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Evolutionary perspective

Experimental approaches to studying the initial evolution of conspicuous aposematic signalling

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Abstract. Aposematism, where prey species conspicuously advertise their unprofitability to predators, is a widespread defensive strategy. One feature of an aposematic anti-predatory strategy that is especially puzzling is conspicuousness. While conspicuousness aids associative learning in predators, it involves being more visible, which probably increases predation risk. Although aposematism is an old evolutionary question, experimental studies to its evolution have been scarce. Only 11 experiments address the potential benefits of conspicuousness, which have successfully manipulated conspicuousness. This is probably because it is difficult to separate conspicuousness from other characters of aposematic prey, e.g. colour. Furthermore since predators and prey species have coexisted for a long time, and there might be special adaptations other than conspicuous signalling, our experimental results might be confounded with, e.g. predatory biases. In this review, I will examine the problems of studying the costs and benefits of conspicuousness as well as the initial evolution of conspicuousness and the recent progress in the study of aposematism.

Key words: aposematism, initial evolution, signal conspicuousness

Evolutionary paradox

The theory of aposematism, where prey species advertise their unprofitability to predators by conspicuous means (for example, through the use of colours, odours and behaviour), dates back to Wallace (1867), and has remained an attractive evolutionary topic ever since (Rowe and Guilford, 2000). The idea that Wallace introduced was that being distasteful was not sufficient as an anti-predatory defence for butterfly larvae, since sampling by predators might fatally injure them. However, if caterpillars were conspicuously coloured and looked as distinct as possible from palatable prey, predators would learn to avoid these individuals in subsequent encounters, and these individuals would be left untouched. Later Poulton (1890) broadened the definition from bright coloration to include any conspicuous warning signal, such as odours and sounds, and introduced the term ‘aposematism’ ($\alpha'\pi o'$; away, $\sigma\eta\mu\alpha$; sign) to distinguish it from crypsis (κρνπτο′ρ; hidden), the other end of the conspicuousness continuum.

Conspicuousness is a common feature of aposematic prey, although there might be some exceptions (see Joron and Mallet, 1998). Typical warning colours such as red, yellow, orange and white, are usually associated with black patterning. These colours give the maximum visibility against brown and grey backgrounds (Cott, 1940), although the conspicuousness of aposematic prey has rarely been measured against their natural backgrounds. Since aposematic prey are advertising their unprofitability to potential predators, the receiver is very important in this signalling system. It should be considered that the way in which receivers perceive colours and contrast is likely to be very different from that of human vision when we test the conspicuousness of aposematic prey (Osorio *et al*., 2001). For example, larvae of the grey shoulder knot (*Lithophane ornitopus*) are cryptic against their natural habitat of oak leaves in the visible spectrum, but not in the ultraviolet (UV) region (Church *et al*., 1998). Conspicuousness in the UV region does not necessarily reveal aposematism (see Lyytinen *et al*., 2001), but this requires further study.

When studying the evolution of conspicuousness, the first question is whether or not conspicuousness is an adaptive feature of warning coloration (see detailed discussion in Guilford, 1988, 1990). It is difficult to know whether conspicuousness is just the by-product of the coloration or whether it is an important component of warning signals. There are basically three experimentally studied hypotheses drawn from predator psychology proposed to explain why it is more beneficial for unpalatable prey to use conspicuous colours rather than cryptic ones as a sign of unprofitability (for more hypotheses, see Endler, 1991):

1. Predators learn to avoid unpalatable prey more rapidly if they are conspicuous rather than cryptic (Gittleman and Harvey, 1980; Gittleman *et al*., 1980; Sillén-Tullberg, 1985a; Roper and Wistow, 1986; Roper and Redston, 1987; Alatalo and Mappes, 1996; Lindström *et al*., 1999a, 2001a).
2. Predators remember this association for longer (Roper, 1994).
3. Predators make fewer recognition errors if the pattern is conspicuous (Guilford, 1986).

