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# The effect of signal appearance and distance on detection risk in an aposematic butterfly larva (*Parnassius apollo*)

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Aposematic coloration commonly involves patterns with contrasting colours. The early larva of *Parnassius apollo* is uniformly black, but the later instars develop two rows of dorsal orange spots. We tested the effect of these orange markings on the conspicuousness of the last-instar larva, by manipulating larval coloration in photographs from a natural habitat and measuring how fast human subjects could discover the larva on a touch screen. In the first experiment we compared the detectability of the natural phenotype with that of manipulated uniformly black and uniformly orange variants at different distances. In the second experiment with another set of photographs we added manipulations involving enlarged and reduced spot sizes. Generally, detection time increased with distance, and decreased with the proportion of orange in the coloration. The uniformly black larvae were surprisingly hard to detect even at the closest distances, suggesting that the young black instar has an efficient camouflage. Furthermore, even a small amount of orange colour increased the conspicuousness of the larva considerably, indicating that the orange markings are costly in terms of detectability. Importantly, as the increase in detection time over distances was larger for the natural coloration than for the orange coloration, we suggest that the natural coloration may involve a distance-dependent switch from conspicuousness to camouflage with increasing distance. Thus, even though the orange markings most probably have a signalling function, the coloration is not maximized for either crypsis or conspicuousness.



*Keywords*: apollo butterfly; aposematism; conspicuousness; crypsis; detection risk; distance dependence; *Parnassius apollo*

Prey animals have evolved a variety of defence strategies. In protective coloration there are two widespread categories: cryptic coloration or camouflage, which decreases the risk of detection, and warning coloration or aposematism, which is used to signal unprofitability (Cott 1940; Edmunds 1974; Evans & Schmidt 1990; Ruxton et al. 2004). Aposematic coloration typically includes conspicuous colours that deviate from those of the natural background of the animal, such as red, orange and yellow. Moreover, such colours often occur together with black, producing a pattern with high contrast.

Aposematic coloration has conventionally been considered to lie at the opposite extreme to crypsis on the conspicuousness continuum. Aposematism involves the cost of a high detection risk (Gittleman & Harvey 1980; Lindström et al. 1999; Riipi et al. 2001; Summers & Clough 2001; Husak et al. 2006) but also a highly conspicuous signal facilitates recognition and avoidance by predators better than less conspicuous signals (e.g. Gamberale-Stille & Tullberg 1999; Lindström et al. 1999; Ruxton et al. 2004). For instance, larger aposematic insects or aggregations produce stronger avoidance than smaller or solitary ones with the same coloration (Gamberale & Tullberg 1996, 1998) and artificial prey with large markings produce stronger avoidance than those with smaller markings (Forsman & Merilaita 1999; Lindström et al. 1999). Therefore, it has often been assumed that aposematic species should maximize the detectability of their warning signal to benefit from the avoidance response it produces.

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Several lines of thought have questioned whether aposematic animals maximize conspicuousness. First, there should be no benefit as such in being detected by a predator, and therefore it should suffice to be recognized as unprofitable when discovered. There is evidence that aposematic prey can evolve to be distinctive from edible prey without simultaneously becoming strikingly different from their visual habitat (i.e. distinctiveness need not be coupled with conspicuousness; Sherratt & Beatty 2003; Merilaita & Ruxton 2007). The European viper, *Vipera berus*, with its zigzag pattern is thought to represent an example of coloration that is distinct but not highly conspicuous (Wüster et al. 2004). Also, the most avoided colours are not necessarily the most contrasting to the background (Gamberale-Stille & Guilford 2003).

The second line of thought questioning maximized conspicuousness is related to the fact that most prey animals have several different predators that may respond differently to the specific prey defence (Exnerová et al. 2003; Endler & Mappes 2004). According to this line of thought a specific protective coloration should be regarded as a compromise between different selective demands from various predators, producing colorations that may combine camouflage and warning signalling functions.

