

THE GENETICS AND EVO–DEVO OF BUTTERFLY WING PATTERNS

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Understanding how the spectacular diversity of colour patterns on butterfly wings is shaped by natural selection, and how particular pattern elements are generated, has been the focus of both evolutionary and developmental biologists. The growing field of evolutionary developmental biology has now begun to provide a link between genetic variation and the phenotypes that are produced by developmental processes and that are sorted by natural selection. Butterfly wing patterns are set to become one of the few examples of morphological diversity to be studied successfully at many levels of biological organization, and thus to yield a more complete picture of adaptive morphological evolution.

PHENOTYPIC PLASTICITY

The variation in phenotype of a given genotype when the individuals complete their development in different environments.

In recent years, there has been renewed interest in bringing together the fields of evolutionary and developmental biology — ‘evo–devo’^{1,2}. Much of this interest was stimulated by advances in developmental genetics and the finding that shared genetic pathways underlie the development of organisms with sometimes very different body plans — the cornerstone example being the involvement of homeobox-containing genes in establishing axial polarity³. Although much work has concentrated on comparing morphologies and developmental mechanisms among distantly related species, the importance of comparisons among closely related species⁴ and studies of variation within species⁵ are being increasingly recognized. Development translates genotypes into phenotypes and generates the morphological variants on which selection can act. The colourful patterns on butterfly wings provide an ideal opportunity to study the interactions between the evolutionary and developmental processes that shape morphological variation both within and among species^{6–9}.

Variation in wing patterns in particular species has provided the material for numerous studies of natural selection, genetic variation and aspects of pattern development (FIG. 1). In this review, we describe how the spectacular morphological diversity in butterfly wing patterns, coupled with studies of their ecological relevance and their underlying developmental basis, places them in an ideal position for an integrated

study of morphological evolution. To make full use of this potential, future work needs to focus on integrating the knowledge obtained at different levels of organization and of different patterns among species from various families.

Evo–devo of butterfly wings

The diverse patterns of colours and shapes that decorate the wings of butterflies have long fascinated biologists and laymen alike. These patterns are interesting from an evolutionary perspective because they represent visually compelling products of selection and often have a known adaptive value. Variation in lepidopteran (moth and butterfly) wing patterns includes some classical examples of adaptation that involve both genetic polymorphisms and PHENOTYPIC PLASTICITY in response to the environment. Some of these examples are discussed below. Butterfly wing-colour patterns are also attractive from a developmental perspective because, compared with many other morphologies, they are structurally simple and are amenable to developmental characterization. Wing patterns are formed by the spatially ordered arrangement of monochromatic scale cells on a single cell layer (BOX 1). This two-dimensional arrangement makes their developmental dissection much simpler relative to three-dimensional morphological structures, such as appendages or bristles. Wing-pattern formation has been characterized at different