These ideas propose that aposematism as a defensive strategy has exploited the fact that predators from different taxa are capable of learning and remembering what is not beneficial to eat and, therefore, it is an adaptive strategy against educated predators (see, e.g. Guilford, 1988, 1990). Since predators learn to avoid an unprofitable prey more quickly if it is conspicuous rather than cryptic, the evolution of aposematism represents a paradox: how can natural selection provide an advantage to a mutation that shifts a lifestyle initially from being cryptic to being highly conspicuous, since, surely, this draws the attention of predators and attracts predation? How can this type of defence have evolved if predators sampled and killed the pioneers?

Experimental approaches

Surprisingly, although aposematism is one of the oldest evolutionary puzzles, the evolution of conspicuous signalling has attracted very few experimental studies. Perhaps this is due to the fact that conspicuousness is not considered to be as important as colours and odours, and that conspicuousness is only a by-product of these characters. It is also very difficult to quantify conspicuousness, and therefore we are also lacking this data from the field. Wickler (1968) thought also that it is impossible to study the initial origin of aposematism, and that we would learn more about warning colours by studying mimicry systems. In addition, the initial evolution happened so long ago that it might be fruitless to study this process (Mallet and Singer, 1987), which is such a rare event (Turner and Mallet, 1996).

Nevertheless, although we might not be able to study the natural appearance of conspicuous aposematic prey, we can study the *relative* selection pressures of different aspects of conspicuous aposematic prey and draw hypotheses from these experiments to explain the evolution of conspicuousness in aposematic signalling species. However, we need to be careful in our experimental approach. First predator and prey species have co-evolved and might have multiple mechanisms to survive an encounter. These mechanisms, e.g. foraging preferences can confound our measures, for instance, of conspicuousness and might not reveal its true costs (see Lindström *et al.*, 2001a). Second, in order to separate parsimonious mechanisms, we need to compare the relative survival values of the aposematic prey to an evolutionarily reasonable alternative. For example, in the case of conspicuousness the comparison should be made between conspicuous unprofitable and cryptic unprofitable prey, to see whether aposematism is a more advantageous strategy, and if so, why (Guilford, 1988, 1990). Finally, we also need to be careful when we generalise our experimental results to the mechanisms explaining the evolution of aposematism. This is important if we draw our conclusion from experiments that are confounded by, for instance, predator biases. If predators have unconditioned aversion towards certain colours (see 'Innate biases of the predators'), our experiments with these colours are likely to reveal more about the present world than give insights to the evolutionary mechanisms.

I will first discuss the problems of coevolution ('Innate biases of the predators' and 'Adaptations of live insect prey to encounters with predators') and its impact on our experiments. Secondly, I will draw attention to use of alternative methods ('Artificial prey') namely the advantage of using artificial prey to overcome these co-evolutionary adaptations. Finally I will discuss how the studies which real insects prey can provide interesting insight to the evolution of aposematism.

Innate biases of the predators

Cott (1940) and Edmunds (1974) argued that predators have no innate aversion against warningly coloured prey, and that each predator has to learn the association between a conspicuous signal and its unprofitability. However, it has now been shown that predators do have unconditioned biases against typical warning colours (Smith, 1975, 1977; Schuler and Hesse, 1985; see Lindström *et al.*, 1999b and references therein). Predators might also have hidden colour biases, which can only be revealed when a second component of the warning display, for example pyrazine odour, is present (Rowe and Guilford, 1996, 1999, 2001). It seems that conspicuousness itself also becomes aversive in the presence of pyrazine, indicating that perhaps in multimodal signalling systems conspicuousness would not necessarily be initially costly (Lindström *et al.*, 2001b).

Predator colour preferences are heritable, and birds can be artificially selected on the basis of their preferences (Kovach, 1978, 1983; Marples and Brakefield, 1995). Biases can also be culturally transmitted: parents do not feed their nestlings with aposematic prey, which might result in a preference for cryptic prey and a bias against aposematic prey in the fledglings. Food preferences can also be learned from parents, who typically show their young what is beneficial to eat rather than what to avoid (Turner, 1964; Avery, 1996). For aposematic prey such biases are beneficial, since naïve predators will not be so naïve when they first encounter aposematic prey. Although naïve birds may experiment and attack warningly coloured prey, colour biases may cause hesitation and allow prey to be sampled, or may subsequently speed up avoidance learning.

