BioEssays
Ideas that Push the Boundaries

Eye genes expressed on wings. This image shows ommatidia containing red-reflecting screening pigments (photo courtesy of Doekela Stavenga) deployed on the wings of an *Heliconius* butterfly (photo courtesy of Chris Jiggins). On pages 181–186 of this issue Antónia Monteiro reviews recent discoveries that suggest that eye-like organs on the wings of Eohelea midges, and red patches on the wings of *Heliconus* butterflies, may have originated from redeployments of the eye gene regulatory network on the wings of these insects. Monteiro also develops an empirical framework to help recognize when such co-option events underlie the origin of novel traits. The doctored image conveys the concept of ectopic expression in the creation of novel traits.
Backcover by A. Monteiro

Gene regulatory networks reused to build novel traits

Co-option of an eye-related gene regulatory network in eye-like organs and red wing patches on insect wings is suggested by *optix* expression

Antónia Monteiro

Co-option of the eye developmental gene regulatory network may have led to the appearance of novel functional traits on the wings of flies and butterflies. The first trait is a recently described wing organ in a species of extinct midge resembling the outer layers of the midge's own compound eye. The second trait is red pigment patches on *Heliconius* butterfly wings connected to the expression of an eye selector gene, *optix*. These examples, as well as others, are discussed regarding the type of empirical evidence and burden of proof that have been used to infer gene network co-option underlying the origin of novel traits. A conceptual framework describing increasing confidence in inference of network co-option is proposed. Novel research directions to facilitate inference of network co-option are also highlighted, especially in cases where the pre-existent and novel traits do not resemble each other.

Keywords:
- co-option; evolution; eyeless; gene regulatory networks; optix

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Abbreviation:
GRN, gene regulatory network.

Introduction

Understanding the origin and evolution of novel complex traits in organisms is arguably one of the most important goals in evolutionary developmental biology [1, 2]. One of the most widely recognized complex traits in organisms, the eye, has been the focus of constant attention. All metazoan eyes appear to share a small orthologous set of genes responsible for initiating eye development, for filtering light and for light reception, but the evolution of eyes and of their underlying gene regulatory networks (GRNs) has proceeded quite independently in different animal lineages [3, 4]. Despite the complexity of some eyes, their evolution and that of their underlying GRN is widely believed to have occurred over a long series of small steps from simpler initial forms and simpler GRNs [5].

But, are all new complex traits built the same way, that is in a gradual fashion taking thousands or millions of years of evolutionary tinkering? Or can novel traits originate rapidly by the co-option of pre-existing and already evolved complex gene networks?

Below I will review two recently described traits that have been, or can be interpreted as originating via instances of gene network co-option, respectively. These studies suggest that an eye network co-option event resulted in the origin of novel and distinct functional organismal traits: red wing pigments used to warn predators of a butterflies' likely unpalatability [6], and a light-reflecting wing organ, possibly used to attract prey [7]. I then briefly review the type of data that has been used in the field of evo-devo to infer gene network co-option, and propose different empirical approaches that can be used to infer co-option with increasing levels of confidence. This empirical framework may be especially useful in cases where the pre-existing and novel trait (resulting from a co-opted network) do not resemble each other.

Eye networks reused to build novel traits

The article of Reed and colleagues, recently published in *Science* [6], as well as a previous article by Dinwiddie and

