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Colour pattern variation affects predation in chrysomeline larvae

Eunice J. Tan [a], [*], Chris A. M. Reid [b], Mark A. Elgar [a]

[a] *School of BioSciences, University of Melbourne, Melbourne, Victoria, Australia*
[b] *Department of Entomology, Australian Museum, Sydney, NSW, Australia*

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Most animals are under strong selection to avoid predation, and several strategies have evolved in response to this selection. The developmental change in colour patterns of toxin-protected chrysomeline larvae provides a system to investigate the potential costs and benefits of conspicuous coloration development in animals. Field experiments in which artificial, palatable prey of various colour patterns were presented to wild avian predators confirmed that warning colours alone are not sufficient to deter predation, but that the spatial distribution of yellow and black coloration may be key to conferring a warning signal.

The risk of predation is pervasive, and numerous antipredator strategies have evolved apparently in response to this selection pressure. The diversity of protective colours and colour patterns are arguably among the most obvious of these strategies, which may reduce prey detection, confuse predators or warn predators of the prey's unpalatability (Cott, 1940; Ruxton, Sherratt, & Speed, 2004; Stevens, 2007). The latter is referred to as aposematism, a defensive mechanism in which a combination of conspicuousness and colour pattern is thought to signal the prey's unprofitability to visual predators (Cott, 1940). Successful aposematic signals therefore benefit both the sender and the receiver, as the former benefits from reduced mortality, while the latter avoids consuming an unpalatable prey (Ruxton et al., 2004). In addition, the strength of the warning signal can also be an indication of the level of toxicity (María Arenas, Walter, & Stevens, 2015).

Investigations of the reaction of predators to aposematism reveal that visual predators associate particular colours (Aronsson & Gamberale-Stille, 2009; Bowdish & Bultman, 1993; Massuda & Trigo, 2009; Sandre, Stevens, and Mappes, 2010; Schuler & Hesse, 1985) and conspicuousness (Alatalo & Mappes, 1996; Tullberg, Leimar, & Stille, 2000) with danger. The mechanism of sensing conspicuousness is less clearly understood, despite evidence that animals are capable of discerning remarkably small differences within mimetic systems. For example, experiments involving artificially manipulated prey with variable ‘eyespots’ suggest that the conspicuousness of the pattern is important in promoting avoidance behaviour in predators (Stevens, Hardman, & Stubbins, 2008). However, other studies (De Bona, Valkonen, López-Sepulcre, & Mappes, 2015; Merilaita et al., 2011) support the antipredator function of eyespots regardless of conspicuousness, and the background may influence the deterring function of eyespots (Vallin, Dimitrova, Kodandaramaiah, & Merilaita, 2011).

While studies of mimicry provide insights into the importance of colour and pattern for both mimic and aposematic model, it is not clear whether the specific patterns on the model derive from coevolutionary processes between the model and its predator, or the model and the mimic. Other studies (Aronsson & Gamberale-Stille, 2009; Exnerova et al., 2008) suggest that the colour patterns of an animal are not important in predator avoidance learning: naïve great tits, *Parus major*, learning to recognize aposematic heteropterans focus on colour and not pattern.

Very dark or black colour, caused by the pigment melanin, is often used in aposematic coloration (Lindstedt, Lindstrom, & Mappes, 2009; Majerus, 1998; Ojala, Lindström, & Mappes, 2007). However, melanization can be physiologically costly. The quality of diet affects melanin-based coloration in butterfly wings (Talloen, Van Dyck, & Lens, 2004) and in the wing spots of damselflies (Hooper, Tsubaki, & Siva-Jothy, 1999). The phenoloxidase cascade that is responsible for insect immunological defences (Rowley, Ratcliffe, Leonard, Richards, & Renwrantz, 1986) is also involved in the synthesis and deposition of melanin (Nijhout, 1991). The immunological response

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[*] Correspondence: E. J. Tan, School of BioSciences, University of Melbourne, Melbourne, Victoria 3010, Australia.
E-mail address: eunice.t@gmail.com (E. J. Tan).

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is also related to the extent of melanization of insects (Nappi, Vass, Frey, & Carton, 1995; Siva–Jothy, 2000). Melanization in lepidopterans (Hegna, Nokelainen, Hegna, & Mappes, 2013; Lindstedt et al., 2009; Watt, 1968) and beetles (Benham, Lonsdale, & Muggleton, 1974; Brakefield & Willmer, 1985) shows that thermoregulatory benefits can lead to increased fitness (also see review in Majerus, 1998). As phytophagous insects, melanin production in chrysomeline beetles may be limited due to their nitrogen intake because the presence of nitrogen is essential to the production of the precursors to melanin (see review by Mattson, 1980).