The third line of thought is that the function of a coloration may be related to the distance from the observer. Thus, some animals may be cryptic from a distance but signalling when seen close up (Papageorgis 1975; Rothschild 1975; Järvi et al. 1981) and there is support for this idea in experiments using image-based detection by human subjects (Tullberg et al. 2005), which showed that the colour pattern of the distasteful larva of the swallowtail butterfly, *Papilio machaon*, has not been maximized for conspicuousness to viewers at longer distances.

The relation between prey conspicuousness and predator avoidance response in nature is far from understood and there is a lack of empirical studies of the conspicuousness of warning colours from the field using natural backgrounds. This is probably because of the problems involved in measuring how conspicuous the aposematic prey are to potential predators, and controlling for distance and predator avoidance of detected prey. The main purpose of the present study was to investigate the detection cost involved as the size of the markings of the signalling pattern of an aposematic species are gradually varied.

We investigated the optimization of conspicuousness of an aposematic animal against its natural background and how this optimization is affected by distance. We specifically investigated the cost in terms of detectability of the orange markings on the black body of the last-instar larva of the apollo butterfly, *Parnassius apollo*. First, we tested the cost of spots by comparing the detectability of the natural coloration with that of uniform black and uniform orange. Second, we tested whether the last-instar larval coloration is maximally conspicuous by comparing it with patterns including larger and smaller spots. Third, we tested how distance affects detectability by comparing detectability of different colour variants in images taken at different distances. To overcome the practical problems involved in measuring detectability and controlling for distance, we used a technique introduced by Tullberg et al. (2005), based on photographic images of prey in their natural habitat, presented to human 'predators', whose task is to search for the prey on a touch-sensitive screen. Tullberg et al. (2005) concluded that the function of the coloration of the *P. machaon* larva is distance dependent. Although highly conspicuous at close range, it is not maximally conspicuous but rather difficult to detect at a longer range. In the present study we investigated another species with a different appearance and habitat to those in Tullberg et al. (2005), to increase our knowledge about the generality of this phenomenon. Moreover, we gradually manipulated the coloration to gain a better understanding of how change in appearance influences detectability at different distances.

## METHODS

### Biology of *Parnassius apollo*

*Parnassius apollo* is a member of the swallowtail family, Papilionidae, a taxon that is easily recognized as most of them have bright coloration (generally assumed to be aposematic) and that includes some of the largest butterflies (Powell 2003). *Parnassius apollo* is distributed in mountainous areas from Spain to southern Fennoscandia and eastwards to central Asia (Tolman & Lewington 1997). The early instars are velvet black with fine setae, but gradually the protuberances on the side of each segment develop a distinct colour, varying from yellow to red according to locality (Tolman & Lewington 1997). The larvae are early spring feeders and sun-basking behaviour is common (Stamp & Bowers 1990), so the black coloration may be important for heat absorbance.

*Parnassius apollo* is oligophagous and the larvae feed mainly on *Sedum* spp. Many lepidopteran species sequester toxins, for example alkaloids, from their host plant as a secondary defence (Nishida 2002), but it is not clear whether the larva of *P. apollo* sequesters any adverse substances from its host plants. However, in trials with naïve chicks the larvae were avoided, either initially or after a few attacks (G. Gamberale-Stille & B.S. Tullberg, unpublished data).

The main distribution of *P. apollo* in Sweden is on the island of Gotland (which has its own subspecies), and along the Baltic coast including the Stockholm archipelago. One of these populations is on the island of Munkö (59°14′05N, 18°43′04E), a nature reserve, where most larvae are found in the sun-exposed southwestern part. On these limestone rocks vegetation is sparse, mainly consisting of mosses, lichens and grasses, and the soil layer is thin. Common herbs are *Geranium sanguineum*, *Allium schoenoprasum*, *Sedum album* and *Sedum telephium*, and the only taller plants are an occasional juniper, *Juniperus communis*, and dog rose, *Rosa dumalis*. The larvae occur singly in the vegetation (Fred & Brommer 2003) and are most often found sun basking in the open, or feeding on *S. telephium* and occasionally on *S. album*.

