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Seasonal plasticity: how do butterfly wing pattern traits evolve environmental responsiveness?

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Phenotypic plasticity in response to environmental cues is common in butterflies, and is a major driver of butterfly wing pattern diversity. The endocrine signal ecdysone has been revealed as a major modulator of plasticity in butterflies. External cues such as day length or temperature are translated internally into variation in ecdysone titers, which in turn lead to alternate phenotypes such as seasonal wing patterns. Here we review the evidence showing that ecdysone-mediated plasticity of different wing pattern features such as wing color and eyespot size can evolve independently. Recent studies show that ecdysone regulates gene expression in *Drosophila melanogaster* via a chromatin remodeling mechanism. We thus propose that environmentally responsive ecdysone titers in butterflies may also function via chromatin regulation to promote different seasonal phenotypes. We present a model of ecdysone response evolution that integrates both gene regulatory architecture and organismal development, and propose a set of testable mechanistic hypotheses for how plastic response profiles of specific genes can evolve.

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Introduction

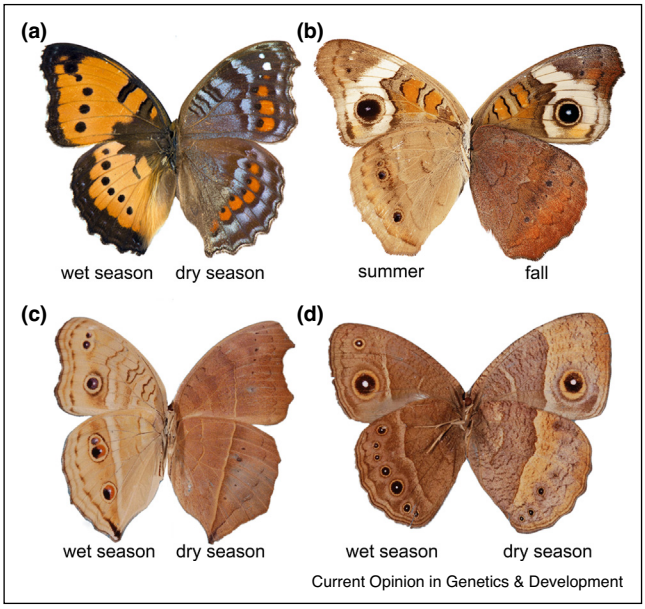
Phenotypic plasticity — the ability to change phenotypes in response to environmental conditions during development — is a major driver of morphological diversity in butterflies [1,2] (Figure 1). During development, environmental cues, such as day length and temperature, are translated into endocrine signals, which in turn activate alternate developmental pathways [3–5]. Wing pattern traits including color, wing shape, and eyespot size can evolve independently to respond to these endocrine cues, allowing for better adaptation to season-specific conditions [2]. Because of this striking morphological diversity, researchers have long tried to understand how phenotypic plasticity in butterfly wings might evolve. Here, we highlight recent major advances from different research fields that have furthered our mechanistic and evolutionary understanding of the evolution of endocrine responsiveness, even though the exact mechanism is still unknown. We present a framework for integrating evolution, development, and gene regulatory dynamics, and propose a series of explicit, testable models of how seasonal plasticity evolves at the level of individual genes.

Environmental cues are mediated by endocrine signaling

In butterflies, endocrine signaling is a major mediator of environmental cues [3]. Different temperatures and light regimes have been shown to affect ecdysone titers in two different butterfly species, *Bicyclus anynana* and *Junonia coenia*, which have emerged as important model species for mechanistic studies of phenotypic plasticity. *B. anynana*, a nymphalid whose range spans much of eastern Africa, displays strong phenotypic plasticity in response to temperature, corresponding to the wet and dry seasons [6]. Wing morphology traits such as color and ventral eyespot size, but also life history traits such as body mass and development time, all show a strong seasonal response [6,7]. Measurements of ecdysone titers throughout larval and pupal development at different temperatures show two periods of a plastic ecdysone signal [4,5]. The first period is during the wandering stage, when the larvae prepares for pupation, where warmer temperatures showed a stronger ecdysone pulse [5]. The second period is during pupal development, where at higher temperatures the ecdysone pulse is earlier in relative development time, while at lower temperatures it occurs later [4]. Interestingly, the magnitude of the pulse does not vary at this time. Ecdysone titer manipulations in this species affect plastic traits, where early manipulations affect wing pattern elements, while later manipulations affect life history traits such as development time [5,7,8]. Not only does ecdysone control seasonal plasticity, intersexual variation of ecdysone signal strength in last instar larvae also results in sexual dimorphism [9], highlighting the complex role for ecdysone during development.