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doi:10.1038/nrg818

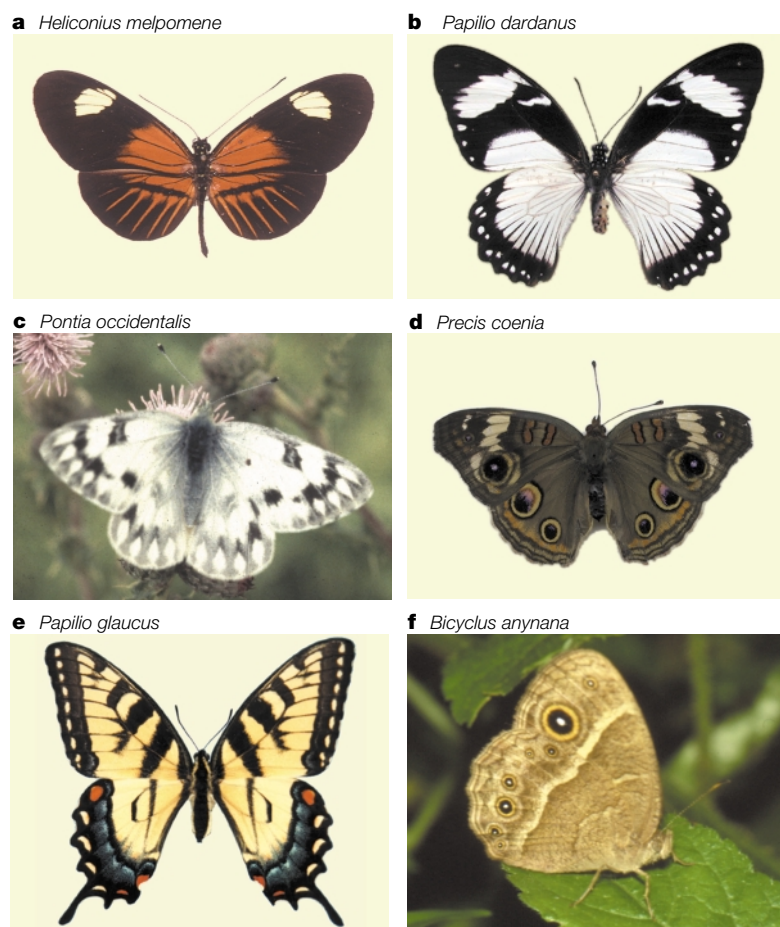


Figure 1 | Examples of model butterfly species. Evo–devo studies of butterfly wing patterns have concentrated on a few model species, which provide different opportunities for research. **a** | *Heliconius melpomene* and **b** | *Papilio dardanus* butterflies are classical examples of species involved in polymorphic anti-predator mimicry systems. **c** | *Pontia occidentalis* has been used to study phenotypic plasticity, which, in this species, has a known ecological relevance. **d** | *Precis coenia* has been the classical model for the developmental dissection of wing-pattern formation. **e** | *Papilio* butterflies, including *P. glaucus*, have a characteristic pigment system and have been a favourite model for characterizing the pigment biosynthesis pathways. **f** | *Bicyclus anynana* has been successfully established in the laboratory and, during the past 12 years, has become the butterfly ‘lab-rat’, as it is amenable to controlled population and organism-level approaches to the study of wing-pattern variation and development. *H. melpomene* courtesy of Jim Mallet, University College London, UK; *P. dardanus* courtesy of Fred Nijhout, Duke University, North Carolina, USA; *P. occidentalis* courtesy of Joel Kingsolver, University of North Carolina, USA; *P. coenia* courtesy of Scott Weatherbee and Sean Carroll, University of Wisconsin, Madison, USA; *P. glaucus* courtesy of Richard ffrench-Constant, University of Bath, UK.

developmental levels, making use of diverse methods (BOX 2). The size of butterflies makes them good model systems for an integrated study of morphological evolution. They are sufficiently small that they can be maintained and examined in large populations (essential for population-level studies), but large enough that they can be individually manipulated at all developmental stages (ideal for organism-level studies). Studies of butterfly wing patterns have traditionally involved morphological descriptions and comparisons among species, and the use of surgical manipulation to characterize pattern formation. In the past decade, other methods have begun to identify the molecules that are involved in wing-pattern formation; these methods

APOSEMATIC

Describes a conspicuous colour pattern that is associated with venomous or distasteful prey and serves as a warning signal to potential predators.

CRYPISIS

The property of those colour patterns that resemble the background as perceived by predators that hunt by sight. Cryptic individuals are well camouflaged.

have resulted in a better understanding of the physiological basis of phenotypic plasticity, pigment biosynthetic pathways and the genetic pathways that are involved in wing patterning. Progress in butterfly wing-pattern research will certainly benefit from advances in the fruitfly *Drosophila melanogaster* and the silk moth *Bombyx mori*, which is a lepidopteran of high economic value and with a fairly advanced genetic toolkit¹⁰, including a genome project and transgenics techniques. Much work on wing-pattern development has focused on particular types of pattern in a small number of species (FIG. 1). Here, we describe these studies and discuss their contribution to our understanding of other types of pattern.