The adults and larvae of numerous chrysomeline beetles are conspicuously patterned and chemically defended, and therefore described as aposematically coloured (Fig. 1). The beetle larvae typically feed diurnally on leaf lamina of the host (Crowson, 1981; Matthews & Reid, 2002) and can be vulnerable to attack by predators and parasitoids while exposed on their host plants (Evans & Hook, 1986; Matthews & Reid, 2002). When physically disturbed, individuals release defensive secretions such as benzaldehyde and hydrocyanic acid from specialized exocrine glands in the pronotum and elytra (Crowson, 1981; Deroe & Pasteels, 1982; Pasteels & Rowell-Rahier, 1989; Pasteels, Rowell-Rahier, Braekman, & Daloze, 1984). Among the Chrysomelinae, adults in the genus *Paropsis* are characterized by extended elytral margins, protecting the appendages (Reid, 2006). *Paropsis atomaria* is typical of this group, and can be commonly found on *Eucalyptus* trees from spring to late summer (Carne, 1966).

The soft-bodied larvae of this group have a pair of eversible secretory glands at the abdominal apex (Pasteels & Rowell-Rahier, 1989) and contrasting colour patterns based on yellow and black that vary according to instar (Carne, 1966). The second- and third-instar larvae of *P. atomaria* are yellow with black head and abdominal apex. The fourth instar has, in addition, a black prothorax and three black dorsal and lateral abdominal stripes. Yellow and black coloration is typical of many aposematic systems (Cott, 1940; Hegna et al., 2013; Kauppinen & Mappes, 2003; María Arenas et al., 2015; Stevens & Ruxton, 2012). In another system, Ojala et al. (2007) showed that the relative size of the warning signal of lepidopteran larvae increases with larval age and nutrition. Other species of insect larvae are also known to undergo ontogenetic changes in colour patterns, and this may be attributed to metabolic constraints that early instar larvae face (Grant, 2007). The ontogenetic change in colour patterns of toxin-protected chrysomeline larvae thus allows us to investigate the evolutionary process of conspicuous coloration development in animals.

Figure 1. *Paropsis atomaria* larvae in the field. A fourth-instar individual with lateral black lines is at rest, and two third-instar larvae are present, with the one on the lower left feeding. The white line represents 10 mm.

Accordingly, the costs associated with any life history change in larval colour pattern may be balanced against the potential protective benefits, which are likely to change as the larvae becomes larger and therefore more conspicuous. Stevens, Castor-Perry, and Price (2009) highlighted the importance of a field setting, as the prey will face predation risk from several species that may have varying perceptual abilities and foraging behaviour. This study was designed to examine the reliability of the larval colour pattern as a signal of unprofitability to potential predators, by conducting field experiments in which we introduced novel variations of colour patterns of the late-instar larvae. Through life history data collection and a series of experiments using models made of flour, we asked: (1) how do the black colour patterns on the larvae change with larval age, and (2) is the life history change in larval colour pattern crucial for conferring protective advantages? In particular, we predicted that models that deviate from the natural pattern of larvae would suffer greater predation because of the optimal coloration of the larvae. We also predicted that greater investment in black coloration would lead to lower disturbance of models because of the greater aposematic signal conferred.

METHODS

Larval Colour Patterns

Paropsis atomaria adults were collected from various locations around Victoria, Australia and maintained in the laboratory with natural daylight conditions and temperatures between 15 and 25 °C. Egg clusters from these adults were collected and the hatched larvae were reared in the laboratory. Larvae from different egg clusters were housed separately and supplied with fresh young *Eucalyptus* leaves ad libitum. Up to three individuals from the same egg cluster were used for the colour pattern analysis. Second- and third-instar measurements are henceforth collectively termed ‘early instar’, as they are similar in colour pattern and differ from fourth-instar larvae (see above). From 20 egg clusters, 23 early instar larvae and 18 fourth-instar larvae were photographed for the analysis. No individual was used more than once for the entire analysis.

Larvae were placed on a white Perspex background with a scale to allow dorsal and lateral views of the larvae to be photographed under the same illumination in laboratory conditions. Digital photographs of the larvae at dorsal and lateral views were then taken using a Sony T77 digital camera in close-up macro mode and the images analysed with ImageJ (Schneider, Rasband, & Eliceiri, 2012), using point-to-point measurements. For the measurement of areas of yellow or black colour in ImageJ, the wand tool was used with legacy mode and a tolerance of 20. The obtained values were then analysed using R (R Development Core Team, 2014) in an initial analysis of covariance, to determine whether there was an interaction term between total larval area and instar in the black area of larvae. Log-transformed values of the total larval area and black area were used for the analyses. As there was a strongly significant interaction term between larval length and instar for the dorsal view (see below), we then performed a linear regression of the black area against total larval area for early and fourth instar separately.

Predator Disturbance and Larval Model Patterns

Prey models are routinely used in experiments to investigate the effect of various visual signals (Cuthill et al., 2005; Edmunds &