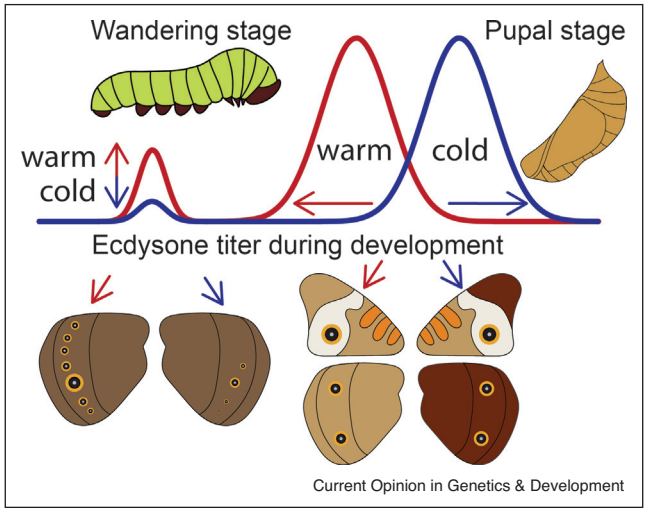
The common buckeye *J. coenia*, a North American nymphalid butterfly, also shows strong seasonal plasticity — a dark red wing color develops when larvae and pupae are reared under cold, short daylength conditions, and a pale

Figure 1



Butterfly wing phenotypic plasticity comes in many forms; wing pattern, wing color, wing shape and eyespot size can all change. Here, we compare different seasonal morphs. **(a)** *Precis Octavia* (specimens (c) Field Museum of Natural History, 124 365, 124 380) [32,33]. **(b)** *Junonia coenia,* **(c)** *Junonia almanac* (specimens courtesy of the Yale Peabody museum of Natural History {YPM, ENT 411934, 411925 [34,35]}), **(d)** *Bicyclus anynana* (YPM, ENT 406041, 406038 [36,37]).

Figure 2



Ecdysone response model.
Different rearing conditions (**(a)** and **(b)**) during development can lead to differences in ecdysone signaling during development, which in turn can lead to changes in wing morphology.

tan color develops when larvae and pupae are reared under warm, long daylength conditions [10]. Similar to *B. anynana*, ecdysone titers show a response to environmental conditions, where cold temperatures cause an ecdysone pulse to occur earlier in relative developmental time [3,11••]. Ecdysone manipulations show that wing color is responsive to ecdysone — when cold-reared butterflies are injected with ecdysone, a pale tan color develops [3]. Thus, ecdysone acts as an important mediator between environmental cues and plastic wing traits in both species (Figure 2). These species are thought to be diverged by ~77my [12], which supports the idea that ecdysone-mediated transduction of environmental cues is a deeply conserved seasonal plasticity mechanism in nymphalid butterflies.

Trait-specific phenotypic plasticity can evolve independently

Decades of research show that numerous traits in butterflies display developmental plasticity, ranging from wing traits, as described above, to larval and pupal coloration, to migration and life history traits [7,13–16]. Indeed, seasonal plasticity is almost always expressed as changes in suite of multiple morphological and behavioral characteristics which varies between species [16]. This leads to the question of to what extent seasonal responsiveness might evolve and adapt independently across various traits, especially given that many of them are likely controlled by the same ecdysone signal. One important recent study directly addressed this question at a macroevolutionary scale by comparing museum specimens from the tribe Junoniini (Lepidoptera: Nymphalidae) collected during different seasons [2]. They found that eyespot size was highly plastic between specimens collected during wet versus dry seasons across the tribe, but that this plastic response varied across species. Notably, wing shape also showed a strong plastic response between seasons in a smaller subset of species, but was not correlated with eyespot size plasticity, showing independent evolution of seasonal response in these traits.