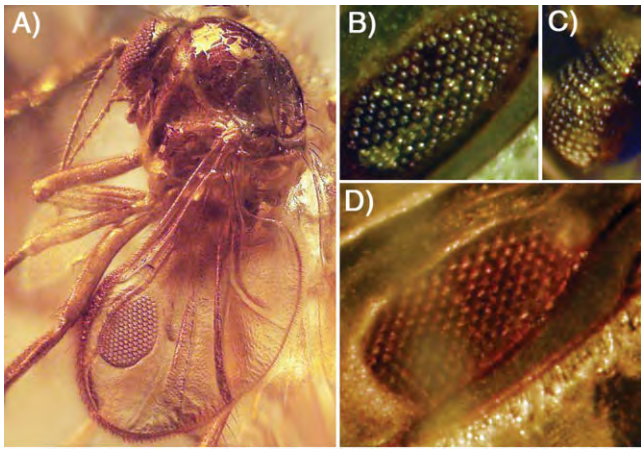

Figure 1. The extinct biting midge, *Eohelea petrunkevitchi*, with a unique wing organ that resembles the surface of its compound eye [7]. **A**: Photo courtesy of Volker Arnold. **B–D**: Photos courtesy of April Dinwiddie. **B**: The dorsal surface of the wing organ. **C**: The midge's compound eye. **D**: The ventral surface of the wing organ.

Rachootin [7], published earlier this year in *Biology Letters*, provide some new and exciting evidence for the possible role of gene network co-option underlying the origin of novel complex traits.

The two articles are, on the surface, very different. One examines a small protruding wing organ present on the wings of very small extinct dipteran insects, midges, who were beautifully preserved in Baltic amber, while the other deals with the identification of a gene, that is involved in the production of red color patterns in three species of tropical *Heliconius* butterflies.

Below the surface, there is more than meets the eye, so to speak. Both the three-dimensional wing organ and the red wing coloration are likely to have evolved from the deployment, in whole or in part, of each insect's eye GRN on its wings, in each case, giving rise to unique novel traits.

In the midge study the work revolved around carefully grinding down and polishing the amber in order to allow for a clear visualization of the embedded insects and their unique wing organs. Females of the species display a slightly protruding organ on the tip of their wings, which has an uncanny resemblance to the eye units, or ommatidia, on the insect's own head. The surface morphology and pigmentation of this oval structure is almost indistinguishable from that of the midge's eyes (Fig. 1). Given the physical resemblance, the authors propose that parts of the eye GRN, but only those parts involved in building apical eye structures, were deployed on the wing to build the novel wing organ.

In the butterfly study, the gene *optix* was identified as the most likely candidate gene associated with the development of red patches of pigmentation on the wings of *Heliconius* butterflies (Fig. 2). Despite this important achievement of linking particular morphologies to particular alleles of a single gene in a non-model organism, no discussion was offered on why *optix*, rather than some other random transcription factor, was the gene found associated with the red wing coloration. Here I offer such a discussion and propose that *optix* was likely co-opted for the control of red pigmentation due to its previously demonstrated connection to eye development in flies [8], which includes the production of red filtering pigments, important for vision [4].

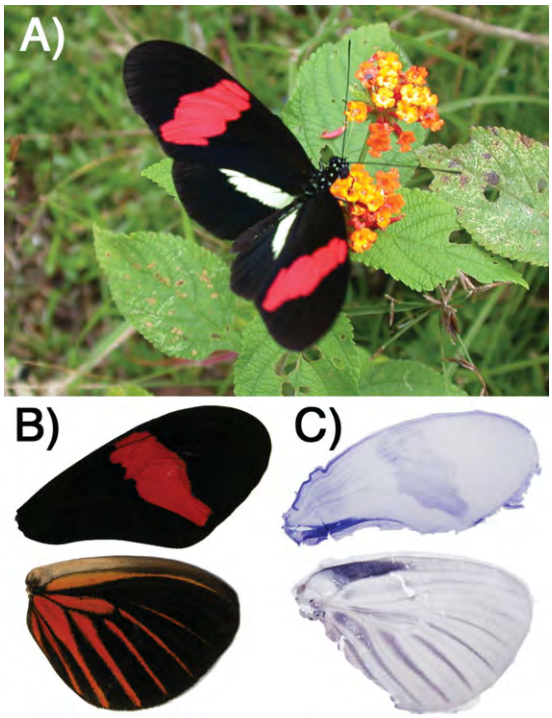

Figure 2. *Heliconius* butterflies express *optix* in wing epidermal cells that will produce red ommochrome pigments. **A**: *Heliconius erato*. **B**: Forewing and hindwing patterns from different races of *H. erato* (top: *H. e. petiverana*; bottom: *H. e. erato*). **C**: Pupal wings expressing *optix* mRNA in a pattern corresponding to the areas of red pigment in wings depicted in (B). Photos courtesy of Robert Reed.

