

Seasonal ontogenetic colour plasticity in the adult striated shieldbug *Graphosoma lineatum* (Heteroptera) and its effect on detectability

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Received: 3 December 2007 / Revised: 13 February 2008 / Accepted: 20 February 2008
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Abstract Camouflage and warning colouration are two important forms of protective colouration. We have studied the detectability of two seasonal colourations in the aposematic striated shieldbug, *Graphosoma lineatum*. The typical colouration of this insect is red and black, which is also the colouration of the reproductive post-hibernation bugs in our study area in south central Sweden. However, the majority of newly eclosed adults in late summer exhibit a ‘pale’ (light brownish, non-red) and black striation, and these bugs appear quite cryptic to the human eye when sitting on the dried stems and umbels of their host plants. In experiments using photographs of prey in the late-summer habitat shown on a computer screen, we compared the time to detection by human subjects of bugs, which had been manipulated to show either of the two typical seasonal colourations. Time to detection was significantly longer for the pale and black than for the red-and-black striation in images with the bug photographed at two different distances. This indicates that the pale pre-hibernation striation may have a cryptic function. In a separate experiment, we tested detectability of striated and non-striated manipulations of bug pre-hibernation colouration against the late-summer background, and found that time to detection was significantly longer for the striated bugs. We discuss potential functional explanations for the seasonal ontogenetic colour plasticity and suggest that the epidermal pale colour in the late summer provides a benefit of increased camouflage.

Keywords Aposematism · Conspicuousness · Camouflage · Crypsis · Detection experiments · Natural background

There are two broad categories of protective colouration in prey animals: cryptic colouration or camouflage, which decreases the risk of detection, and warning or aposematic colouration, which is used to signal unprofitability in defended prey (Poulton 1890; Cott 1940; Edmunds 1974; Evans and Schmidt 1990; Ruxton et al. 2004). Both of them function to decrease predation risk.

There are a number of studies that have demonstrated the function and efficiency of various types of protective colouration (reviewed in Ruxton et al. 2004). Less well understood, however, are the ecological circumstances that facilitate the evolution of either camouflage or aposematism. One study, using a combination of evolutionary simulations and phylogenetic comparisons, showed that the evolution of conspicuous warning colouration is facilitated by constraints on camouflage (Merilaita and Tullberg 2005). In that study, habitat heterogeneity and diurnal activity were identified as potential constraints on camouflage. Another study focussed on the potential facilitation of evolution of aposematism by the opportunity costs that crypsis entails (Speed and Ruxton 2005).

In insects, both endo- and exopterygotes, it is common to find warning colouration in one instar and crypsis in another instar of the same species. For instance, many butterfly species have aposematic larval and adult stages, whereas the pupa is cryptic (Wiklund and Sillén-Tullberg 1985), and different functional colourations may also be found among the different larval instars in the same species (Nylin et al. 2001; Grant 2007). Such intra-specific changes in protective strategies, although poorly understood, may be related to changes in availability of various options or in cost and benefits of different strategies. For example,

Communicated by D. Gwynne

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change in lifestyle between different developmental stages, such as change of habitat or in mobility, may affect the costs and benefits of camouflage and aposematism (Merilaita and Tullberg 2005; Speed and Ruxton 2005). Availability of defence substances may open the possibility to switch to aposematism: Some grasshoppers of the genus *Schistocerca* show phenotypic plasticity with regard to colouration and distastefulness to predators, and individuals change to host plants providing distasteful substances and from cryptic to conspicuous colouration during a moult in response to high density (Sword 2002; Despland and Simpson 2005). Finally, seasonal variation in the appearance of the habitat or in constitution of the community of potential predators or alternative prey (Poulton 1890, p.179) may change the optimal defence strategy in time.

Stink bugs of the order Heteroptera are chemically protected and store protective substances in dorsal abdominal (larvae) or metathoracic scent glands (adults; Remold 1963; Scudder and Duffey 1972; Aldrich 1988). Several heteropteran families contain red-and-black species (e.g. Pyrrhocoridae, Lygaeidae, Pentatomidae), some of which have been subjects in experiments and comparative studies of aposematism (Berenbaum and Miliczky 1984; Paradise and Stamp 1991; Zrzavý 1994; Zrzavý and Nedved 1999; Exnerova et al. 2003a, 2006; Gamberale-Stille and Tullberg 1999; Vesely et al. 2006). One of these is a species widely distributed in Europe: the striated shieldbug *Graphosoma lineatum* L. (Heteroptera, Pentatomidae).

