

# Convergent evolution of neuroendocrine control of phenotypic plasticity in pupal colour in butterflies

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Phenotypic plasticity in pupal colour occurs in three families of butterflies (the Nymphalidae, Papilionidae and Pieridae), typically in species whose pupation sites vary unpredictably in colour. In all species studied to date, larvae ready for pupation respond to environmental cues associated with the colour of their pupation sites and moult into cryptic light (yellow–green) or dark (brown–black) pupae. In nymphalids and pierids, pupal colour is controlled by a neuroendocrine factor, pupal melanization-reducing factor (PMRF), the release of which inhibits the melanization of the pupal cuticle resulting in light pupae. In contrast, the neuroendocrine factor controlling pupal colour in papilionid butterflies results in the production of brown pupae. PMRF was extracted from the ventral nerve chains of the peacock butterfly *Inachis io* (Nymphalidae) and black swallowtail butterfly *Papilio polyxenes* (Papilionidae). When injected into pre-pupae, the extracts resulted in yellow pupae in *I. io* but brown pupae in *P. polyxenes*. These results suggest that the same neuroendocrine factor controls the plasticity in pupal colour, but that plasticity in pupal colour in these species has evolved independently (convergently).

**Keywords:** phenotypic plasticity; pupal colour; colour adaptation; environmentally cued dimorphism; butterfly; peptide hormone

## 1. INTRODUCTION

The proximate mechanisms underlying the control of phenotypic plasticity are of interest because, among other things, they shed light on the evolution of such traits. For example, if the proximate control of a plastic trait in related taxa is similar then it is more likely that the plastic trait is homologous in the two taxa. Alternatively, distinct differences in the proximate control argue for the independent (convergent) evolution of plasticity (Futuyma 1998).

Among insects, phenotypically plastic morphological traits are often influenced by hormones (Brakefield *et al.* 1998). For example, the correlation between variation in pupal colour in some species of Lepidoptera and the colour of their surroundings, which was first described by Wood (1867), is known to be under neuroendocrine control (Hidaka 1961a,b; Smith 1978, 1980; Awiti & Hidaka 1982; Bückmann & Maisch 1987; Starnecker 1997) and occurs in three lepidopteran families. So impressed was Wood (1867) by the phenomenon that he postulated that the integument of the pupa was like that of a photographic emulsion and, thus, was able to reproduce the colour of its surroundings. It was not until 20 years later that Poulton (1887) provided evidence that the colour of pupae was influenced by the colour of their pupation sites. In this century, numerous investigations have confirmed and extended Poulton's (1887) work (reviewed in Hazel 1995).

Plasticity in pupal colour is most likely to occur in species whose larvae have evolved preferences for pupation sites which vary in colour (West & Hazel 1979, 1996; Hazel & West 1996). As a result, green or yellow pupae are typically produced when pupation is on yellow–green surfaces, while pupation on brown or black surfaces results in brown or almost black pupae, although the larval rearing photoperiod and pupation site texture also have effects on pupal colour in some species (Brecher 1919, 1924; Wiklund 1972; Smith 1978, 1980; Hazel & West 1979, 1983; West & Hazel 1985). Therefore, it is generally assumed that the adaptive significance of plasticity in pupal colour has to do with crypsis (Baker 1970; Wiklund 1975; Hazel *et al.* 1998).

In the peacock butterfly *Inachis io* (Nymphalidae) and the large white butterfly *Pieris brassicae* (Pieridae) plasticity in pupal colour is achieved by a reduction in melanization and stimulation of lutein incorporation into the cuticle, resulting in yellow and/or green pupae on a yellow background (Kayser 1974; Maisch & Bückmann 1987; Starnecker 1997). Both pigmentation effects are controlled by a neuropeptide (Starnecker 1997) named, according to its first effect, as pupal melanization-reducing factor (PMRF) (Bückmann & Maisch 1987). PMRF is located throughout the entire central nervous system (Starnecker *et al.* 1994), but its release during the pre-pupal stage is controlled by nervous stimulation from the brain (Bückmann 1969). When pupation is on dark backgrounds, PMRF fails to be released and melanized pupae

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are produced. In contrast, in the swallowtails *Papilio xuthus* (Hidaka 1961a,b; Awiti & Hidaka 1983), *Papilio demoleus*, *Papilio polytes* (Smith 1978), *Papilio polyxenes*, *Papilio glaucus*, *Papilio troilus*, *Eurytides marcellus* and *Battus philenor* (W. Hazel, unpublished observations) a 'browning factor' is released when pupation is on dark backgrounds resulting in the formation of a brown pupa. Failure to release this factor results in a green pupa.