Optix and other master regulators function in eye development

Optix is expressed in a variety of tissues during *Drosophila* development [8–10] but its functional role so far has only been connected to eye development. Mis-expression of *optix* in the imaginal discs of flies leads to the development of ectopic eyes at those locations [8] (Fig. 3A). *Optix*, however, is not the only known selector gene with these properties. The most famous of these genes is *eyeless*, which was the first eye "master regulatory gene" to be discovered in flies [11]. By mis-expressing *eyeless* on the wings of flies using the Gal4-UAS system, bulging red-colored ommatidia appeared on the fly's wings as well as on the legs and antenna (Fig. 3B). In one of the GAL4 lines described in this study, GAL4 was induced at low levels on the wing, leading presumably to weaker expression of *eyeless* on the wing, and only red pigment but no other eye structures were formed. So, depending on levels of *eyeless* activation, either the complete eye or components of the eye, red pigments, formed on the wing. Unlike the *eyeless* experiments, however, no weak ectopic *optix* expression lines were investigated [8]. It is thus unclear whether ectopic *optix* would, as with *eyeless*, merely lead to the production of red

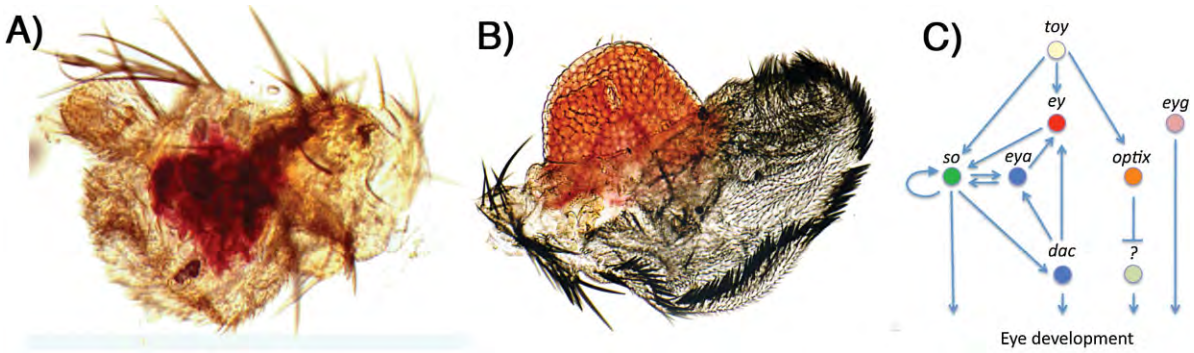

Figure 3. Ectopic expression of eye regulatory network genes in flies. **A**: Ectopic expression of *optix* in antennal discs leads to ectopic red pigment in adult antennae [8] (photo courtesy of Makiko Seimiya and Walter Gehring). **B**: Ectopic expression of *eyeless* in wing discs leads to ectopic eyes [11] (photo courtesy of Georg Halder). **C**: The pattern of gene connections between several top regulatory genes in the eye network (adapted from [5]).

pigments with no associated additional eye structures if expressed at low levels on the fly's wing.

Is the eye gene regulatory network composed of smaller sub-networks?

The differential behavior of the fly's eye GRN to levels of *eyeless* suggests two things: (1) that different parts of the network are modular [12], and can be activated directly by this top regulatory gene, independently of the development of other modules and (2) that different modules inside the larger gene network require different thresholds of activation by the *eyeless* master regulator. These eye network properties, if conserved from flies across butterflies, can potentially explain why the co-option of a top eye master regulatory gene may be causally and directly implicated in the evolution of red pigmentation on the wings of butterflies. A modular sub-network structure may equally explain why the cuticle facets present in the apical part of each ommatidium also appeared on the wings of the extinct midge, instead of a complete eye.