In this study, we report an ontogenetic change in colouration and conspicuousness of the adult striated shieldbug. In south central Sweden, the majority of newly eclosed adults appearing in the late summer have no or very little red pigmentation. Instead, they exhibit a pale, light brownish (epidermis) and black (melanised cuticula) striation. In this study, we refer to this colouration as pale or pale striation. These bugs are usually found against a background of dried umbels and stems of their host plants. They leave their host plants for hibernation in the ground, and when they appear again on their flowering host plants in early summer, the bugs show the typical red-and-black striation. Thus, the pale stripes turn red sometime before the reproductive period.

The two broad functions of protective colouration, camouflage and warning colouration, need not be mutually exclusive. For instance, it has been suggested that warning patterns may be used by animals that match the background in colour (Wüster et al. 2004) and cryptic and aposematic functions may be combined in a distance-dependent manner (Tullberg et al. 2005). Furthermore, it has been suggested that the red-and-black striation of *G. lineatum* may be difficult to detect through a disruptive effect of the patterning, at least against some backgrounds (Vesely et al. 2006).

In this paper, we compare the detectability of the pale-striated and the red-striated colouration of adult *G. lineatum* in the pre-hibernation habitat. We test the idea that the pale striation improves camouflage in the newly eclosed bug against its late-summer natural background. Moreover, we test the idea that a striated pattern in the pale form may increase camouflage compared to a non-striated colouration. There are two obvious problems involved in the measurement of the detection of a distasteful prey by its natural predators in its natural habitat. First, it is very difficult to know when the predator has detected the prey if it is unwilling to attack it. Second, it is difficult to control the distance at which the prey will be presented to the predator. To solve these problems, we measure detectability of insect colouration against its natural background by using human subjects searching for the colour-manipulated insect in a photographic image shown on a touch screen (cf. Tullberg et al. 2005).

Materials and methods

Biology of *Graphosoma lineatum* L. (Heteroptera, Pentatomidae)

G. lineatum feeds on the flowers and seeds of various species of Apiaceae. In south central Sweden, where this study has been carried out, it is most often found in sun-exposed meadows on *Anthriscus sylvestris* (wild chervil) and frequently also on *Aegopodium podagraria* (ground elder). In this area, it has also been recorded on a number of other apiaceaeous plants, e.g. *Angelica sylvestris*, *Angelica archangelica* and *Myrrhis odorata* (cicely).

The species has a facultative adult diapause induced by a short-day photoperiod (Nakamura et al. 1998). A second generation may be produced in southern Europe and parts of Russia and Ukraine (Musolin and Saulich 2001; see references in Slachta et al. 2002), but there is only one generation per year in the northernmost part of the species' distribution including Sweden (Larsson 1986). Adults emerge from hibernation sites and occupy their host plants when these start to bloom, usually in middle or late May. In these plants, they reproduce during June and July, and larvae are found from early July and newly eclosed adults from August. The new adults hibernate singly under leafy litter in the ground at forest edges close to breeding sites (Stehlik 1984; Coulianos, personal communication).

Colouration of *G. lineatum*

Usually, adults of *G. lineatum* are described as red and black (e.g. Tietz and Zrzavý 1996; Vesely et al. 2006), but colour variation, including specimens exhibiting little or no

red pigmentation, has been reported from Central and Southern Europe (Wagner 1956; Stichel 1960). However, there is no mention about the seasonal timing of this colour variation. In our study area, there is a clear seasonal variation in colouration: All post-hibernation adults exhibit the typical red stripes, whereas in the pre-hibernation adults, these stripes are generally pale to light brownish and non-red (see below under “[Reflectance spectra of seasonal variants](#)”). In our south central Swedish population, there is little if any variation among individuals with regard to patterning: The black and lighter stripes are approximately equally wide and do not vary in number. The five larval instars are all coloured in various shades of brown and black and appear quite cryptic when feeding on seeds in the dried umbels of a host plant like *A. sylvestris*.