Pierids and nymphalids are more closely related than either is to the papilionids (Martin & Pashley 1992). The differences in the neuroendocrine control of plasticity in pupal colour in the nymphalids and pierids relative to the papilionids would seem to suggest convergent evolution of the plasticity in pupal colour in the two lineages. We have examined this hypothesis by determining whether pupal colour in a representative papilionid and nymphalid species results from the action of two different hormones or a single hormone that shows functional cross-reactivity. Therefore, purified extracts from the dissected ganglia chains (GCs) of pre-pupal larvae of *I. io* and *P. polyxenes* were tested for their effect on pupal colour when injected into *P. polyxenes* and *I. io* pre-pupae. For *I. io*, PMRF activity would be indicated when an injection into a pre-pupa which should normally moult into a black pupa (e.g. when pupation is on a black background) results in a yellow pupa. For *P. polyxenes*, the formation of a brown pupal cuticle following injection when a green cuticle would normally be expected (e.g. when pupation is on a yellow background) would indicate browning factor activity.

## 2. MATERIAL AND METHODS

### (a) Bioassays

Larvae of *I. io* were fed stinging nettles (*Urtica dioica*) and *P. polyxenes* larvae were fed Queen Anne's lace (*Daucus carota*) and reared under a 15 h photophase at 25 °C and/or 30 °C. For the *I. io* bioassay wandering larvae were kept singly in glass dishes whose bottoms and outer sides were lined with black and/or yellow cardboard. The larvae were permanently illuminated from the top. Three-hour-old pre-pupae adapted to a black background (which normally develop into strongly melanized pupae) were injected with 10 µl of test solution or water as a control using a Hamilton syringe. The degree of melanization of the resulting pupae was determined by a scoring system of five classes based on the size and form of certain black spots on the dorsal abdomen of the pupae and their presence and absence. For the *P. polyxenes* assay wandering larvae were placed in yellow construction paper cylinders with clear plastic tops. Pupae formed on such surfaces are invariably green. Pre-pupae were injected as described above at 12 h after the release of the pre-pupal pro-legs from the substrate, as the factor responsible for browning of the cuticle is released at this time (W. Hazel, unpublished data). Pupae of both species were scored into five classes, with class 5 containing the darkest and class 1 containing the lightest pupae.

### (b) Preparation of ganglionic extracts

The ventral GCs of the wandering larvae of *I. io* and *P. polyxenes* were dissected and stored frozen in pure acetone until homogenization. The ganglia were homogenized three times in acetone with a Branson sonifier (W250). After centrifugation (10 min at 15 000 g) the supernatant was discarded and the

pellet extracted under sonication three times with ice-cold 80% (v/v) ethanol in water and 2 M acetic acid each. The ethanol was removed in a high-speed vacuum concentrator. The water phase was combined with the 2 M acetic acid extract and applied to RP-C18 solid-phase extraction cartridges (Waters Associates, Milford, MA, USA) for purification. The cartridge was eluted with 25 and 75% acetonitrile and 0.1% trifluoroacetic acid. Based on previous experiments, the latter solution, which is known to contain *I. io* PMRF (Starnecker 1997), was dried and dissolved in water for subsequent bioassays.

## 3. RESULTS

When injected into *I. io* pre-pupae, the extracts derived from *I. io* caused a dose-dependent production of yellow pupae (Kruskal–Wallis ANOVA on ranks, d.f.=7,  $H=37.59$  and  $p<0.001$ ) indicating the presence of PMRF (figure 1a). A dose-dependent reduction in melanization and stimulation of lutein incorporation into the cuticle was also achieved when ganglionic extracts derived from *P. polyxenes* were injected into *I. io* pre-pupae (Kruskal–Wallis ANOVA on ranks, d.f.=8,  $H=23.93$  and  $p<0.002$ ). Even the low dose of 0.5 GC equivalent resulted in yellow pupae (figure 1b), indicating the presence of PMRF in the *P. polyxenes* extracts.