Ecology and lepidopteran wing patterns

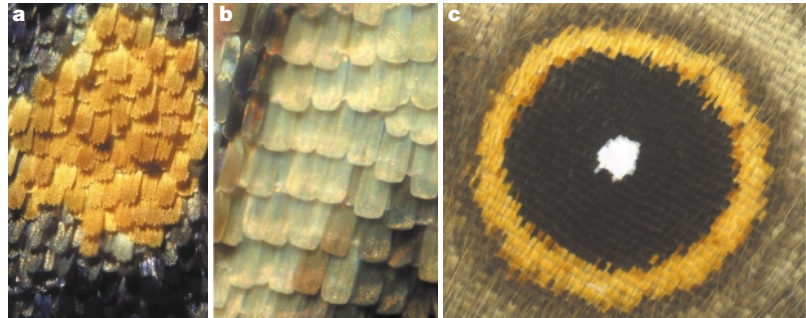
The colour patterns on butterfly wings often function in intra- or interspecific visual communication, or in relation to the physical environment. Many examples of visual communication that are based on wing-colour patterns involve interactions with predators¹¹, especially through APOSEMATIC warning coloration¹² or CRYPISIS^{13,14}. Examples of other types of ecological interaction that are mediated by colour patterns include intraspecific recognition, mate localization and SEXUAL SELECTION^{15–18}.

Genetic polymorphisms. Probably the most popular example of adaptive evolution of wing patterns involves the peppered moth *Biston betularia*. Industrial melanism in this species has long been a favourite textbook example of adaptive evolution in response to man-made effects on the environment. Changes in the frequency of paler and darker forms of *B. betularia* have occurred in response to changes in the levels of air pollution^{19,20}. This occurs, at least in part, owing to the darkening effects of pollution on the resting background of the moth and concomitant changes in its relative crypsis for escaping the attention of predators²¹. Another ‘showcase for Darwinian selection’ is the evolution of anti-predator visual mimicry^{22,23}. Predatory birds can learn to associate particular bright and colourful patterns with distasteful prey; by sharing the same aposematic colour pattern with distasteful butterflies, mimetic species gain additional protection from predatory birds. Classical examples include the swallowtail *Papilio dardanus*, whose palatable females have morphs that mimic unpalatable ‘model’ species, and distasteful species of the genus *Heliconius*, who share colour patterns, but for whom the mimetic pattern can vary in different geographical regions.

Seasonal polyphenisms. Butterflies also include examples of marked variation in wing-colour patterns in response to the environment. In some butterflies, this phenotypic plasticity occurs in the form of seasonal polyphenism, in which alternative phenotypes are adapted to the particular seasonal environment in which they spend their adult life²⁴ (BOX 3). The environmental variable that is experienced at an early stage of development acts as a predictable cue for the environment that the organism will encounter in the adult

Box 1 | **Butterfly wing scales: the evolutionary novelty that underlies colour patterns**

The Lepidoptera (butterflies and moths) are so named because of their scale-covered wings. Both the dorsal and ventral surface of an adult wing is covered by a mosaic of partially overlapping scales (see panels a, b), the cuticle structure and pigmentation of which produces the visible



patterns of colour. Late in wing development, some epidermal cells produce scales in which a single colour pigment is deposited. Scales of different colours differ also in ultrastructure⁶¹, and their pigments are laid down at different stages shortly before adult emergence^{53,78}. The molecular nature of some of the pigments and of the enzymes that are responsible for their synthesis has been partly described^{79–81}. These coloured scale cells are the unit basis of colour patterns, such as the eyespot (see panel c); they have been a key innovation in early lepidopteran evolution and have been fundamental for the later diversification in wing-colour patterns.

On the basis of morphological and molecular evidence, it has been proposed that lepidopteran wing scales are homologous structures to the sensory bristles of the insect peripheral nervous system, which has been a favourite model in both developmental and quantitative-genetic studies. A homologue of one of the *Drosophila melanogaster* *Achaete-scute Complex* (ASC) genes, which encode transcription factors that promote neural-precursor formation, was cloned in the butterfly *Precis coenia* (*ASH1*, ASC homologue)⁸². In butterfly larvae, *ASH1* is expressed in the putative sensory mother cells of the wing IMAGINAL DISCS and, in the pupa, it is found in each of the evenly spaced cells that contributes to the development of an individual wing scale. The use of a common genetic circuitry in sensory bristles and wing scales supports the contention that these structures are homologous. Galant *et al.*⁸² propose that, during the evolution of the basal Lepidoptera, the basic function of ASC genes in the development of non-innervated bristles was maintained. However, genetic changes in the control of cell lineage also occurred, so that the neuron and glial cells that are normally associated with bristles were lost, and bristles covered the entire wing. As the lepidopteran lineage evolved, genes that were downstream of ASC are thought to have changed the cytoarchitecture and pigmentation as bristles were modified into flattened, pigmented scales. Images courtesy of Sean Carroll, Steve Paddock, Mike Serfas and Scott Weatherbee, University of Wisconsin, Madison, USA.