Another study compared seasonal reaction norms across five mycalesine species from a wide range of environmental conditions, spanning Africa, Madagascar, and Asia [17]. Researchers exposed developing larvae to four different temperature conditions and measured life history traits — mass, growth rate, and development time — as well as wing pattern elements — wing area, eyespot size, and color stripe width on both dorsal and ventral surfaces. Most traits showed a significant response to environmental conditions, but the reaction norm shape and response varied widely across species. The majority of traits were correlated in their response to developmental conditions, and this collective response was consistent with ecdysone manipulations in *B. anynana* [5,7,8], which suggests that the plastic ecdysone pulse is the underlying regulator of phenotypic plasticity in mycalesines. However, phenotypic correlations in some traits, such as fat content and

dorsal eyespot size, were notably absent in some species. The most striking result was that the Cu1 dorsal eyespot size could be either negatively or positively correlated with other wing pattern elements, depending on the species. The overall size of the ventral eyespot is responsive to ecdysone manipulations in *B. anynana*, but dorsal eyespot size is not. Interestingly, although overall size of the dorsal eyespot in *B. anynana* does not change between seasons, the eyespot center is seasonally plastic, and responsive to ecdysone. Taken together, this study shows that traits can independently evolve their response profiles to seasonal conditions and ecdysone signaling in a highly specific manner.

Cue detection and hormonal response are highly conserved

The evolution of ecdysone responsiveness of ventral eyespot size plasticity was more thoroughly explored in a recent study comparing 13 butterfly species representing a wide range of nymphalid subfamilies [18••]. In most species measured, the strength of the pre-pupal ecdysone pulse responded to temperature differences during development in a predicted fashion, providing strong support for the model that this pulse is a deeply ancestral seasonal response mechanism in nymphalids. Ventral eyespot size was plastic in eight of these species. Interestingly, even though Ecdysone Receptor (EcR), was expressed in most eyespot centers during development, only one species, *B. anynana*, showed a strong eyespot size effect in response to ecdysone manipulation experiments. EcR expression levels and timing in eyespots centers do vary between forewings and hindwings in *B. anynana*, and this corresponds to variation in the plastic response of these eyespots. Taken together, these results show that gain of EcR expression alone does not result in ecdysone responsive plasticity, but if ecdysone responsiveness is present, modulation of EcR expression can result in variation in plasticity. Coincidentally, ecdysone responsive plasticity corresponded to a different direction of the reaction norm relative to the other butterflies (*B. anynana* was the only species where eyespots became larger in warmer conditions), and also a much larger change in size between conditions. This case study provides a striking example of how a deeply conserved trait can independently gain strong responsiveness to the seasonal ecdysone signal.

Reaction norms can evolve rapidly

If the ability to respond to plastic ecdysone titers is a major mechanism of seasonal plasticity across different independent traits, then how does ecdysone responsiveness evolve? Although the precise genetic mechanism of increasing ecdysone responsiveness is still unknown, one study we recently published does show how a reduction of ecdysone responsiveness may be attributed to *cis*-regulatory changes in downstream effector genes [11••]. *J. coenia* shows strong seasonal plasticity for wing color, and there is variation for the reaction norm across different populations [10,19]. Using lines selected for increased and reduced plasticity, respectively, we showed that the genetic assimilation of the autumnal red wing color was associated with sequence variation near three differentially expressed genes: *cortex*, *trehalase*, and *herfst*. Targeted knockouts in each of these genes resulted in a loss of the red color, thus validating that these genes are required for production of seasonal pigmentation. Changes in environmental responsiveness of these genes appeared to be independent of endocrine signaling, because ecdysone titers were not different between selection lines. We also observed that reaction norm-associated sequence variation was almost entirely in cis-regulatory elements near the causal genes, and that several candidate regulatory elements showed selection line-specific patterns of chromatin accessibility. Together, these findings led us to propose a model where different cis-regulatory alleles of downstream color pattern genes vary in their responsiveness to the post-pupal ecdysone pulse, and that selection for these different alleles can cause the evolution of color pattern reaction norms.

