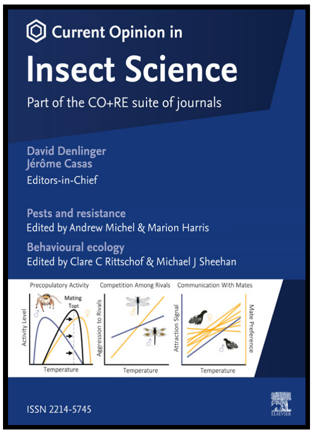

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Eco-evo-devo advances on seasonal plasticity of butterfly eyespots

Short Title: eco-evo-devo of eyespot plasticity;

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Abstract

Seasonal variation in butterfly wing color patterns is a classic model system of adaptive phenotypic plasticity. While decades of studies provided insights on the underlying ecological roles and physiological regulation of this adaptation, recent advances detail how plasticity develops at the transcriptomic level and evolves at the genomic level. Here, we synthesize these recent advances focusing on wing eyespot size plasticity in the subfamily Satyrinae (Lepidoptera, Nymphalidae) and the model species *Bicyclus anynana*, the most intensively studied system. We also propose future directions in the field.

Keywords: plasticity; butterfly; eco-evo-devo; evolution; eyespots

Introduction

Phenotypic plasticity describes the ability of organisms to produce divergent phenotypes in response to environmental heterogeneity, such as alternating seasons [1-3]. Seasonal variation in butterfly wing color patterns is a classic example of phenotypic plasticity. Here, environmental cues experienced during development produce season-specific modifications in a butterfly's wing appearance, including changes in background wing color, color contrast of pattern elements, and/or size of pattern elements such as eyespots [4-10]. These changes are adaptive to seasonal changes in resting background and predator guilds [11-13], mating strategies [14], and/or the regulation of body temperatures [15, 16].

Recent studies on eyespots in satyrid butterflies have deepened our understanding of this plastic system from an integrated ecological, evolutionary, and developmental (Eco-Evo-Devo) perspective. These studies have investigated the cues that can trigger the plastic size response, improved our understanding of systemic and eyespot-specific developmental responses to these cues, pinpointed the origin of the plastic response on a phylogeny, identified genomic changes behind the origination and adjustment of plastic responses, and explored the conservation potential of plasticity to cope with climate change. Below we summarize our current understanding of eyespot size plasticity and highlight recent discoveries.

Ecological significance

In satyrid butterflies, eyespots on the ventral (exposed) hindwing surfaces exhibit seasonal plasticity in their size, a classic example of a seasonal adaptation. In the model *B. anynana*, this seasonal plasticity is triggered primarily by environmental temperature associated with alternating seasons in tropical Africa [4, 17]. During the warm wet season, eyespots are large, and during the cool dry season, eyespots are small [18, 19] (Fig. 1a). This plasticity is adaptive: Large eyespots help deflect prevalent invertebrate predator attacks to the wing margin in the wet season, and small eyespots are cryptic to prevalent vertebrate predators in the dry season, helping the butterfly remain undetected against brown leaf litter [11, 12].

Across satyrids, hidden eyespots on both sides of the forewing and those on dorsal surfaces show different patterns of size plasticity [18, 20-22] and also number plasticity [23], that are best explained by their role in mate signaling [14]. Ventral forewing (hidden) eyespots are less plastic in *B. anynana* [18], and those on the hidden dorsal side of the wing can have opposite patterns of size plasticity in *Mycalesis* butterflies - becoming smaller with increasing temperature [20]. In addition, eyespot number is also plastic in *B. anynana* females, where higher temperature leads to fewer eyespots on dorsal hindwings; males adjust to these changes by learning to prefer females with fewer eyespots [23].

