird vision)** | **(-)-adaline (ng/mg)** | **Mass (mg)** | **Elytral Luminance (Bird vision)** | **Elytral Saturation (Bird vision)** | **Elytral Luminance (Ladybird vision)** | **Elytral Saturation (Ladybird vision)** |
| **Response variables** | **Egg (-)-adaline (ng/mg)** |  | X12= 3.88 | X12= 0.76 | *NA* | X12= 0.71 | **X12= 39.34** | *NA* | *NA* | *NA* | X12= 3.75 | *NA* | *NA* | *NA* | *NA* | *NA* |
|  | *P* =0.33 | *P* =0.68 | *P* =0.95 | ***P* <0.001** | *P* =0.053 |
| **Egg volume (mm3)** |  | X12= 4.17 | X12= 1.97 | *NA* | X12= 1.42 | ***NA*** | X12= 7.77 | *NA* | *NA* | *NA* | X12= 1.19 | *NA* | *NA* | *NA* | *NA* |
|  | *P* = 0.12 | *P* = 0.37 | *P* = 0.84 | *P* <0.01 | *P* = 0.28 |
| **Egg luminance** |  | **X12= 19.32** | X12= 0.56 | **X12= 30.34** | X12= 2.27 | *NA* | *NA* | X12= 0.001 | *NA* | *NA* | *NA* | **X12= 11.40** | *NA* | **X12= 5.57** | *NA* |
|  | ***P* <0.001** | *P* = 0.76 | ***P* <0.001** | *P* = 0.69 | *P* = 0.98 | ***P* <0.001** | ***P* = 0.018** |
| **Egg saturation** |  | **X12= 11.69** | X12= 0.18 | **X12= 8.86** | X12= 1.95 | *NA* | *NA* | *NA* | X12= 0.01 | *NA* | *NA* | *NA* | X12= 2.57 | *NA* | X12= 0.38 |
|  | ***P* < 0.01** | *P* = 0.92 | ***P* < 0.01** | *P* = 0.74 | *P* = 0.98 | *P* = 0.11 | *P* = 0.54 |

The analysis for egg luminance and egg saturation were carried out twice once using paternal values of luminance and saturation obtained from models of bird vision and a second time using paternal values of luminance and saturation obtained from models of ladybird vision. The results reported are the parameter estimates and *P*-values for the mixed effects models outlined in the methods, calculated using likelihood ratio tests comparing models with and without the term in question (drop 1 method) (Crawley, 2014; Bates et al., 2015).