These visual biases in predators are problematic in experiments that explore both the benefits of conspicuousness itself and the initial evolution of aposematism. For instance, conspicuousness might not be necessary for enhancing avoidance learning but the colour red is (Sillén-Tullberg, 1985b; Roper, 1990). It is, therefore, very difficult to separate the effects of pure conspicuousness from those of colour unless we control for coloration in our experiments. If we offer naïve predators a choice between edible mealworm larvae and a yellow and black monarch butterfly larvae presented on a brown background, the results are likely to be confounded by foraging biases. If the predators readily eat all the brown larvae but only a few attack the conspicuous monarch butterfly larvae, what can we conclude about conspicuousness? The results would tend to indicate that there is no cost of being conspicuous, but can we really conclude that there isn't a high visibility risk for the conspicuous aposematic prey? And if the monarch butterflies survive predator attacks, can we then conclude that conspicuousness promotes prey survival, since conspicuousness makes birds initially more hesitant to attack? This result, where all brown prey are eaten and only on a few occasions do predators eat aposematic prey leads to the more interesting question of why predators only attack very few aposematic prey. The answer may lie in the foraging preferences of the predators: if predators prefer brown food over yellow food, then by using these colours in our experiments we might overestimate the survival values of unpalatable yellow prey, since the predator already has a tendency to avoid this colour. This unconditioned aversion towards the colour could be additionally reinforced by the unprofitability of the prey, and thus the predator might only eat a single monarch larva. One way to overcome this problem experimentally is to have the colours reversed, for example, brown indicating unpalatability and yellow indicating palatability (see, e.g. Brower, 1960). However, given the difficulties of accurately reversing the coloration of live prey, experiments often resort to the use of artificial prey. The advantage of using artificial prey is that we can more easily control for the predatory biases towards colours as well as manipulate conspicuousness. For example, we can use symbols instead of colours and test the selective advantages of different levels of conspicuousness. I will discuss this at length in 'Artificial prey'.

Adaptations of live insect prey to encounters with predators

An additional problem with studying the initial origin of conspicuous signalling using real aposematic insect prey is that these prey species might have evolved other mechanisms to survive encounters with predators. These adaptations might be characters which we, as unnatural predators of these aposematic individuals, may consider meaningless. For instance, some insects use multicomponent signalling, where odours, sounds and behaviour are associated with conspicuous coloration (Cott, 1940; Edmunds, 1974); each of these components can interfere with conspicuousness. A moving aposematic insect might enhance avoidance learning by a predator, but then conspicuousness is only one part of aposematic strategy. It has been shown that the survival of an aposematic insect is best when all these components are presented simultaneously, but some characters like coloration in the defence is more important than others (Marples *et al.*, 1994). Thus, when we use real insect prey to test the costs and benefits of conspicuousness, we might introduce adaptations that have an impact on the result unless we control for them. Therefore, interpreting our measures of survival in such experiments might be problematic.

A good example is a tough cuticle that some insects have, which allows them to survive even when attacked (Järvi *et al.*, 1981; Sillén-Tullberg *et al.*, 1982; Marples *et al.*, 1994). Given that a toughened cuticle can enhance survivorship of prey, we need to know: (1) How did the tough cuticle evolve? (Endler, 1991) (2) Can predators taste the unpalatability or noxiousness without eating the prey? (Kassarov 1999) (3) Are there any interactions between coloration and toughness, i.e. would aposematically coloured individuals with a tough cuticle be more easily avoided than if they were brown? Ideally we should answer these questions prior to suggesting, for instance, a mechanism (see Evans, 1987) for the evolution of aposematic signalling. However, in the absence of these data, we should ensure that when we measure the role of conspicuousness we are not confounding the results with either predator biases or adaptations of living insects.