Ecdysone is known to affect the epigenetic landscape

To understand how specific traits might gain or lose responsiveness to seasonal ecdysone signaling, we must consider how traits develop in the first place, and how ecdysone affects this process. The manifestation of specific phenotypic traits is determined by cell fate, which in turn is influenced by changes in the chromatin accessibility that modulate when and where fate-determining transcription factors are able to bind to DNA [20]. Furthermore, even if chromatin accessibility does not change, different histone modifications or transcription factor occupancy profiles could also affect gene expression [21,22]. Thus, a combination of chromatin accessibility, chromatin modification, and chromatin occupancy at regulatory elements is important to determine cell fate. We then accordingly hypothesize that seasonal plasticity is regulated by changes in chromatin regulation in response to environmental conditions. In support of this, several studies have suggested a role for histone modifiers in trait plasticity [23,24]. However, more functional studies are needed to investigate the precise role of chromatin landscape changes in phenotypic plasticity.

In terms of seasonal plasticity in butterflies, we can focus our mechanistic questions specifically on how the plastic ecdysone pulse influences seasonal variation in gene expression. Importantly, a series of recent studies show that ecdysone is an important regulator of chromatin structure [25,26•,27••]. Uyehara *et al.* showed that ecdysone-induced transcription factor E93 is a major regulator of chromatin accessibility during *Drosophila melanogaster* development [26•,27••]. Another study from the same lab showed that EcR directly regulates many genes involved

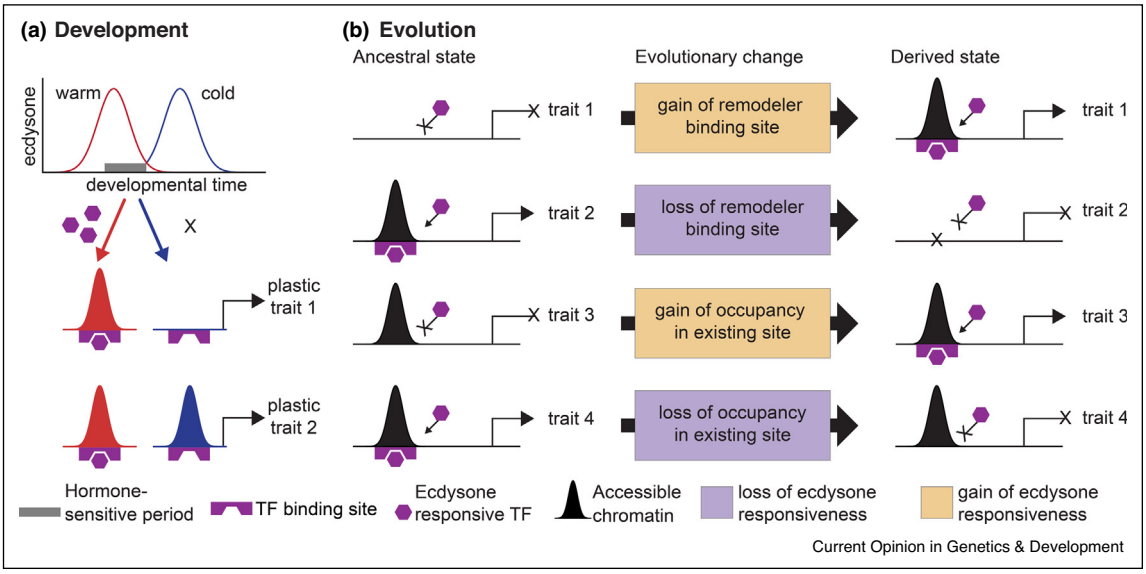
in wing metamorphosis [28•]. In our previous study of chromatin accessibility changes during *J. coenia* wing development, we found that ecdysone-responsive nuclear hormone receptors, such as *Hr4* and *ftz-f1*, are strongly predictive of large changes in chromatin accessibility during development [29••]. EcR occupancy, however, was highly stable throughout development, and was not associated with changes in chromatin accessibility. It is clear that the hormone ecdysone is a major regulator of development, but the precise molecular mechanisms through which ecdysone acts in butterflies needs more investigation. In conclusion, three major lines of evidence collectively suggest a potential mechanism for how ecdysone may regulate seasonal plasticity of cell fates: (1) environmental modulation of ecdysone titers underlie trait plasticity, (2) ecdysone is a major regulator of changes in the chromatin landscape, (3) chromatin modifications determine cell fate (Figure 3a). Although direct evidence is still lacking, we suspect this chain of observations will soon be linked, and it will become clearer how ecdysone mediates seasonal plasticity by altering the chromatin landscape in response to environmental cues.