## Photography and Image Manipulations

The principal methods of photography, image manipulation and presentation of the images to the subjects are similar to those described in Tullberg et al. (2005). The same methods were used in both experiments 1 and 2 with only slight modifications. We took the photographs in June 2005 on the island of Munkö (see above), with a 5.0 megapixel digital camera (Canon Power Shot G5) using the shortest focal length of its zoom lens (corresponding to 35 mm focal length in 135 film format cameras). Last-instar larvae were photographed as they were found in their natural habitat, sun basking and thus immobile. Photographs were taken at an approximately 45° angle from above so that the whole photograph was filled with the background habitat (below the horizon). Photographs were taken so that the larva was placed not in the centre but in the middle of one of the four quadrants of an image. The distances were selected within a range where the sizes of the natural larvae were noticeably different but still possible to detect by a human observer, which in this case was from 20 to 80 cm. Accordingly, in the more distant photographs more was shown of the background consisting of a mixture of bare rock, soil, mosses, lichens, grasses and herbs as described above. To control for exact larval position and habitat, we selected images of two different larvae, one for each experiment. The images were selected with regard to photographic quality and how well they represented the species and its habitat.

### *Experiment 1*

We used four photographs of the same larva (3.5 cm body length) taken at four distances: 20, 40, 60 and 80 cm. Because of the wide-angle lens, the distances in the images appeared longer to the human eye, and are thus referred to as distances 1, 2, 3 and 4, respectively. These distances correspond to larvae measuring 4.5, 3, 1.5 and 1 cm in length in the 17-inch (36 × 45 cm) images used in the experiment.

*Image manipulation.* The photographs with the natural larva were manipulated in two ways to create uniformly black and uniformly orange larvae (as in Fig. 1a, e). Accordingly, in this experiment subjects were presented with three larval colorations, black, natural and orange, at four distances. The photographs were manipulated with the image-editing software Adobe PhotoShop 7.0 (Adobe Systems Inc., San Jose, CA, U.S.A.).

In the image manipulations we used only colours that are naturally present in the *P. apollo* larva. To produce the black larva, we covered the orange spots with pixels copied from the nearest surrounding black areas. The making of a uniformly orange larva was more complicated because the larva would have lost its natural texture in the photograph if it had simply been covered by pixels copied from the orange spots, and would have appeared highly unrealistic and consequently easily detected in the image. Thus, for the orange larvae we increased the brightness to change the area of the black colour to a light grey shade that maintained the texture. Then, we increased the balance for the red and yellow colours and decreased the hue until the originally black area had a similar colour to the orange spots. Hence, an orange larva was created without losing the original texture (Fig. 1e).

### *Experiment 2*

In experiment 2 we used one photograph of a larva (4.0 cm body length) taken at a distance of 80 cm. The photograph was used to create five different images: one with the natural black and orange coloration and four with manipulated variants (Fig. 1). To produce two closer views of the larva than in the original photograph, we enlarged the five images to 200% and 150% of the original photograph. Thus, in experiment 2 there were three distances for each image, referred to as distances 1 (200%), 2 (150%) and 3 (100%). These distances corresponded to larvae measuring 3.5, 2 and 1.5 cm in the images used in the experiment (and thus the size categories of experiments 1 and 2 are not directly comparable).

*Image manipulation.* The photographs were manipulated with the image-editing software GIMP 2.2.13 (Free Software Foundation Inc., Boston, MA, U.S.A.). The uniform variants were created in the same manner as in experiment 1. In addition to the black and orange variants, we also included one coloration with the area of the orange spots enlarged by 30% and another with the spot area reduced by 30%. The percentage of orange was chosen in order not to make the enlarged spots too big and overlap, and hence reduce the number of spots. For the enlargement, we covered parts of the surrounding black area with orange pixels from the spots, and for the reduction, the orange spots were partly covered with pixels copied from the surrounding black areas. Thus we ended up with five images representing an order of increasing proportion of orange in the larval coloration; black, small spots, natural, large spots and orange (Fig. 1), at three distances.