Modularity of sub-networks inside a larger eye network offers distinct paths to the origin of novel traits that rely only on sub-network function. One possibility, highlighted above regarding *optix*, involves the co-option of the master regulator into a novel context, but at a different expression level from the endogenous eye context, leading to the activation of only those sub-networks that require lower levels of master gene expression to pass an activation threshold. The second possibility is the co-option of the master regulator at normal expression levels, followed by the ''shedding'' of sub-modules that are not required for the network's novel ecological function.

Did all red wing pigments come from eyes?

The presence of red pigmentation in insect's wings is widespread and this raises the question of whether red pigments in other insect species are also associated with *optix*. The answer, partly addressed in the Reed et al. study [6], suggests that this is not the case. A distantly related butterfly species, *Vanessa cardui*, did not express *optix* associated with regions of red pigment of its wings. Future research in this area could potentially explore the extent that other master regulators of the eye GRN could have been co-opted in these other species. The eye GRN in flies consists of a series of interconnected master regulators that when ectopically expressed are sufficient, in a somewhat tissue-dependent manner, to induce red ectopic eyes [5] (Fig. 3C). These other genes, including *eyeless*, should be seen as novel candidate transcription factors associated with red pigmentation in insect wings.

To what extent many of the pigments seen today on the wings of butterflies were first deployed in eye development, as screening pigments, is unknown. But it is possible that the eye has been a source of many such pigments. Previous work characterized the red pigments in *Heliconius* wings as ommochromes [13–15], and proposed that the ommochrome biosynthetic pathway originally deployed in the eye, had been co-opted to the wing [15]. It appears that this was indeed the case, but the co-option was not done in a stepwise fashion. Instead a single gene, that is known to regulate all the elements of this pathway as well as other eye components, *optix*, was co-opted to the wing.

While work in *Drosophila* has identified the pterine and the ommochrome pathways involved in building the red and the brown pigments deposited in the eyes of flies, respectively [16, 17], little is currently known about the type of screening pigments that butterflies and other insects have in their eyes [4]. It is known, however, that the different colors that butterfly eyes exhibit are a combination of screening pigments, mostly black or red, often apically covered by other pigments that may participate in the overall camouflage of the animals [17]. A biochemical characterization of the types of screening pigments present across a wide variety of insect species is a necessary first step in advancing any type of work that involves evolutionary analysis of insect pigmentation and the order of the evolutionary co-option of these pathways onto the insect's body, or conversely, from the body to the eye.

Co-option is usually followed by network modification

Co-option of a master regulator to a precise stripe of cells on the *Heliconius* butterfly wing should lead to the expression of

the downstream target genes, such as ommochrome pigment synthesis genes, in similar tightly regulated expression patterns. This prediction, however, is only partly supported in *Heliconius*. There is co-expression of necessary enzyme-coding genes for the production of ommochrome pigments at the stripe location in *Heliconius* wings [18], but the domains of expression of these genes are not perfectly associated with the *optix* expression domain. This suggests that after a co-option event, the expression domains of individual components of the regulatory network can be modified. For instance, in the red-banded *Heliconius erato cyrbia*, *vermillion* is expressed not only along the band region, but also in more proximal and distal regions of the wing [18]. This gene expression expansion, probably due to neutral processes, carries no phenotypic consequence, as the other necessary ommochrome synthesis enzymes are absent from these wing regions. In *Heliconius erato himera*, a race with a yellow band instead of a red band, *vermillion* expression has been lost [18], indicating an interruption to the complete biosynthetic pathway ancestrally deployed at that location, and the appearance of the respective intermediate pigments in the ommochrome biosynthetic pathway.

Loss of expression in genes coding for individual members of the melanin pigment synthesis pathway has also been previously described for members of the genus *Drosophila*. Here loss of *yellow* expression via modifications to its *cis*-regulatory region led to loss of black wing spots in two independent fly lineages [19]. In *Drosophila*, however, the master control gene that leads to the co-expression of all the necessary melanin pathway genes in spots on the wing has yet to be discovered.