stage, when the patterns are visible and selection occurs. For example, alternative wing-pattern phenotypes are associated with different seasonal strategies for thermoregulation in *Pontia occidentalis*^{25,26} and for predator avoidance in *Bicyclus anynana*⁸.

Diversity of wing patterns

Most of the more than 17,000 species of butterfly can be distinguished by their wing colours⁶. Wing patterns can also differ markedly within species that have genetic polymorphisms^{27,28} or seasonal polyphenism²⁴. Furthermore, many species are sexually dimorphic, and others contain individuals with different patterns on their dorsal and ventral wing surfaces (for examples, see REF. 6).

Pattern elements and the nymphalid groundplan.

According to a classical model that describes wing-pattern morphology, all diverse patterns can be viewed as derivations of an idealized 'nymphalid groundplan' (popularized by the works of Fred Nijhout⁶). This groundplan is composed of different types of pattern element (for example, chevrons, eyespots and bands) that are arranged in several parallel rows of homologous pattern elements; these are serially repeated in defined wing compartments that are circumscribed by veins

(FIG. 2). More or less profound changes in the number, colour, position, shape and size of individual elements can explain the derivation of different wing patterns from this basic groundplan⁶. The groundplan has been extremely useful to interpret and compare wing patterns through the establishment of homologies between pattern elements found within and among species^{6,29,30}. However, the nymphalid groundplan does not explain all the described wing morphologies and might not be as universal a description of butterfly wing patterns as supposed³¹. Furthermore, the developmental dissection of wing-pattern formation described below indicates that the homologies across different types of pattern based on morphological data are unlikely to correspond to homology in terms of development.

Modularity and evolutionary flexibility. It has been proposed that the spectacular diversification in butterfly wing patterns has been possible owing to the evolutionary and developmental flexibility that arises from their organization in different series (or types) and from their distribution in individual wing regions^{32,33}. Studies of variation in wing patterns within and among species have shown that different types of pattern element (that is, those belonging to different series) are independent, but also that there are correlations across homologous

SEXUAL SELECTION

The selection that results from differential mating success. It includes competition for mates (usually among males) and mate choice (usually by females).

IMAGINAL DISC

Sac-like infolding of the epithelium in the larva. They give rise to most of the external structures of the adult. Imaginal disc cells are set aside in the embryo and continue to divide until pupation, when they differentiate.

Box 2 | **Toolbox for evo-devo laboratory research on butterfly wing patterns**

Various methods have been used to analyse the two-dimensional patterns on butterfly wings. These have taken advantage of the tractable size of butterfly life stages and knowledge from insect model systems.

Comparative method

Most early work on butterfly wing patterns involved comparing morphologies among species⁶. Such studies resulted in the description of an idealized wing pattern, the nymphalid groundplan (see main text), which has been useful for many more comparative studies. The current availability of phylogenies for different butterfly taxa^{83–87} provides a solid framework for comparing patterns and their developmental bases.

Experimental crosses

Mass matings or single-pair crosses between butterflies with distinct wing patterns have been classically used to characterize the Mendelian genetics that underlie such diversity^{40–42}. The phenotypic variation that is observed in the progeny from such crosses gives valuable information about the number of loci that are involved and about their dominance and epistasis relationships⁴⁴. Controlled crosses are also essential for many gene-mapping approaches.