## Humans as Predators

Many studies on protective coloration are based on human colour perception (e.g. Knill & Allen 1995; Glanville & Allen 1997; Summers & Clough 2001; Beatty et al. 2004; McGuire et al. 2006), which differs from the visual systems of natural predators such as birds. Birds perceive colours differently from us and many species are sensitive to ultraviolet (UV) wavelengths (reviewed in Bennett & Cuthill 1994), which are invisible to humans. However, spectrophotometric measurements of the black and orange body areas on *P. apollo* larvae show no reflectance peaks in the UV range (unpublished data).

## Image Presentation

In both experiments the images were presented on a touch screen to volunteer human subjects, high-school students, 16–19 years of age. Each subject received written and oral instructions before the experiment. These included information on the general purpose of the experiment, but not about the specific hypothesis concerning

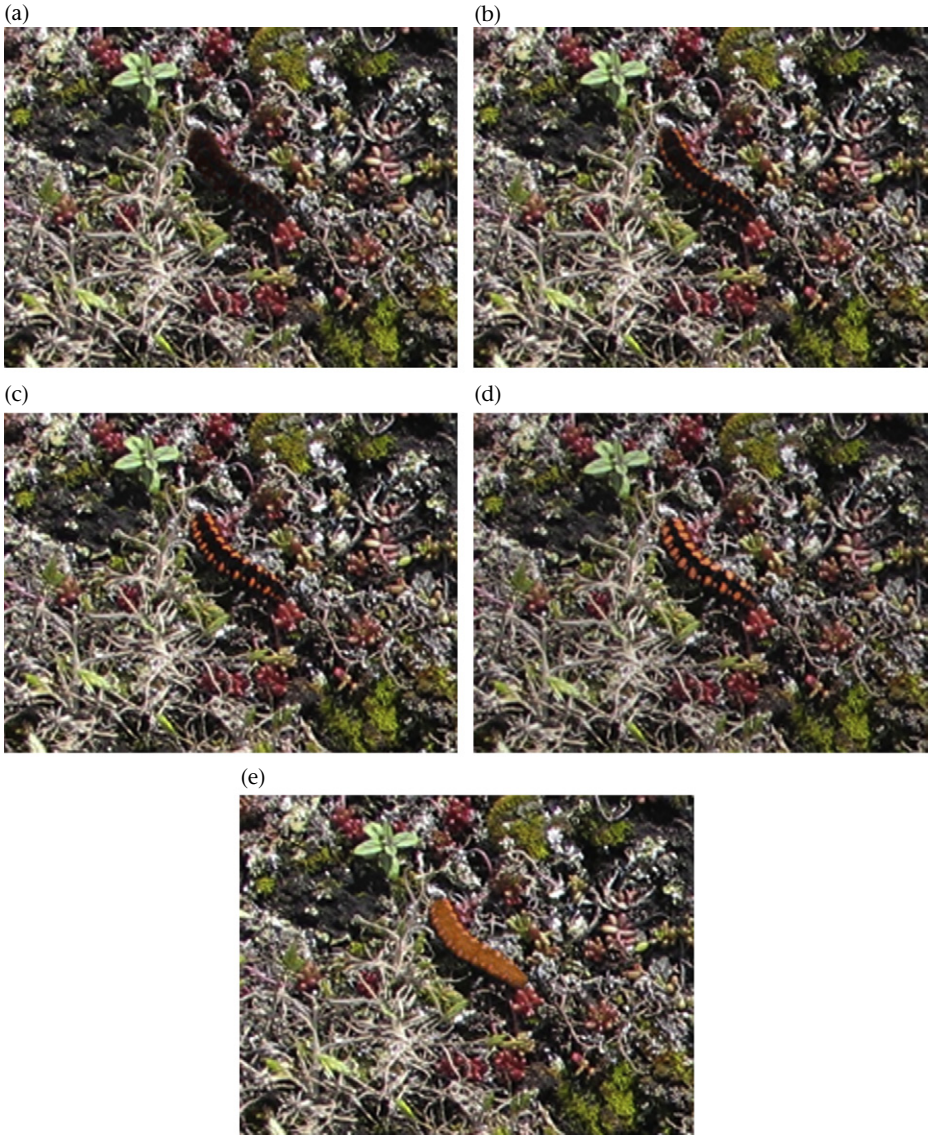


**Figure 1.** Images (enlarged details of original) of the natural and the four manipulations of *P. apollo* larval coloration used in experiment 2. (a) Black, (b) small spots, (c) natural, (d) large spots and (e) orange. In experiment 1, colorations (a), (c) and (e) were used with similar images.