Artificial prey

Since predators have unlearned preferences and live insect prey might have multiple defence strategies, applying the results of experiments using live prey or traditional warning colours (e.g. red, yellow, orange, unless the colours are controlled for) directly to the puzzle of the initial or even secondary evolution of conspicuousness is problematic to say the least. Therefore, to study the predation pressures that make conspicuousness a more favourable strategy than crypsis, it is necessary to conduct experiments where biases for certain colours and prey adaptations are taken into consideration.

The benefits of using an artificial system is that it is possible to control for the adaptations of live insect prey, and it is also easier to control for the predator biases against certain colours. An additional benefit is the ability to create different levels of conspicuousness. One powerful experimental method to study the benefits of conspicuousness, as well as the initial evolution of aposematism, is the use of a 'novel world' – a method created by Alatalo and Mappes (1996). This approach attempted to overcome the problems of predator biases by using a system where both the background 'environment' and prey 'species' consisted of artificial symbols. The landscape consisted of crosses, and prey animals were either cryptic, in that they had the background symbol printed on them, or they were made to contrast with the background by having a different symbol. By introducing different symbols on the prey, it was possible to manipulate the level of conspicuousness of the aposematic prey. The major advantage of this method is that it was now possible to use wild birds and regard them as evolutionarily naïve predators. In this way we can begin to assess the possible selective forces naïve predators might have generated to newly evolving conspicuous unpalatable prey. An obvious disadvantage is that we cannot directly relate selection pressures uncovered in these experiments to coloration, since it may be that some colours more easily account for the evolution of aposematism without the costs of conspicuousness (for example, the colour red, Sillén-Tullberg, 1985b).

Real insect prey

Experiments with real insect prey can provide us with valuable insights into the costs and benefits of existing aposematic defence strategies. There are at least two fascinating systems where prey can have two morphs, one with a conspicuous signal and another with a more cryptic signal, indicating that there are differences in predation or there is a cost of producing the conspicuous signal (see Sillén-Tullberg, 1985a, b; Sword, 1999). *Lygaeus equestris* bugs can be either grey or bright red: the red form has a selective advantage over the grey morph since predators are more hesitant to attack red bugs and predators also learn to avoid red individuals more rapidly than grey morphs (Sillén-Tullberg, 1985a, b). The grasshopper *Schistocerca emarginata* has a yellow and black morph that lives gregariously at high densities which also has an advantage over the low-density cryptic morph in relation to predation (Sword, 1999). These differences allow us to look more closely into the benefits and costs of *producing* the conspicuous signal, which cannot be studied with artificial prey.

These examples also highlight the potential importance of considering the life-histories of aposematic prey, since the animals' coloration reflects the end result of a combination of different selection pressures. For example, a colour polymorphism of ladybirds might be the result of a balance between predation and parasitism (Losey *et al.*, 1997), or perhaps it is dependent upon their larval diet and is thus a result of plant–herbivore interactions (Grill and Moore, 1998; see also Tullberg *et al.*, 2000a). Of course, predation pressures might change between life-history stages of prey. Presumably by taking the life-histories into account it would also give insights into evolutionary pathways: for example, whether conspicuous coloration is selected for adaptive defence or whether it was initially selected for other functions (see Mallet and Singer, 1987; Guilford, 1988, 1990; Endler 1991; Turner and Mallet, 1996; McMillan *et al.*, 1997; Summers *et al.*, 1997; Mallet and Joron, 2000). Whether we should then invoke the theory of aposematism to explain these adaptations has been questioned (Guilford, 1988, 1990).