Evolutionary patterns

The evolutionary history of eyespot plasticity has been reconstructed by placing evidence from field observations and common garden experiments on a well-resolved butterfly phylogeny. Based on field collections and museum vouchers, multiple recent studies aligned the observed eyespot size variation with complex seasonal variation in natural habitats, and showed that seasonal plasticity in eyespot size is widely observed within the *Bicyclus* genus [24, 25]. Common garden experiments further elucidated that such variation can be triggered by a common environmental cue, temperature, and such temperature-mediated plasticity was estimated to have originated ~60 million years ago in a single clade, Satyrinae [19, 20, 26-28], the largest nymphalid subfamily with ~2700 extant species [29, 30] (Fig 1b). This type of plasticity is conserved even among species from aseasonal habitats without obvious temperature fluctuations [20, 27], suggesting that eyespot plasticity might have originated as an ancestral adaptation with strong fitness benefits, preventing it from degenerating easily in populations that subsequently colonized weakly seasonal habitats [27]. In addition, stronger regional seasonality is associated with higher plasticity levels [24, 31], suggesting that plasticity can evolve quantitatively in response to local conditions.

Physiological regulation

Inductive cues. The development of plasticity requires the perception and integration of environmental cues. While temperature is generally used to regulate eyespot size plasticity across satyrids [32], a series of recent studies demonstrate that a complex suit of other cues, such as humidity, host plants, food quality, day/night temperature alternations, and/or their interactions, also play a role [25, 28, 33-36]. Alternative cue use appears population/species-specific [28, 33, 34], and in certain species, such as *Melanitis leda*, well correlated with cue reliability in predicting upcoming seasons [34]. These studies reveal a complex network of interacting factors underlying satyrid eyespot size plasticity, and highlight how species, and populations within single species have gained, lost, and/or integrated sensitivities to different cues to alter eyespot development.

Ecdysone signaling. In butterflies, external cues are often integrated via endocrine signaling which coordinates the development of multiple plastic traits (reviewed in [37]). In the model satyrid *B. anynana*, temperature affects titers of a steroid hormone, 20-hydroxyecdysone (20E), during the larval-pupal transition, which mediates the development of eyespot size plasticity [18, 22, 38] (Fig. 1c). The expression of *Ecdysone Receptor* (*EcR*) in eyespot center cells during the larval stage may, via the promotion of local cell division, explain localized effects of 20E signaling in eyespot development [18, 22]. The absence of *EcR* expression in the centre of forewing eyespots, during the 20E-sensitive late larval wandering stage, may contribute to the different sensitivity to 20E and lower levels of plasticity observed in these eyespots in this species [18]. However, temperature-induced 20E dynamics, as well as the expression of *EcR* in eyespots, are pre-adaptations, but insufficient to explain how temperature-mediated eyespot size plasticity evolved on a phylogeny [19]. Additional genetic mechanisms are required to sensitize eyespots to 20E signaling to effect localized developmental responses.

Developmental response

Molecular responses. External cues, via endocrine signaling, instruct development by inducing extensive downstream molecular responses (reviewed in [39]). Recent studies in butterflies uncovered cue-induced molecular changes across various morphological [40-42], physiological [43, 44], life-history [45], and behavioral traits [46-48]. While alterations in gene expression levels is a well-known response to environmental cues, recent studies started to describe less explored changes in post-transcriptional [40, 43-45] and post-translational [41] processes. For example, extensive changes in gene expression, alternative splicing, and microRNA (miRNA)-mediated gene silencing are taking place in whole wings in response to temperature in *B. anynana* [40] (Fig. 1d). In addition, largely non-overlapping, functionally divergent sets of genes are either differentially expressed or differentially spliced across seasonal regimes (Fig. 1d), emphasizing complementary rather than redundant roles of gene expression and alternative splicing in developmental plasticity [40, 43], consistent with other plastic systems [49].