detectability and signal size. The subject was instructed to try to find one larva on the screen and touch it as soon as he/she detected it. In experiment 1 the participants were simply informed that the object to find was a larva and that it could be of any size and coloration. In experiment 2, the subjects were allowed to look at an illustration with 23 different butterfly larvae chosen from different taxonomic groups, varying in size and colour (modified from Carter & Hargreaves 1986), to give them an idea of the general appearance and variability of butterfly larvae.

Each subject was presented with one image only, shown on a 17″ touch-sensitive LCD screen (NEC AccuSync LCD 52 VM, Display Solutions, Ltd., Tokyo, Japan). The experiment was conducted in a dark room to avoid reflections on the screen. The subjects were tested one at the time, in random order, under supervision of one person (T.B. or B.S.T.) during the presentation. A purpose-written program, responding only to touches on the larva, recorded the time elapsed from start to detection. The subject started the image presentation by following instructions on the screen. The maximum presentation time of the image was 120 s after which the image disappeared, had the larva not been detected before that. The computer program presented the images so that all combinations of larval coloration and distance were shown approximately the same number of times in each experiment (experiment 1: $N = 10–11$; experiment 2: $N = 11–13$). In experiment 1 the series of 12 images was shown to 124 students at Norra Real Gymnasium in Stockholm and in experiment 2 the series of 15 images was shown to 184 students at Kungsholmens Gymnasium in Stockholm.

## Statistical Analyses

Because our data on detection time contained censored values, we used the survival analysis in STATA 9.0 (StatacorpLP, College Station, TX, U.S.A.). To compare detection time between colorations and distances we used

the Cox proportional hazards model (Cox regression). We used the log-rank test for trend to check for differences between distances within colorations, as well as for trends between colorations that had been ranked with respect to signal size (amount of orange) within a given distance (Klein & Moeschberger 2003).

## RESULTS

### Experiment 1

Detection time differed between the three colour variants of the *P. apollo* larva. The orange coloration was generally easiest to detect and the black coloration most difficult (Fig. 2). For each colour variant, detection time increased significantly with distance (Fig. 2).

A Cox regression disclosed differences in detection time between the three colorations at distance 1. In comparison to the natural coloration, detection time of the black coloration was significantly longer ($z_2 = 3.31$, $P = 0.001$), and the detection time for the orange coloration was significantly shorter ($z_2 = -2.01$, $P = 0.044$). Thus, the larva became more conspicuous or more cryptic depending on the amount of orange colour in its pattern.

The detection time of the black coloration had a sharp rise after distance 1 (mean = 46.52 s) and approached the upper time limit even at distance 2 (mean = 105.39 s). However, the overall change in detectability of the natural and orange colorations increased continuously at a lower rate over the distances used (Fig. 2). To investigate whether the natural coloration had a distance-dependent function by being signalling at a short distance and increasingly cryptic at longer distances, we compared the change in detection time from the closest to the longest distance for the natural and orange colorations (Fig. 2). We did not compare the change in detection time between the natural and the black coloration over distances, as their detection times differed considerably even at the shortest distance (see above; Fig. 2a, b). The detection time for the natural coloration increased almost seven times (distance 1: mean = 3.68 s; distance 4: mean = 25.38 s), whereas the increase for the orange coloration was only threefold (distance 1: mean = 2.25 s; distance 4: mean = 6.68 s). Thus, the detectability decreased at a considerably higher rate with distance for the natural than for the orange coloration.