The post-embryonic development of the dorsoventral colour pattern in the adult has been studied by Tietz and Zrzavý (1996). These authors conclude that the epidermis is uniformly red in the adults and that the “complex black-and-red colour pattern is formed exclusively by different melanization of cuticular areas.” Thus, the red areas have a transparent cuticle, whereas the black areas are melanised. The pigments involved in the reddish colouration have been studied in several heteropteran species, and pteridines have been identified in the pyrrhocorid genera *Dysdercus* (Melber and Schmidt 1994) and *Pyrrhocoris* (Socha and Nemeč 1996) and in the pentatomid bug *Halyomorpha brevis* (Niva and Takeda 2002). However, the pigments involved in the pale or red colours of *Graphosoma* species have not yet been identified.

Chemical defence against birds in *G. lineatum*

The stink glands of *G. lineatum* produce a number of volatile compounds identified by Stransky et al. (1998), and the defensive ability of the red-and-black adult *G. lineatum* against birds has been verified in experimental studies (Schlee 1986; Exnerová et al., in preparation; Vesely et al. 2006). Moreover, in areas where the bug is common, it is rarely found in the diet of wild birds (Exnerova et al. 2003b). In this study, we wanted to check whether the newly emerged pale-striated adults are distasteful to bird predators. In early September 2006, we individually presented 13 1-week-old chicks (*Gallus gallus*) with a shieldbug for 30 s. The birds had no prior experience of insects apart from mealworms. Only five of the chicks attacked the bug, although all chicks readily consumed a mealworm directly after being presented with a bug. Three of the five attacking chicks abstained from attacking the bug when presented again, whereas two of the attacking chicks attacked a shieldbug in two more trials before refusing. Each of the attacking chicks consumed mealworms in between presentations with shieldbugs. However,

they never consumed any bugs but only pecked at them, and none of the bugs were killed in attacks. Bugs frequently discharged obnoxious secretions. From this bioassay, we conclude that the pale-striated pre-hibernation adult is indeed defended and avoided by predators.

Photography

We used a 5.0-megapixel digital camera (Canon Power Shot G5) with 7.2 mm focal length to photograph adult *G. lineatum* on its host plant *A. sylvestris*. Photographs were taken at several occasions, in June of the post-hibernation reproductive red-and-black adults, and in August of the newly eclosed pale-and-black pre-hibernation adults. The bugs were photographed in their typical more or less vertical position in an umbel of the plant. We took photographs at several distances and under various light conditions in sun-exposed meadows with grasses and *A. sylvestris* as the dominating herb. The photographs were taken at about a 45° angle from above so that the whole image was filled with the typical habitat background below the horizon. We then selected two photographs from late summer, August, comparable with regards to light conditions, both taken at midday in sunlight softened by a slight mist. The photos were selected on basis of high quality, and because of the small variation in bug size and pattern, we regard these photos as highly representative of *G. lineatum* in its habitat in our geographic area. The two photographs were taken at different distances, and these were selected because the detection task was judged to be not too easy or too difficult for a human subject. In the first photograph, the bug had been photographed at a distance of 40 cm and measured 12 mm when presented on the 17-in. screen (image size 36×45 cm), and in the other, it had been photographed at 60 cm and measured 9 mm. It should be noted that because of the wide-angle lens, a bug appeared farther away in the images than the actual distance at which the photograph was taken.

Image manipulations

Bugs in the photographs were manipulated using the software GIMP 2.2 (Free Software Foundation, Boston, MA, USA). A similar method has been used in Tullberg et al. (2005). In this method, for each of the two photographs to be manipulated, we selected a similar photograph of the same bug taken within a minute before or after the original photograph. Further, we selected photographs of red-and-black bugs taken in June at the same distances and under similar light conditions. Pixels from bugs in these secondarily chosen photographs, referred to as ‘donor’ photographs, were copied and pasted onto the bug in the original photographs at approximately the same area. This was done

to ensure that all bugs in the experiments described below were manipulated so that any possible overall effect of image manipulation as such was controlled for.