Systemic vs trait-specific responses. Recent studies have started to dissect how different tissues in the same body respond to the same environmental cue at the molecular level. One study sampled laser-dissected eyespot central cells and adjacent control wing tissue across seasonal forms of *B. anynana* [40], and found that most genes responded systemically across both tissue types, whilst a smaller subset was eyespot-specific (Fig. 1e). One of these eyespot-specific plastic genes, *Antennapedia* (*Antp*), which had not been picked up from a previous whole wing transcriptome study [40], turned out to be a key regulator of eyespot size plasticity - demonstrated by reduced plasticity levels when *Antp* was disrupted across two satyrids [50] (Fig. 1e). Contrary to the findings above, largely trait-specific responses were observed across distant body parts, such as adult thorax versus abdomen [44, 51]. Overall, these studies suggest that molecular plasticity is largely systemic across cells within a similar tissue, while trait-specific molecular responses (within tissues) are more likely to be consequential for the trait of interest. This highlights the importance of resolving molecular plasticity at a trait-specific resolution to pinpoint genes with localized functional relevance.

Genomic drivers

Intraspecific variation. Identifying genomic loci associated with intraspecific variation in plasticity levels is essential to understand the evolutionary origin and genetic underpinnings of plasticity (reviewed in [39]), but recent genotype-phenotype association studies in butterflies have had mixed outcomes [31, 52]. A recent study focusing on diverging levels of eyespot size plasticity among *B. anynana* populations across an ecotone-rainforest gradient found no obvious genome-wide variation, probably due to frequent gene flow among the habitats that homogenized most parts of the genome [31, 53]. This limitation was overcome in a study on *Junonia coenia*, a non-satyrid with seasonal plasticity in background wing color, not eyespot size. In this species, artificial selection was performed on a base population to accentuate plasticity levels in wing color – to flatten and increase reaction norms [52]. Subsequent crosses and a genome-wide association study identified multiple causal loci, including a hotspot miRNA locus [52, 54-56]. These studies suggest that association studies are feasible to map plasticity loci, but artificial selection may be required to enrich causal alleles, as plasticity is often polygenic [57, 58]. It is uncertain, however, whether genetic results from studies that used artificial selection actually inform us about how plasticity evolves in natural populations.

Interspecific variation. A long-standing question in the field is how plasticity originates on a phylogeny – where a clade of species is plastic relative to the non-plastic outgroup. *Antp* was identified to boost the level of temperature-mediated eyespot size plasticity in Satyrinae [50]. Notably, the origin of temperature-mediated eyespot size plasticity at the base of Satyrinae [19] coincides with the evolutionary origin of *Antp* expression in butterfly eyespots [59-61]. In addition, *Antp* recruitment to eyespots coincides with the origin of a novel promoter in satyrid genomes that activates *Antp* expression specifically in eyespots [50] (Fig. 1f). This illustrates that a taxon-specific cis-regulatory innovation on a conserved developmental gene may have fueled or enabled the evolution of adaptive phenotypic plasticity in a large phylogenic clade of organisms, the satyrids (Fig. 1f).

Conservation potential

Little is known whether adaptive phenotypic plasticity can facilitate adaptation to climate change. Combining transcriptomic and genomic analysis, two recent studies on *B. anynana* [43, 44], and one on *Pieris napi* [45] suggest that in seasonal habitats, strong purifying selection that aligns plastic genes with recurring seasonality leads to erosion of genetic variation in those genes. This could limit the evolutionary potential of these populations to cope with further environmental change. On the flipside, relaxed selection in aseasonal habitats may allow populations with inherent plasticity to accumulate additional genetic variation [27]. Unexpected environmental perturbations may release such inherent plasticity to cope with climate change [39]. Future studies are needed to better understand how plasticity impacts short- and long-term survival of populations with diverse evolutionary histories in the era of ongoing climate change.

Conclusions & Future directions

An Eco-Evo-Devo model of plasticity. Past and recent advances on eyespot size plasticity have illustrated how plasticity can be created by genomic innovations, how these produce downstream molecular responses, and how these responses are physiologically regulated and modulated by diverse ecological conditions, making it one of the most well-studied Eco-Evo-Devo model systems of adaptive phenotypic plasticity. Swiftly evolving technologies and methodologies could help understand proximate and ultimate mechanisms in this model system at an unprecedented resolution. Below we propose some future directions, focusing on Evo-Devo aspects.