When the extracts from *P. polyxenes* were injected into *P. polyxenes* pre-pupae the result was a dose-dependent increase in the darkness of the pupae (Kruskal–Wallis ANOVA on ranks, d.f.=6,  $H=23.11$  and  $p<0.001$ ) (figure 1c) demonstrating the presence of 'browning factor' activity in *P. polyxenes* ganglia. Injection of the extracts derived from *I. io* also resulted in the production of dark pupae when injected into *P. polyxenes*, although the dose had to be increased to 40 GC equivalents to show a marked increase in browning (Kruskal–Wallis ANOVA on ranks, d.f.=2,  $H=9.42$  and  $p=0.011$ ) (figure 1d). The injection of water had no effect in either species.

## 4. DISCUSSION

Our results show that ganglionic extracts of both *I. io* and *P. polyxenes* show 'browning factor' activity when injected into *P. polyxenes* and PMRF activity when injected into *I. io*. It is possible that the ganglia of each species contain both PMRF and 'browning factor', with PMRF controlling pupal colour in *I. io* and 'browning factor' doing so in *P. polyxenes*. However, it is more likely that the opposite effects of the injections in the two species are the result of the action of a single hormone (PMRF) which stimulates the incorporation of different carotenoids into the pupal cuticle. In *I. io*, yellow pupal colour is due to lutein in the cuticle (Maisch & Bückmann 1987; Starnecker 1997), whereas in *P. polyxenes*,  $\beta$ -carotene and its derivatives are possibly transported into the cuticle to result in brown pupal colour as in *P. xuthus* (Ohnishi 1959; Harashima *et al.* 1972). If this hypothesis is correct, then the lower activity of the *I. io* extract when injected into *P. polyxenes* might suggest that this hormone (or its putative receptor) differs slightly in the two species. Similarly, PMRF from *P. brassicae* also shows lower activity in *I. io* (Koch *et al.* 1990). However, *P. polyxenes* pupae are nearly twice the size of those of *I. io* and this difference could