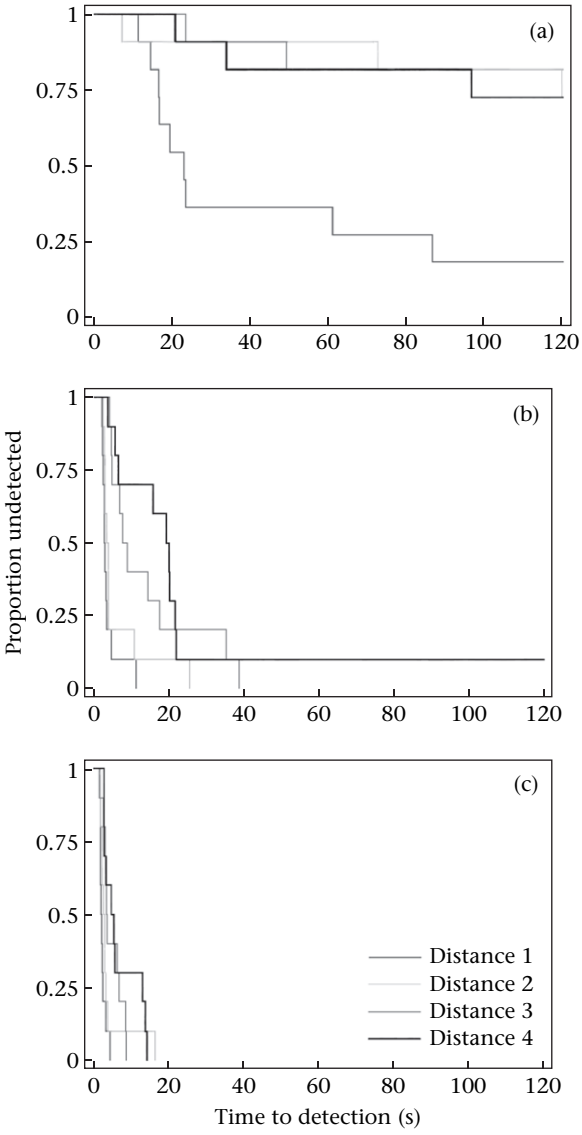


**Figure 2.** Experiment 1. Detection time (s) for the natural coloration and for the two uniform manipulations of *P. apollo* larval colorations at four distances. The proportion of undetected larvae illustrates the time to detection. (a) Black ($\chi^2_1 = 8.88$, $P = 0.003$), (b) natural ($\chi^2_1 = 78.74$, $P < 0.001$) and (c) orange ($\chi^2_1 = 10.19$, $P = 0.001$). Statistics for each larval coloration refer to the log-rank test for trend of the dependence of detection time on distance.

### Experiment 2

The five colour variants in experiment 2 also showed noticeable variation in detection time between the colorations (Fig. 3). Detection time increased significantly with distance for three of the five colorations, small spots, natural and orange, but not for the black and large spots (Fig. 3). There was a distinct trend showing that detectability increased with the proportion of orange in the coloration. Thus, when the manipulations were ordered with respect to proportional amount of orange colour, from black, to small spots, natural, and large spots, ending with orange, there was a significant trend in decreasing detection time for each of the three distances (log-rank test for trend: distance 1: $\chi^2_4 = 35.05$, $P < 0.001$; distance 2: $\chi^2_4 = 47.31$, $P < 0.001$ distance 3: $\chi^2_4 = 66.81$, $P < 0.001$). Accordingly, as the proportion of orange increased the larval coloration became more conspicuous.

To be able to compare the results with those from experiment 1, we tested in experiment 2 whether the detection time of the natural coloration differed from the two uniform colorations at distance 1. In comparison to the natural coloration, detection time was significantly longer for the black coloration (Cox regression: $z_1 = 4.92$, $P < 0.001$), and significantly shorter for the orange coloration ($z_1 = -2.60$, $P = 0.009$).