Physiological regulation. It is still poorly understood how different cues are integrated to produce similar plastic responses in eyespot size. A recent study performed shift-experiments and mapped a humidity-sensitive stage in a satyrid, *Mycalesis mineus*, to the short larval-pupal transition, which is the same window of sensitivity to temperature in *B. anynana* [33]. This suggests that titers of the same (or a different) hormone, are likely altered via different cues within the same developmental window across Satyrinae. To test this, it is important to measure the titers of 20E and/or other insect hormones at the identified window, and test whether these changes are causal using hormone agonist/antagonist injections. These experiments can be performed across multiple satyrids with diverse cue use strategies to understand the extent of conservation of such physiological regulations on a phylogeny.

Genomic loci of plasticity. Little is known about the genomic loci underlying diverging levels of plasticity in eyespot size in any satyrid. Genotype-phenotype association analysis could potentially map the genomic loci underlying intraspecific variation in plasticity levels in eyespot size. This could be performed in parallel across deeply diverged satyrids to understand whether any hotspot loci appear as recurrent targets of selection. Similar experiments can focus on alternative cues, elucidating how genetic mechanisms of plasticity diverge in response to different ecological conditions. Recent advances in color pattern quantification using convolutional neural networks offer great potential to facilitate large-scale morphological measurements in such association studies [62-64].

Evolution of plasticity GRNs. Understanding gene regulatory networks (GRNs) underlying plasticity is essential to unravel the detailed genetic mechanisms of how plasticity evolves. The expression of *Antp* in satyrid eyespots is conserved but its effects on eyespot plasticity levels differ across the clade [50], suggesting evolution in the underlying GRNs. Rapid advances in single-cell multi-omics and deep learning models [65] provide new powerful tools to resolve how molecular profiles (gene expression, open chromatin) can be assembled in cell-type-specific GRNs, such as in *Antp*-positive eyespot center cells, and how such GRNs respond to cues. It is possible that temperature-sensitive expression of *Antp* is mediated by 20E signaling. To test this, *EcR* binding profiles can be generated using ChIP-seq. Gene expression responsiveness of *Antp* can be tested following 20E agonist/antagonist injections. Regulatory connections between *EcR* and *Antp* can be examined by disrupting one gene and examining the expression of the other. Comparative analyses, spanning satyrid species, or different eyespots from the same satyrid with different patterns of plasticity [18, 20], can shed light on how environmental responsiveness of eyespot GRNs evolve under different selective regimes.

Broader study systems. Many butterflies from the tribe *Junoniini* (Lepidoptera: Nymphalidae) also display taxon/species-specific seasonal plasticity in a suit of morphological traits including eyespot size [19, 66]. Investigations in these systems can highlight the extent that molecular mechanisms of eyespot size plasticity in Satyrinae are used in *Junoniini*. Also, in both *Junoniini* and Satyrinae, seasonal plasticity usually involves coordinated responses across morphological, behavioral, and life-history traits [20, 46, 66]. The extent that evolution of plasticity across multiple traits can be achieved via changes on small-effect modular loci, or large-effect pleiotropic loci is unknown. Future studies on these extended systems should be undertaken using parallel approaches.