Artificial selection

By selecting butterflies with trait values that are either above or below a certain threshold across consecutive generations of laboratory breeding, it has been possible to produce lines with divergent wing patterns. Artificial selection in *Bicyclus anynana* has shown that there is ADDITIVE GENETIC VARIANCE for several pattern phenotypes^{35,36,39,46,63,88}. By measuring the strength of the selection applied and the amount of response obtained, the HERITABILITY of the selected traits can be estimated (as defined in REF. 89).

Surgical manipulations of pupal wings

The wings lie underneath a hardened pupal cuticle and are readily available to experimental manipulation. The effect of grafting (transplant) or cautery (damage) on adult wing patterns has been classically used to characterize components of the cellular interactions that underlie pattern formation^{50,51,90}.

Biochemistry

Classical biochemical techniques have been used to identify the molecular nature of the pigments that are deposited on butterfly wing scales and the enzymes that are involved in their production^{78–81,91,92}.

Physiology

The physiological basis of wing-pattern development and of its plastic properties has been characterized by measuring hormone titres during pupal development and by studying the effect of hormone injections, infusions or brain extirpations^{45,93–95}.

Gene-expression patterns

It is technically straightforward to dissect wing imaginal discs for morphological inspection. This has been used together with *in situ* hybridization and antibody staining to study the developmental pathways that are involved in wing patterning^{46,54–56}. Such studies have been guided by our knowledge of wing development in *Drosophila melanogaster* and have focused on the genetic pathways that are described in this system.

Gene mapping

In recent years, the genetic dissection of many quantitative (and other) traits has benefited from technological developments that have surveyed molecular variation in many different species. Once polymorphic molecular markers have been found, either in known candidate genes or randomly distributed throughout the genome, their segregation with different phenotypic traits can be examined⁸⁹. Candidate genes include those that are involved in *D. melanogaster* wing development, some of which are associated with butterfly wing patterning⁶⁵. A less-biased genetic dissection of butterfly wing-pattern evolution will require genome-wide searches and involve building linkage maps for different butterfly species.

Genetic manipulation

The ability to manipulate gene expression will be essential to study the function of the genes, the expression patterns of which indicate their involvement in wing-pattern formation. There are several methods by which to manipulate gene expression, including viral ectopic expression vectors, RNA INTERFERENCE and MORPHOLINOS. So far, local ectopic gene expression in butterflies has been possible using the Sindbis virus⁹⁶; however, these experiments have had limited success. The development of germ-line transformation techniques will be fundamental for future research and will hopefully build on advances in the model lepidopteran *Bombyx mori* (the silk moth)⁹⁷.

pattern elements in a series (for example, eyespots)^{30,34–36}. Such results inspired the suggestion that pattern elements in a series might constitute a module, or individual unit, for which evolution and development occurs independently from other modules³², but in which traits tend to change in a concerted manner^{37,38}. A recent study has directly tested such ideas by taking advantage of the

laboratory population-level studies that are possible with *B. anynana* butterflies. Artificial selection experiments have shown that, despite the genetic correlations and developmental coupling between eyespots in these butterflies, there is much flexibility for the size of eyespots to evolve independently³⁹. Butterflies responded to selection in the laboratory by producing all patterns found in

ADDITIVE GENETIC VARIANCE
The genetic variance that can be statistically associated with the linear relationship between mean offspring and mid-parent values. This is the component of variance that contributes to the response to selection.

HERITABILITY
The proportion of the total phenotypic variation in a given characteristic that can be attributed to additive genetic effects.

RNA INTERFERENCE (RNAi). A process by which double-stranded RNA specifically silences the expression of homologous genes through interference with their cognate mRNA.