The concept of co-option is evolving from single genes to whole gene regulatory networks

Gene network co-option is a likely mechanism underlying the origin of novel complex traits, but the empirical evidence gathered to support this mechanism has been quite variable across studies, including those described above. In the early days of evo-devo, the focus was more on single gene co-option than on the co-option of larger GRNs, and many of these genes were end products of GRNs [20]. For instance, the co-option of enzymes into becoming lens crystalins in a variety of eyes, was offered as an example of such a mechanism [21]. Later, the co-option of regulatory genes began to be described. Early in the field, the mere discovery of one or two common transcription factors, signaling pathways, or gene circuits, that were expressed in the early development of two non-homologous traits in the same organism [22–24], or in doubtful orthologous traits in different organisms [25], was usually sufficient to invoke gene network co-option underlying the origin of the newest trait. Now inference of gene network co-option is involving information from many more shared genes [22, 26–32]. Information about their pattern of functional connectivity, however, the actual shared "network", is still usually lacking.

Inferring the origin of novel traits via gene network co-option can also be complicated if the appearance of the novel trait does not immediately resemble any of the other pre-existent traits in the organism. One possibility for the lack of similarity in traits that originate from similar networks, is that only modular parts of pre-existing networks are co-opted, and then these parts are later re-wired to other modules to produce entirely novel and hybrid traits.

When two distinct-looking traits are suspected of sharing similar sub-networks, it becomes important to investigate the details of these sub-networks at the molecular level to determine whether or not they are process homologous [33, 34].

How can we infer gene network co-option?

Regulatory connections between genes create a network. These connections consist of transcription factors activating other transcription factors or members of cell-signaling pathways. When a modular network is co-opted into a novel location in the body, via the recruitment of a top regulatory gene, the network has the potential to be activated at the novel location using the *same exact* genetic architecture. This means that not only should the same genes be present in both contexts, they should also be activated in the same temporal order, retain their activating or repressing functions, and retain the same *cis*-regulatory elements that now function both in the ancestral as well as in the novel developmental contexts [2]. Inference of gene network co-option can be done by examining similarities between the ancestral and the derived networks at each of these levels of analysis (Fig. 4). The first level involves discovering similarities in sub-sets of genes expressed during the development of two traits in the same organism. The second level examines whether the shared genes are expressed in similar relative temporal order during development of the two traits. The third level examines the pattern of functional interactions between genes, that is whether gene A up-regulates gene B during the development of both traits. The fourth level examines whether the *cis*-regulatory elements of genes that lie in the middle of the putative co-opted network drive gene expression in both traits 1 and 2 (Fig. 4) [2]. Each of these comparisons, on its own, is useful to infer network co-option with some level of confidence, but data from all the levels will provide the largest degree of confidence that co-option did indeed take place and the networks are homologous. It is important to realize, however, that given enough time a co-opted network will be gradually modified in the novel developmental context where it became active, adapting it to its novel function, and it is unreasonable to assume perfect conservation with the ancestral network at each of the levels proposed above [33].

Analogous (convergent) networks may originate by a de novo network building process, gene by gene, in the novel developmental context. These networks may possess similarities perhaps at the level of gene composition, temporal gene expression, or even gene function, but it is hard to imagine the evolution of a convergent network with the exact same gene regulatory connections, that is *cis*-regulatory elements involved in driving gene expression in the two separate developmental contexts. Shared *cis*-regulatory elements should be the distinctive hallmark of underlying gene network