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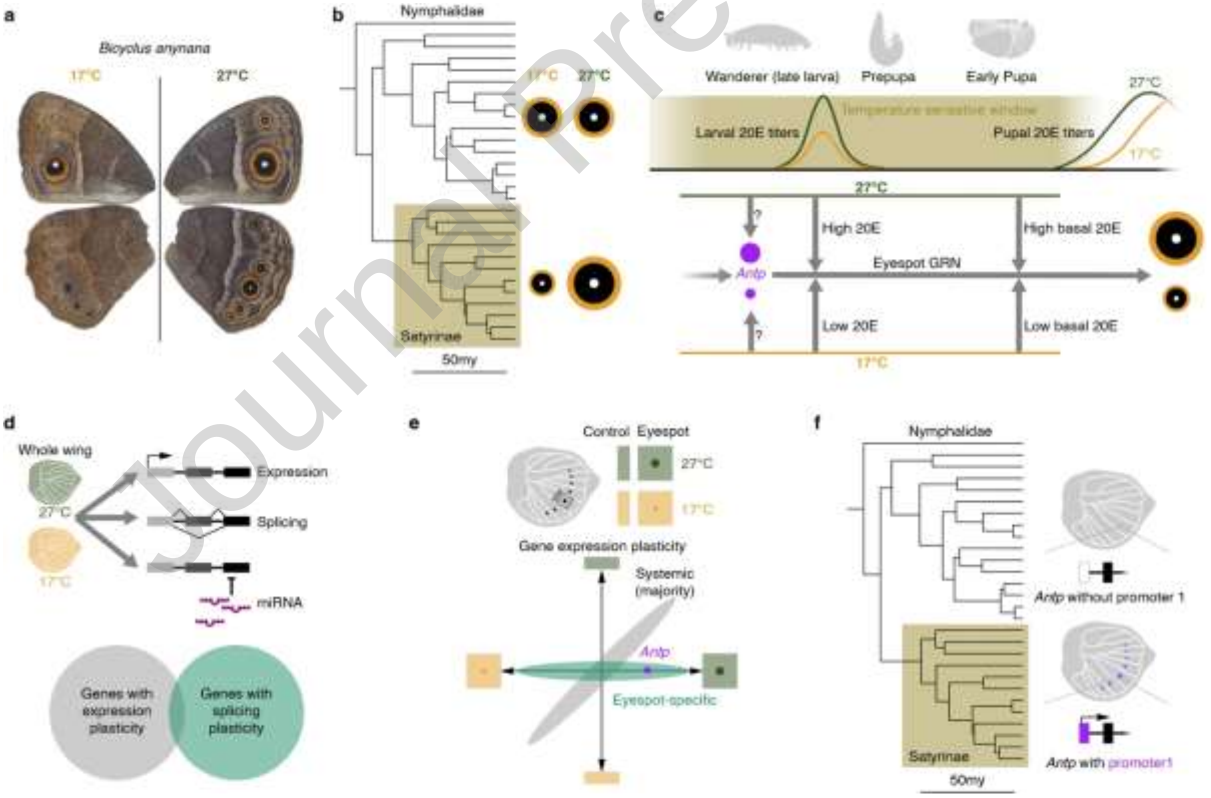

Fig. 1. Eyespot size plasticity in satyrid butterflies. (a) A model satyrid butterfly, *Bicyclus anynana*, exhibits plasticity in the size of eyespots, especially those from ventral hindwings, in response to temperature [18]. **(b)** This mode of temperature-mediated plasticity in hindwing eyespot size is estimated to have evolved ~60 million years ago in the Satyrinae subfamily. [19]. **(c)** Temperatures experienced during the short larval-pupal transition (shaded) alter titers

of the hormone 20-hydroxyecdysone (20E), which mediate the development of eyespot plasticity [18, 38, 50]. A key eyespot size regulator, *Antennapedia* (*Antp*), shows expression plasticity specifically in eyespots early in this transition [50]. GRN, gene-regulatory network. **(d)** Temperature imposes extensive transcriptional and post-transcriptional molecular responses during whole wing development, with largely non-overlapping sets of genes exhibiting responses in gene expression and alternative splicing [40]. **(e)** When micro-dissections of eyespot and control tissues are performed it becomes clear that most of these responses are systemic; *Antp* is among the smaller set of genes showing eyespot-specific expression plasticity [50]. **(f)** A novel promoter (promoter 1) discovered in satyrid genomes activates the expression of *Antp* specifically in eyespots, and it functionally boosts temperature-mediated plasticity levels in the model *B. anynana* [50].

Declaration of interest

Both authors declare no conflict of interest in writing this review.