Comparison of red striation and pale striation in the pre-hibernation habitat

For each photograph, pixels from the bug in the donor August photograph and the donor June photograph were pasted onto the bug in the manipulated photograph producing a pale-striated and a red-striated August image, respectively (Fig. 1). This ensured that all bugs were manipulated to the same degree. The images with manipulated bugs were compared with regards to time to detection.

Effect of striated pattern in pre-hibernation adults

The photograph taken at 40 cm was used to produce four manipulated images to investigate whether the striation affects detectability in the pale pre-hibernation adults. The photograph was manipulated so that pixels from the bug in the donor August photograph were pasted onto the bug in the original photograph, producing a striated bug similar to the original. Then, non-striated bugs were created in three different ways. Firstly, by only taking pixels from the black stripes, we created a black bug, and secondly, by only taking pixels from the pale stripes in the donor photograph, we created a pale bug. Thirdly, we created a bug with a colour that contained a mixture of the pale and black stripes and had the same net brightness as the naturally striated bug. This was done by repeatedly averaging the colour

(RGB system) and brightness parameters over a group of (3×3) adjacent black and pale pixels in the donor photograph and pasting this on the manipulated image. This way, we created photographic images with non-striated insects being black, pale (light brown) and dark brown, respectively, the last of which represented an average of the pale and dark stripes.

Image presentation

To test the relative detectability, the images were presented on a touch-sensitive screen to volunteer human subjects, 16–19 years of age, each viewing one single image. The first experiment consisted of four images, two for each distance, manipulated with respect to pale- and red-striated colouration in the August seasonal environment. There were 53 subjects participating in this experiment. The second experiment, with 54 subjects, also consisted of four images, one striated and three non-striated against the August background.

The images were presented on a 17-in. touch-sensitive liquid crystal display screen (NEC AccuSync LCD 52 VM, NEC Display Solutions, Japan). Before the image presentation, the students were given a short introduction to the experiment and its general purpose. The students were also allowed to look at a picture with 20 drawn images chosen from different taxonomic groups of Heteroptera (modified from Saunders 1892) to get an idea of what form of insect to search for and to gain an understanding of the variability of colour and size. The experiment was conducted in a dark room with one subject at a time together with one person supervising the presentation. The subject was instructed to touch the bug on the screen as soon as it had been discovered. The subject started the display of the image by touching a start button that appeared on the screen. A purpose-written presentation programme responded only to touches on the bug. It recorded the time elapsed between start of the image display and touch on the insect, allowing a maximum of 120 s search time after which the image disappeared.

Reflectance spectra of seasonal variants

In contrast to the trichromatic vision of humans, many passerine birds have a fourth class of photoreceptors that are sensitive to light in the UV part of the spectrum (e.g. Cuthill et al. 2000; Ödén and Håstad 2003). Therefore, if a bug seen against a given background appears cryptic to humans, it is not necessarily cryptic for passerine birds, if the background and the bug differ in their reflectance of short wavelengths (below 400 nm). Because such birds are potential predators on our insects, we wanted to find out how the exclusion of UV in our experiment, due to use of

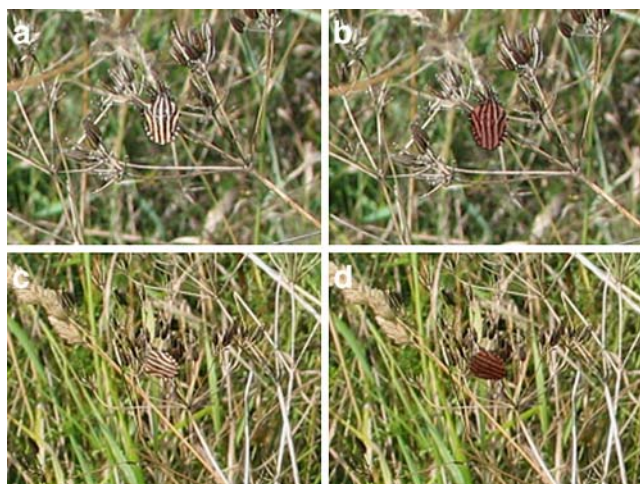


Fig. 1 *Graphosoma lineatum* on the host plant *Anthriscus sylvestris* in late summer (August). Figures represent an enlarged section of images presented in the experiments with two manipulations of bug coloration reflecting the typical pre-hibernation pale striation and the post-hibernation red striation. **a** 40-cm pale striation, **b** 40-cm red striation, **c** 60-cm pale striation, **d** 60-cm red striation