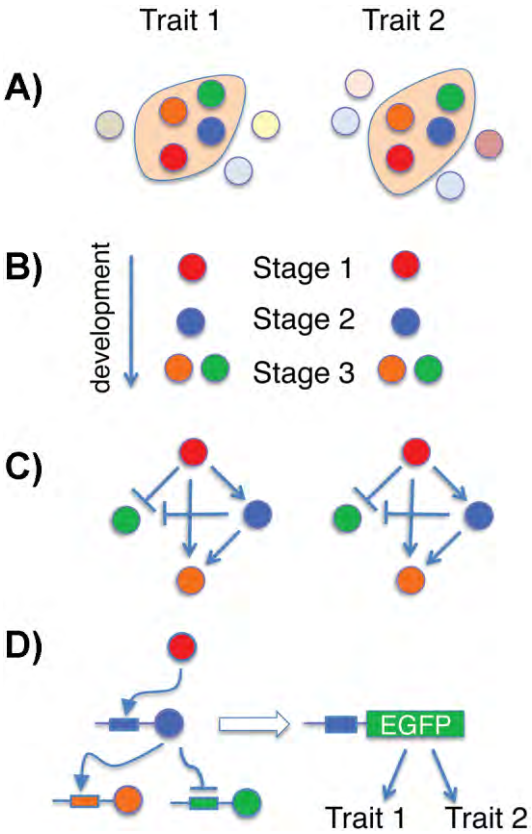

Figure 4. Distinct types of data that can be used of infer whether the same gene network underlies the development of two traits in the same organism. **A**: A common set of genes (encircled) is expressed during the development of the two traits. **B**: The genes are expressed in a similar temporal order. **C**: The genes display the same type of regulatory interactions (red represses green, blue activates orange, etc. Note that the regulatory interactions inferred may be direct or indirect). **D**: Genes internal to the shared set (expressed at developmental stages 2 or 3, but not stage 1) may contain unique *cis*-regulatory elements that drive gene expression in the two different developmental contexts. This is depicted by the isolation of the blue *cis*-regulatory element, attaching it to a reporter gene (EGFP), transforming the genome of the organism with this construct, and observing EGFP expression in the tissue precursors of the two traits.

co-option [2]. Given enough time, however, the set of shared *cis*-regulatory elements driving gene expression in the two developmental contexts may eventually duplicate or sub-functionalize [35], gradually erasing the signal used to infer shared network ancestry.

Evidence for shared *cis*-regulatory elements is present in the *yellow* gene that was likely co-opted as part of the larger melanin pigmentation pathway in the evolution of novel pigmentation spots in two distinct *Drosophila* lineages. The *cis*-regulatory elements controlling spot expression are the same elements that also drive *yellow* expression along the veins and along the entire wing epidermis, respectively [19]. The origin of the novel black spots, however, was not interpreted as being the result of a large gene network co-option event, where *yellow* is just part of a modular GRN. The origin of the spots was interpreted as being the result of piecemeal gene co-option, with evolution at *yellow* being responsible for the co-option event. This is highly unlikely, given that *yellow* expression alone at the spot location is insufficient to produce black pigmentation [36, 37].

Conclusions and prospects

There may essentially be two distinct ways for complex traits to evolve in organisms – a gradual way, where novel traits evolve through the gradual addition of elements to basic regulatory gene networks – and a saltational way, where novel traits evolve by the co-option of whole or parts of pre-existent networks, deployed in novel locations of the body and later modified to perform novel functions. Traits likely to have evolved under the gradual mechanism include the multiple different eye networks across the Metazoa. Traits likely to have evolved under the saltational mechanism include the expression of red pigments on butterfly wings, and the eye-like organ on the wings of an extinct species of midge. Both of these latter traits may have originated from the co-option of the pre-existent eye regulatory gene network into the wings, but additional work is required to test this hypothesis more rigorously. In order to detect such instances of gene network co-option, it is important to rigorously distinguish homologous from convergent gene networks. Here I proposed an empirical framework for detecting co-option and distinguishing it from convergence. The framework includes describing similarities across gene networks in different parts of the body focusing on distinct levels of network organization: (1) gene composition; (2) gene temporal expression order; (3) gene function; and (4) conservation of pleiotropic *cis*-regulatory elements in genes deployed in the middle of the network. Similarities detected across all these levels would imply that a homologous gene network was deployed to build a non-homologous novel complex trait.

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