The black coloration was difficult to detect even at distance 1 (mean = 71.68 s), and distance did not

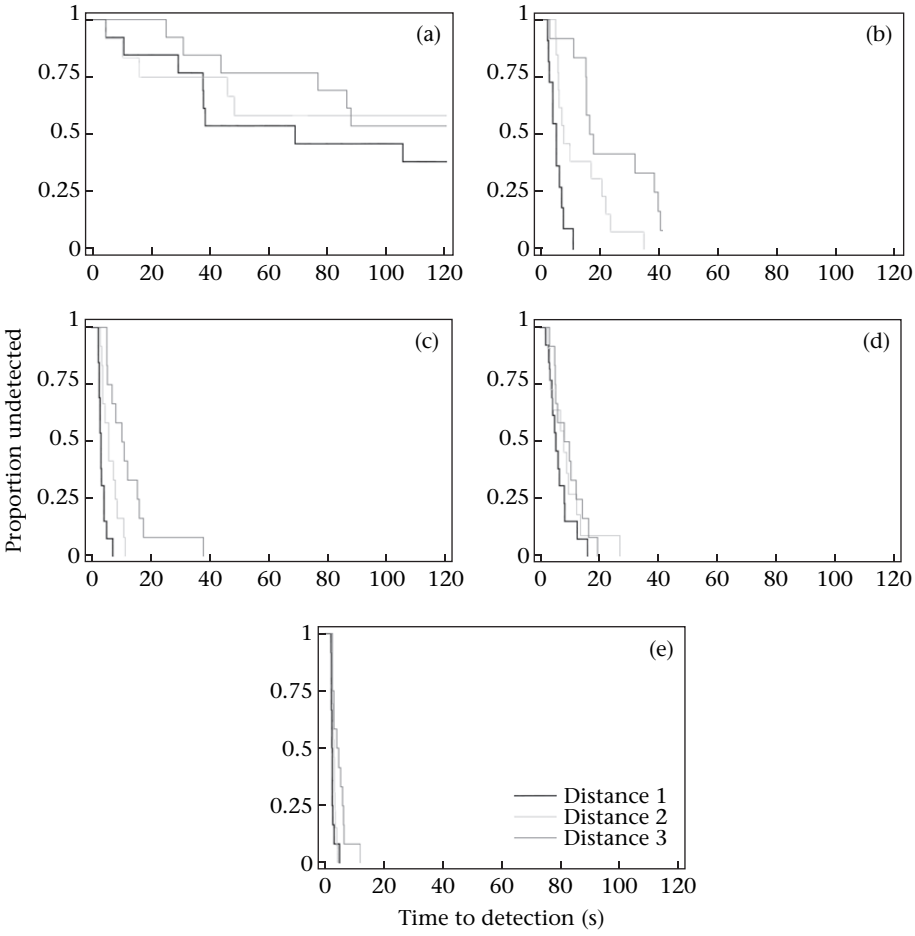


**Figure 3.** Experiment 2. Detection time (s) for the natural coloration and for the four manipulations of *P. apollo* larval colorations at three distances. The proportion of undetected larvae illustrates the time to detection. (a) Black ($\chi^2_1 = 1.09$, $P = 0.297$), (b) small spots ($\chi^2_1 = 23.03$, $P < 0.001$), (c) natural ($\chi^2_1 = 29.86$, $P < 0.001$), (d) large spots ($\chi^2_1 = 1.57$, $P = 0.455$) and (e) orange ($\chi^2_1 = 9.18$, $P = 0.002$). Statistics for each larval coloration refer to the log-rank test for trend of the dependence of detection time on distance.

significantly affect detectability for this coloration (Fig. 3a). As in experiment 1, we compared the change in detection time from the closest to the longest distance between the natural and the orange coloration to check for a distance-dependent function in the natural coloration. Again, we did not test the change in detection time between the natural and the black coloration over distances as there was a significant difference between the black and natural coloration even at distance 1, and distance did not affect the detectability of the black coloration. The detection time for the natural coloration increased almost four times (distance 1: mean = 3.36 s; distance 3: mean =12.43 s), whereas the detection time for the orange coloration did not even double (distance 1: mean = 2.43 s; distance 3: mean = 4.59 s). Thus, also in this experiment, detectability decreased at a higher rate with distance for the natural than for the orange coloration.