Experimental results

As we now know, it is not straightforward to study the evolution of aposematism or the benefits of conspicuousness. In this paper, I review only 11 experiments, which test the survival of conspicuous unpalatable prey (i.e. 'aposematic' prey) against a cryptic alternative prey. There are several experiments which compare the survival of aposematic prey against that of conspicuous palatable prey, thus in these experiments conspicuousness rarely possess a risk since alternative prey is easy to find (e.g. Järvi *et al.*, 1981; Wiklund and Järvi, 1982; Tullrot and Sundberg, 1991; Gagliardo and

Guilford, 1993; Gamberale and Tullberg, 1996a, b, 1998; Mappes and Alatalo, 1997; Tullrot, 1998), and I have excluded them from this review. Moreover, if we are interested in revealing the mechanism, which can account for the evolution of conspicuous signalling, adding contrast to the experiments is no longer sufficient. Instead, we should also compare the selective value of a conspicuous prey, for example, by making comparisons between solitary and aggregated prey, or comparing the survival of novel versus familiar conspicuous prey.

Surprisingly, all our experimental evidence on the benefits of conspicuousness comes from laboratory experiments (Gittleman and Harvey, 1980; Gittleman *et al.*, 1980; Sillén-Tullberg, 1985a, b; Roper and Wistow, 1986; Roper and Redston, 1987; Roper, 1994; Alatalo and Mappes, 1996; Lindström *et al.*, 1999a, 2001a; Tullberg *et al.*, 2000b). Most of these experiments have used artificial prey (except Sillén-Tullberg, 1985a, b). Nearly all these experiments conclude that conspicuousness is initially costly, but that this is later beneficial through more effective avoidance learning. Conspicuousness also seems to be more risky if the prey is a colour, which is not a traditional warning colour (Sillen-Tullberg, 1985b). This could indicate that there is nothing special about conspicuousness *per se*, but that it is the combination of colour and contrast that enhances the learning process in predators. Instead, perhaps conspicuousness acts as an attractor that draws the attention of the predator. On the other hand, whether predators could use conspicuousness as a signal for unpalatability and generalise this to other conspicuous prey irrespective of the colour, has not been experimentally tested.

The major difference among experiments that investigate avoidance learning in predators is whether they also include palatable prey. Some experiments have tested the learning function of predators in the absence of palatable prey (Gittleman and Harvey, 1980; Sillén-Tullberg, 1985a; Roper and Wistow, 1986; Roper and Redston, 1987; Roper, 1994; Tullberg *et al.*, 2000a), whereas others have included palatable prey (Gittleman *et al.*, 1980; Sillén-Tullberg, 1985b; Alatalo and Mappes, 1996; Lindström *et al.*, 1999a, 2001a). We should bear in mind that the interpretations of the results differ. In the absence of palatable prey, the predators need to learn not to eat, whereas in the presence of palatable prey predators need to discriminate, and learn what to eat as well as what not to eat. This difference in experimental design can lead to differences in results and in the interpretations (Alatalo and Mappes, 1996, 2000; Tullberg *et al.*, 2000a). It should also be noted that all experiments except one (Alatalo and Mappes, 1996) have used a frequency of 50% conspicuous and 50% cryptic prey. Since it is likely that conspicuous aposematic prey evolved from cryptic ancestors and were initially very rare, the possible anti-apostatic selection forces on rare conspicuous prey could influence our results and diminish or increase the costs of conspicuousness. This can only be modelled until we have more experimental data on predator behaviour towards rare conspicuous prey.

It is very difficult to draw conclusions from our experiments investigating the evolution of conspicuous aposematic prey. Our experimental evidence is too scarce, since most experiments have tested the costs and benefits of conspicuousness and not the evolution of aposematism. There are two hypotheses, which have been experimentally tested: either conspicuousness evolved gradually (Yachi and Higashi, 1998, 1999; Lindström *et al.*, 1999a) or gregariousness allowed major mutations in conspicuousness (Alatalo and Mappes, 1996). Gradual evolution requires that a slight mutation in conspicuousness is selectively advantageous compared to a cryptic prey. This could be possible if predators would learn to avoid slightly conspicuous prey, which would not suffer from visibility risks but still gain from avoidance learning. Later this learned avoidance would be transferred to an avoidance of even more conspicuous prey and conspicuousness could evolve through peak-shift mechanisms (Yachi and Higashi, 1998). However, predators only learn to avoid rather conspicuous prey, and furthermore peak-shift only seems to be advantageous when predators have learned to avoid rather conspicuous prey (Lindström *et al.*, 1999a). Nevertheless, gradual evolution might be possible through drift when there is no cost of being unpalatable, with predators eating slightly conspicuous prey in a similar way to palatable prey (Lindström *et al.*, 1999a).