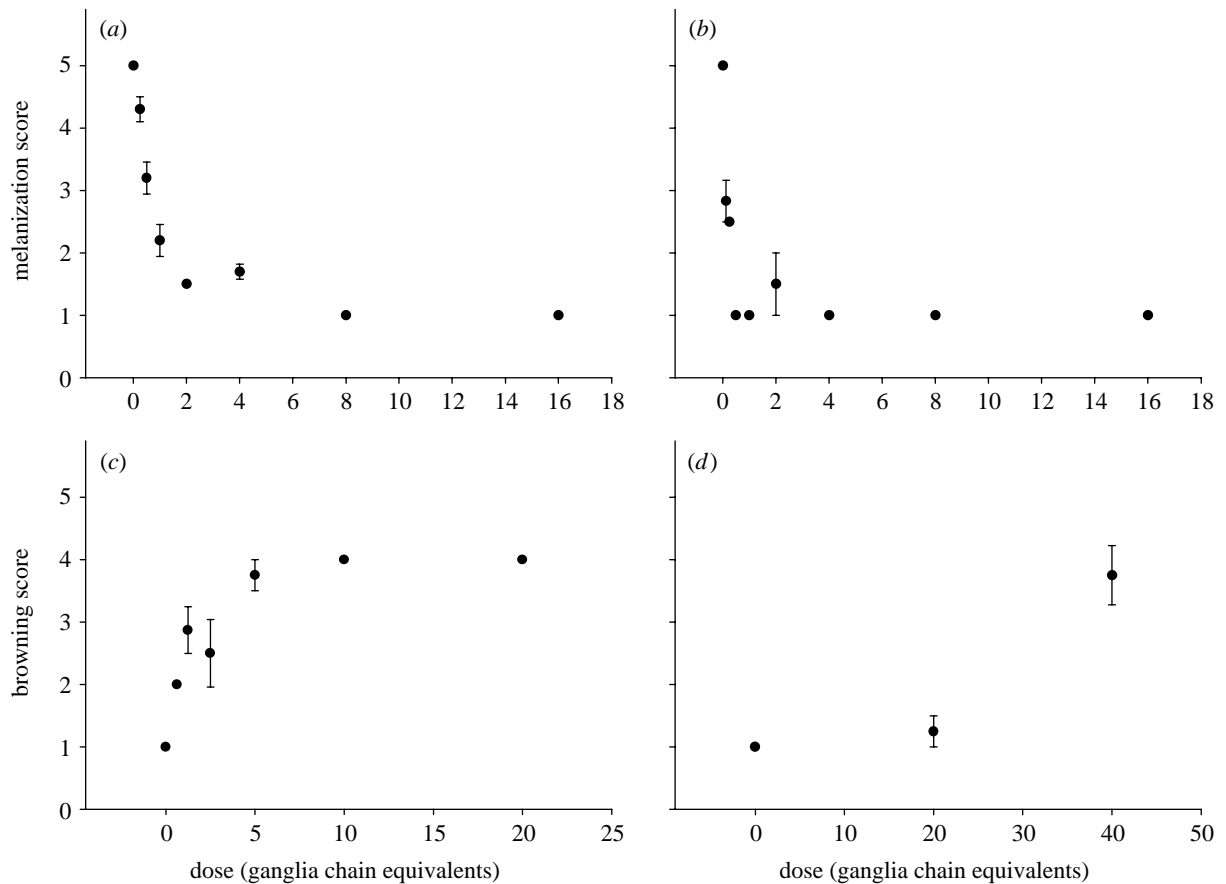


Figure 1. Effects of injections of increasing doses of GC extracts from *I. io* and *P. polyxenes* on pupal colour. Black (*I. io*) or dark brown (*P. polyxenes*) pupae received melanization or browning scores of 5. Yellow (*I. io*) or green (*P. polyxenes*) pupae received scores of 1. (a) Pupal colour responses of *I. io* to injected *I. io* extract ( $n=5$ ) showing the effect of PMRF on pupal colour. (b) Pupal colour responses of *I. io* to injected *P. polyxenes* extract ( $n=3$ ) showing an effect similar to that of PMRF. (c) Pupal colour responses of *P. polyxenes* to injected *P. polyxenes* extract ( $n=4$ ) demonstrating the effect of browning factor on pupal colour. (d) Pupal colour responses of *P. polyxenes* to injected *I. io* extract ( $n=4$ ) showing an effect similar to that of browning factor. Dose 0 = water injection.  $n$ , number of injected animals per dose. Mean values are given with standard errors of the mean (s.e.m.).

account in part for the reduced response of *P. polyxenes* to injections of *I. io* extracts.

PMRF or PMRF-like molecules are widespread in the Lepidoptera. In addition to occurring in species exhibiting plasticity in pupal colour, such as *Aglais urticae*, *Vanessa (Cynthia) cardui* and *P. brassicae*, PMRF activity is also found in species lacking plasticity in pupal colour, such as the buckeye *Precis coenia* and the African satyrid *Bicyclus anynana*. It has even been found in the moth species *Manduca sexta*, *Bombyx mori* and *Galleria mellonella* which pupate in cocoons (Starnecker 1996). However, PMRF activity was not detectable in ganglia extracts dissected from four species of non-lepidopteran insects (Starnecker 1996). In *I. io*, PMRF activity is present during each developmental stage, including freshly hatched larvae, pupae and adults (Starnecker & Bückmann 1997). These observations suggest that PMRF has multiple physiological functions in lepidopteran insects in addition to its control of pupal colour and that these functions predate the divergence of the lineages leading the nymphalids (and pierids) and papilionids.

If, as our data suggest, the plasticity in pupal colour in *I. io* and *P. polyxenes* is controlled by PMRF, then the neural control of the hormone's release would have to be different in the two species. In *I. io* (and *P. brassicae*) the hormone is released when pupation is on or in green

vegetation, while in *P. polyxenes* it is released when pupation is on a brown surface.

Molecular evidence indicates that the nymphalids, pierids and papilionids form two distinct clades, with the pierids and nymphalids sharing a more recent common ancestor than either does with the papilionids (Martin & Pashley 1992). Our results indicate that the neuroendocrine control of the plasticity in pupal colour is controlled by the same neuroendocrine factor (PMRF), with opposite effects on pupal colour in the two groups. This result suggests that the plasticity in pupal colour evolved independently in the papilionids and nymphalids (and pierids) via the co-option of the same neuroendocrine factor (PMRF). Therefore, the evolution of plasticity in pupal colour in these lineages is both convergent and parallel.

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