humans as predators, affects our conclusions. For this purpose, we measured the reflectance as the percentage of the reflectance of a white standard from the two seasonal morphs of *G. lineatum* and the late-summer host plant, using an Ocean Optics (Dunedin, FL, USA) USB2000 spectrophotometer and PX-2 pulsed xenon light source (Fig. 2). The peak of UV sensitivity in passerine birds ranges from 355 to 376 nm (Cuthill et al. 2000, Hart 2001). Within the range invisible to humans but visible to some birds, from 355 to 380 nm, the reflectance of colours of the bugs and their host plant were very similar (Fig. 2). Generally, these reflectances were low compared to the reflectances within the range visible to humans, except for the black stripes, which had a very low reflectance even within that range. In sum, there appears not to be any dramatic differences in the UV range that could make seemingly cryptic elements of the pattern conspicuous to passerine birds.

Statistics

Our data contained censored values, and we used survival analysis in the program STATA 9.0 for Macintosh (Statacorp LP, Texas, USA). Using Cox regression models, we tested whether there was a difference in time to detection between the 40- and 60-cm-distance images. Further, we tested whether there was a difference in time to detection between the pale- and red-striated manipulations at each distance. Last, we tested whether time to detection differed between striated and non-striated bugs in the 40-cm image.

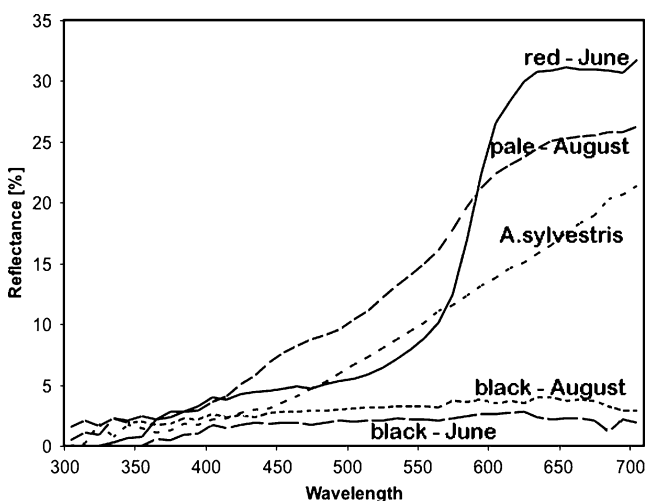


Fig. 2 The reflectance spectra of the red and black stripes of the bug, *Graphosoma lineatum*, measured in June, and the pale-and-black stripes of the bug and the host plant, *Anthriscus sylvestris*, measured in August. Each line is the average of three measurements

Results

When comparing time to detection of the bug between the images taken at 40 and 60 cm, it took a longer time to detect the bug at 60 cm; however, the difference was not significant (Cox proportional hazards model: $z=-1.71$, $p=0.088$; Fig. 3).

In the 40-cm-distance image ($N=27$), there was a significant difference between the two colourations, and it took on average more than twice the time to detect the pale (mean=14.4 s; $N=13$) than the red (mean=6.3 s; $N=14$) bug (Cox proportional hazards model: $z=2.55$, $p=0.011$; Fig. 3). Similarly, in the 60-cm-distance image ($N=26$), it took about four times longer to detect the pale (mean=40.3 s; $N=13$) than the red (mean=9.3 s; $N=13$) bug (Cox proportional hazards model: $z=2.61$, $p=0.009$; Fig. 3).