Alternatively, unpalatability in cryptic prey might have selected for gregariousness as Fisher (1930) suggested. Once the gregarious lifestyle had been attained, conspicuous signalling might have been selected for, since conspicuousness risk is diluted between individuals in a group (Alatalo and Mappes, 1996, 2000; but see Tullberg *et al.*, 2000a). Additionally predators learn faster to avoid gregarious rather than solitary aposematic prey (Gagliardo and Guilford, 1993, Gamberale and Tullberg, 1996, 1998), and thus more individuals survive encounters with predators, which might have been essential when aposematic prey have been very rare. However, the benefits of gregariousness have been under explored, since we do not have a measure of how conspicuousness works in groups of prey. It has also been suggested that gregariousness might act as an signal itself (Cott, 1940; Gamberale and Tullberg, 1996; but see Lindström *et al.*, 1999b), but this has not been studied experimentally. It has also been suggested that gregariousness has evolved in prey that is already aposematic (Sillén-Tullberg, 1988, 1993). However, there might be very strong selection against aggregations: for instance, although *Lygaeus equestris* bugs have a lower predation risk when aggregated (Tullberg *et al.*, 2000b), in nature they are rarely seen in groups. Perhaps the cost from competition over resources might have consequently prevented a gregarious lifestyle (Bertram, 1978). Therefore, our phylogenetic evidence of the evolution of aposematism in butterfly larvae might reflect this strong selection against aggregations, although in other species gregariousness might have been a prerequisite for aposematic signalling. Additionally there could also be different selection pressures among different life-stages of a particular species. It has been suggested that gregariousness might explain larval aggregations but individual selection would explain why adults are conspicuous (Sillén-Tullberg *et al.*, 1982). Until we know more about aposematic prey under natural conditions, our suggestions on the mechanisms on the evolution are purely conjecture.

There are other factors that could have influenced the initial evolution of aposematism. Novelty effects, which are known to affect the food choices of generalist predators, might play a role in the evolution of aposematism (Lindström *et al.*, 2001a; see Marples and Kelly, 2001). However, it has been pointed out that novel signals might be selected against in an aposematic signalling system: it is likely that frequency-dependent disadvantages on a new rare morph relative to the common aposematic signal would cause predators to experiment more readily on them (Mallet and Singer, 1987; Mallet and Turner, 1996). However, novel appearance might not be unfavourable, since novel conspicuous prey might benefit from peak-shift mechanisms, where predators bias their avoidance towards more conspicuous prey than those which they have already learned to avoid (Lindström *et al.*, 1999a; Gamberale and Tullberg, 2000). Curiously, novelty effects do not appear to lead to more rapid avoidance learning (see Lindström *et al.*, 2001a). Our evidence of predatory responses towards novel, rare aposematic prey is very scarce, and we need more experimental data before we can fully understand their effects on aposematic signal evolution.

It still remains relevant as Guilford (1989) wrote that we need more controlled laboratory experiments to study both the selective values of conspicuous aposematic prey and the evolution of aposematism. Alternatives to the experimental approach are studying the evolution of conspicuousness using phylogenetic analyses (of prey: Sillén-Tullberg, 1988, 1993; Tullberg and Hunter, 1996; Read and Nee, 1997; but also of predators, Motychak *et al.*, 1999) or by mathematical modelling (Harvey et al., 1982; Sillén-Tullberg and Bryant, 1983; Leimar *et al.*, 1986; Mallet and Singer 1987; Endler 1988; Sillén-Tullberg and Leimar, 1988; Leimar and Tuomi 1998; Yachi and Higashi 1998, 1999; Speed, 2000). In the future, we need to focus on the particular question we are asking, and whether our experiments, phylogenetic studies or models are confounded with e.g. predator biases or multiple defensive mechanisms of aposematic prey.

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