## DISCUSSION

The experiments in this study show that the detectability of the larva of *P. apollo* is affected by both coloration and distance to the viewer. Moreover, we found that the relative change in detectability with increasing distance differed between colorations. The orange markings significantly increased larval conspicuousness, which implies that *P. apollo* is better camouflaged in its first larval instar, which lack these markings. Moreover, the natural coloration could be manipulated to be more conspicuous in both experiments and this indicates, in accordance with a study on the *P. machaon* larva (Tullberg et al. 2005), that the function of the coloration is not maximized with regard to conspicuousness.

In both experiments there was a striking difference in detectability between the uniformly black and all the other colorations. This strongly concealing effect of black is somewhat surprising, because the background was not predominantly black but also consisted of other colours, such as grey lichen, green mosses and dry grass (Fig. 1). Experiment 2 shows that even a small proportion of orange (small spots) amplified the conspicuousness of the larva significantly (Fig. 3). On the other hand, had the apollo larva evolved towards maximal camouflage, then we would expect it to be black rather than orange-spotted.

The black larva was difficult to detect even at the closest experimental distance and remained so at the longer distances, whereas detectability of the other colorations decreased continuously with distance (Figs 2, 3). In both experiments the detection time of the natural coloration, as well as that of the manipulated orange coloration, increased significantly with distance, but this change in detectability was more pronounced for the natural coloration. This indicates that the protective coloration of the last-instar *P. apollo* larva may involve a distance-dependent combination of functions, that is, signalling close up and crypsis at longer distances. These results are in accordance with the findings on the *P. machaon* larva (Tullberg et al. 2005).

In this study, each experiment was based on images of a single larva to ensure that only coloration or size of the larva and no other factor varied between the images. Although one might argue that this limits the generality of our results, the similarity in results between the two experiments does indeed support the idea that our results describe a general pattern in this species.

Coloration may also have functions not related to predation. Thus, the black colour of the *P. apollo* larva may not only be selected for because of its concealing properties. Black colour absorbs heat better than other colours (Watt 1968; Porter 1984; Forsman 2000; Hazel 2002; Nice & Fordyce 2006) and sun-basking behaviour occurs at all stages of many lepidopteran larvae (Stamp & Bowers 1990) including *P. apollo*. So the black colour in the apollo larva may have a thermoregulatory function. There is also a growing body of evidence that melanin is closely linked to the immune system and enhances disease resistance (reviewed in Wilson et al. 2001).

If we assume that black colour is optimal from the point of concealment, thermoregulation or immunology, what is the function of the orange markings? We find it highly likely that they have a warning function, and that this function is certainly associated with a cost in detectability as our study shows. This does not exclude the possibility that the black colour of the larva also improves its warning function (Vaughan 1983; Roper & Cook 1989), although at little or no cost in terms of detectability.

Many lepidopteran larvae, including *P. apollo*, change colour during development (Booth 1990), and it is common to find crypsis in the early instars and aposematic colorations in later instars (e.g. Clark & Faeth 1997; Nylin et al. 2001; Inouye & Johnson 2005; Grant 2007). Such colour change may be related to a shift in cost–benefit ratio of changes in defence strategies (e.g. Stamp 1986) or foraging behaviour during development (Reavey 1993, and references therein). The smallest instars may have no or too weak a defence to make signalling advantageous, and there may be an increase in defence with larval age as in the comma butterfly larva, *Polygonia c-album* (e.g. Nylin et al. 2001). Thus, advertising unprofitability may be more advantageous in the later instars. In addition, as the signal strength is proportional to body size, warning signals may be ineffective in small larvae (Mänd et al. 2007). Even if small larvae are defended, their vulnerability in a predator attack may be too high making the signal too costly. Furthermore, the change in protective coloration may also be related to an alteration in predator species with increasing size (Dempster 1967; Bernays & Montllor 1989).

To summarize, this study provides evidence that both coloration and distance are important factors that affect the detectability of the *P. apollo* larva. The uniformly black coloration is highly cryptic in the natural environment and the orange markings increase conspicuousness, suggesting a signalling function. However, we conclude that the *P. apollo* larva is not maximally conspicuous and the results suggest that the natural coloration involves a distance-dependent dual function of warning signal and camouflage.

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