In the experiment with manipulated striated and non-striated bug colourations ($N=54$), the time to detection was significantly longer for the striated image than for the three non-striated images (Chi-square=21.31, $p<0.001$; Fig. 4). Furthermore, in separate comparisons between the striated and each of the non-striated manipulations, it took a significantly longer time to detect the striated bug (mean=27.7; $N=14$; Cox proportional hazards model; ‘average’ manipulation [mean=8.4 s; $N=13$]: $z=2.82$, $p=0.005$; ‘pale’ manipulation [mean=3.7 s; $N=13$]: $z=4.30$, $p<0.001$; ‘black’ manipulation [mean=5.1 s; $N=14$]: $z=3.15$, $p=0.002$).

Discussion

In this paper, we have described a seasonal polyphenism that is based on an ontogenetic colour change that takes

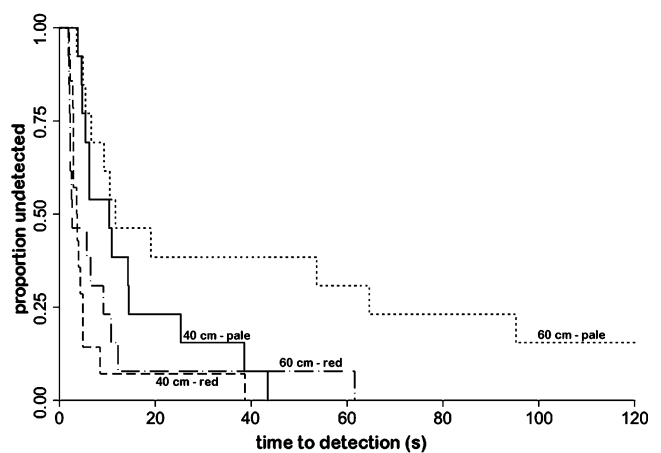


Fig. 3 Detection time as illustrated by proportion of undetected bugs in experiments with human subjects searching for a bug in a touch screen image. Images represent two distances to the bug, 40 and 60 cm, manipulated to represent two seasonal colorations, pale and red striations. Results of statistical analyses in text

place in adult *G. lineatum* from the pre- to post-hibernation stage. Furthermore, we have investigated the detectability of the two adult colourations against the natural late-summer background to shed light on the function of the pale-striated late-summer colouration. In both images with manipulated seasonal colouration, pale-striated bugs were harder to detect than red-striated ones. This supports the hypothesis that pale colouration in the young adults may serve for increased camouflage and that ontogenetic colour plasticity in the adult is related to detectability to potential predators.

The experiment with pattern manipulation further suggests that the striation increases camouflage in the late-summer adult (Fig. 4). This may be due to pattern matching between the striation and the many linearly shaped objects (stems and umbels) in the environment, but the stripes may also disrupt the body shape (Merilaita 1998; Schaefer and Stobbe 2006; Stevens et al. 2006).

Although our study suggests that in the late summer, the pale striation is more cryptic than the red striation, this does not exclude that this form can have an aposematic effect if discovered by predators. This is supported by our survey with chicks as predators (“Materials and methods”). It has been suggested that aposematism does not necessarily require maximisation of conspicuousness and that distinctiveness from edible prey may be more important (Sherratt and Beatty 2003, Merilaita and Ruxton 2007). Thus, it is possible that the stripes of the late-summer phenotype make the bug distinctive and that this, together with the distastefulness, is sufficient to result in benefit from aposematism. For instance, predators can distinguish between non-chromatic patterns of distasteful prey (e.g. Lindström et al 1999). On the other hand, in experiments with colour mutants of *Pyrrhocoris apterus*, passerine birds needed colour to recognise prey as aposematic, and the

melanin pattern alone was not sufficient (Exnerová et al. 2006). In addition, recent experiments indicate that colour may override the pattern in avoidance learning of distasteful prey (Aronsson and Gamberale-Stille 2008). In conclusion, our study suggests that the stripes increase the camouflage of the fall phenotype, but their relative contribution to an aposematic function is unknown.