Phenotypic plasticity can evolve by changing chromatin responsiveness to ecdysone signaling

Ecdysone has been established as a mediator between environmental cues and plastic trait response, and also as an important determinant of the chromatin landscape during development. Thus, we hypothesize that one mechanism by which butterfly wing morphology might gain or lose ecdysone responsiveness is by changes in how individual trait-determining genes respond to ecdysone signaling; either through changes in chromatin accessibility, or changes in transcription factor occupancy. Here we propose a model through which ecdysone responsiveness of an individual gene can change, with a set of hypotheses that can be tested in two steps (Figure 3b). First, we can test whether a gain or loss of ecdysone responsiveness is due to a gain or loss of a binding site for an ecdysone-induced chromatin remodeler, which would lead to a change in accessibility. We can test this by comparing the chromatin landscape in different conditions of closely related species that vary in their plastic response [30]. Regulatory elements that show a change in accessibility between rearing conditions, but only in the ecdysone responsive species would be strong candidates for involvement in regulating a plastic response. *In vivo* ecdysone manipulations and subsequent ATAC-seq would show whether or not these sites indeed respond to ecdysone, and CRISPR/Cas9 gene knock-out of variable accessibility sites would validate that these regulatory elements are indeed causal for the trait of interest [31].

The second mechanism through which ecdysone responsiveness can evolve is through a change in transcription factor occupancy in existing accessible sites. A gain or loss of responsiveness can evolve by acquiring or losing occupancy of an ecdysone-induced transcription factor in a

Figure 3



Gene-specific ecdysone responsiveness model.
We propose that ecdysone responsiveness of an individual gene can evolve via chromatin landscape changes. **(a)** During development, a change in ecdysone titers during a sensitive period leads to a change in activity of ecdysone-responsive transcription factors (TFs), which in turn alter the chromatin landscape through influencing accessibility or occupancy. **(b)** Evolutionary change can lead to a gain or loss of binding sites for ecdysone-responsive TFs, which in turn leads to changes in ecdysone responsiveness, either through changes in accessibility or occupancy.

trait-specific regulatory element, without changing the accessibility of that site. Testing this hypothesis would involve a comparison of occupancy by known ecdysone-induced transcription factors using ChIP-seq, which is more technically challenging. However, to understand how butterfly wing pattern plasticity evolves at a mechanistic level, we believe such experiments are necessary.

Conclusions

Recent advances show that phenotypic plasticity in butterflies is widespread, and can affect many traits. Surveys of wing color, wing shape, and eyespot size show that plastic response profiles of these traits can evolve both rapidly and independently. Studies across multiple butterfly species suggest that environmentally induced changes in ecdysone titers are a key determinant of color pattern plasticity. Because recent studies in *Drosophila* show a role for ecdysone in chromatin remodeling, we hypothesize that wing pattern plasticity may be regulated through ecdysone-mediated chromatin remodeling. We also speculate the evolution of wing pattern plasticity may be traceable to gene-specific changes in this process. With the availability of new functional genomics tools in non-model butterfly species, we can now start to investigate how ecdysone responsiveness might evolve to allow reaction norms to evolve on a trait-by-trait basis.

Conflict of interest statement

Nothing declared.

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