In the light of our study, it is easy to understand the benefits of the pale-striated pre-hibernation phenotype in the dry late-summer environment. However, the use of epidermal red colour against a background of the green leaves and white flowers of the host plants after hibernation warrants an explanation. First, it is important to note that in addition to pale and red epidermal colours, other colours such as green, which potentially could match the early-summer background, have never been reported in the genus *Graphosoma* (Wagner 1956; Stichel 1960). If the seasonal difference in colouration is adaptive, then its underlying reason has to be a difference in the cost–benefit ratio of the different protective functions of the colouration. This cost–benefit ratio is influenced by several factors. First, even if the pale-striated colouration is aposematic, the red striation could elicit a stronger avoidance response, but this needs experimental testing. The only experiments set out to compare *Graphosoma* colourations showed more attacks by great tits on painted brown non-striated than on natural red-striated *Graphosoma* (Vesely et al. 2006). Secondly, the difference in detection risk between the two phenotypes could be smaller in the green and white early-summer environment than in the brownish late-summer environment. Third, because reproductive activities, i.e. mating and oviposition, occur in the post-hibernation adults, constraints on crypsis (sensu Merilaita and Tullberg 2005) or opportunity costs of crypsis (sensu Speed and Ruxton 2005) ought to be higher during the early summer season. This could facilitate the evolution of aposematic colouration. Furthermore, the post-hibernation adults may occur more aggregated as they tend to leave hibernation sites synchronously. This may further facilitate an aposematic function or decrease the opportunity for crypsis. Finally, there are seasonal differences in the age composition of predator populations. Thus, the birds in the early summer environment are adults that are likely to have previous experience of aposematic insects, whereas the later season is characterised by high numbers of inexperienced young birds, the type of predators that probably are most dangerous for aposematic prey (Ruxton et al. 2004). These three explanations are not mutually exclusive and need further investigation.

Phenotypic plasticity that involves ontogenetic change in colouration is quite common in Heteroptera. For instance, some pentatomid bugs switch between green and brown colour (Kotaki 1998; Musolin and Numata 2003), and this

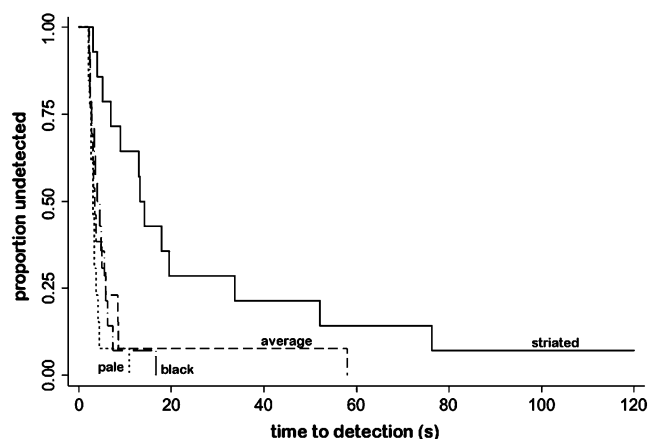


Fig. 4 Detection time as illustrated by proportion of undetected bugs in experiments with human subjects searching for a bug in a touch screen image. Images represent four bug colour manipulations, one striated and three non-striated. See “Materials and methods” for further explanation and “Results” for statistical analyses

may be functionally related to seasonal background matching. Furthermore, age-related colour change has been reported in the conspicuously coloured *Eurydema oleracea* (Fasulati 1979), but the function, if any, is not understood. We suggest that the difference in colouration between the pre- and post-hibernation adult stage *G. lineatum* is related to the differential possibilities to achieve camouflage or to benefit from it and to gain protection from signalling during the different seasons. Specifically, our results suggest that the plastic response involves a cryptic colour against the late-season background. As mentioned above, pale-striped colourations have been reported in other, more southern *G. lineatum* populations (Wagner 1956; Stichel 1960), but whether this represents seasonal plasticity is not known. Further geographic and experimental investigation of the extent and nature of colour variation in this species would shed light on the circumstances that facilitate aposematic or cryptic colouration.

Acknowledgements We are indebted to Dr. Olle Brick, Felicia Dinnetz and Rasmus Neideman for their invaluable help with the organisation of the photo image experiments. We are especially grateful to the many students at Norra Real for their participation as subjects. We thank Carl-Cedric Coulianos, Alice Exnerová, Pavel Stys and Christer Wiklund for valuable comments on earlier drafts of the manuscript. This study was financially supported by the Swedish Research Council (to B.S.T., G.G.S. and S.M.). The experiments in this study fully comply with current Swedish legislation.

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