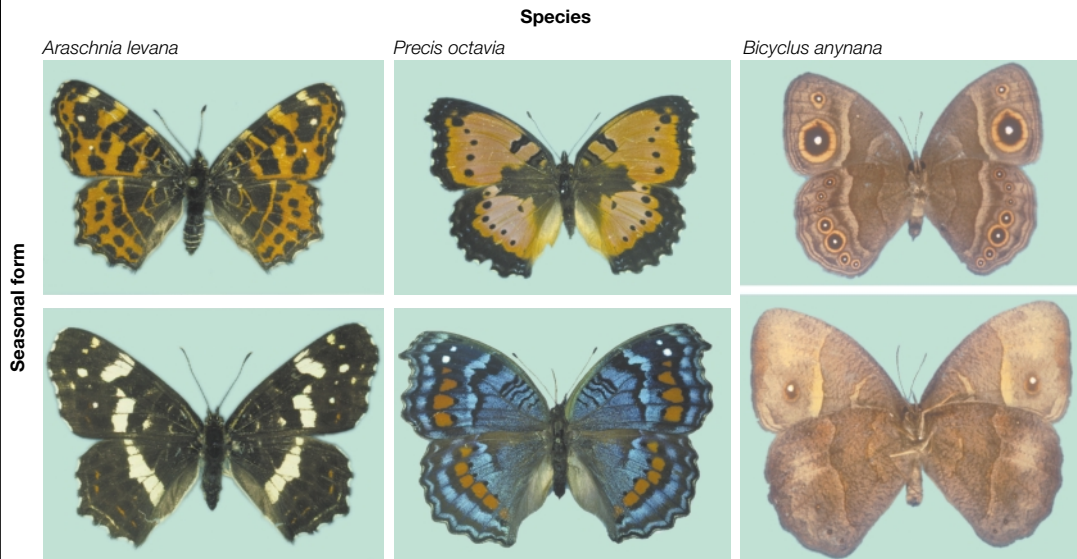
MORPHOLINO
A chemically modified oligonucleotide that behaves as an antisense RNA analogue and is therefore used to interfere with gene function.

Box 3 | **Plasticity in wing-pattern development**

In some butterfly species, genetically identical individuals can have strikingly different wing patterns, depending on the environment in which they complete their development. Phenotypic plasticity refers to such variation in the phenotype (morphological, but also life history or behaviour) of a given genotype, and represents a potential means of adapting to divergent environments. The figure shows alternative seasonal phenotypes in the species *Araschnia levana*, *Precis octavia* and *Bicyclus anynana*. These phenotypes are produced in response to the different environmental variables, such as temperature or photoperiod, that are experienced during larval development.

Phenotypic plasticity provides the potential for exploring the control of development and the interactions between gene-expression and environmental cues. Little is known about the genetic basis of plastic responses in butterflies, including whether there are specific regulatory genes for plasticity^{44,88,98}. Animals of a particular genetic composition lose the ability to produce both seasonal forms and constitutively express one of these. This effect has been described as the result of gradual responses to artificial selection in *B. anynana*⁴⁶ or of a single spontaneously occurring mutation in *Precis coenia*⁹⁹.

Ecdysteroid (EC) hormones regulate seasonal polyphenism in several butterfly species^{45,93,95,100}; alternative seasonal forms being controlled by differences in the time at which the hormone is released after pupation. The complex formed by EC and its receptor regulates gene transcription and has mainly been described in association with metamorphosis¹⁰¹. In wing-colour formation, EC interacts with the pigment biochemical pathways^{80,94}, but little else is known about the pathways on which EC impinges. Exciting challenges for the future will be to understand fully the links between sensing the larval environment and changing hormone titres in early pupae, and how ecdysteroid dynamics interact with the developmental genes that are involved in wing-pattern formation. *A. levana* and *P. octavia* photos courtesy of Fred Nijhout, Duke University, North Carolina, USA. (Reproduced with permission from REF. 6 © F. Nijhout.)



the genus and even a candidate ‘forbidden morphology’ that is not represented in any of the 80 or so *Bicyclus* species described³⁹. This work illustrates how butterfly wing patterns can be used to analyse experimentally the extent to which the evolutionary trajectories of adaptive traits are shaped not only by natural selection, but also by the range of phenotypic variants that can be generated. The developmental repertoire for forming butterfly wing patterns seems to be extraordinarily flexible. This flexibility is presumably the result of the compartmentalization of pattern elements in individual wing regions (the wing cells) and might result from the lack of physical communication between them and/or from the wing-cell-specific genetic composition that might regulate eyespot-forming genes^{9,33}. However, these potential mechanisms await experimental support. Understanding how this flexibility has evolved *per se* is fascinating, but it is equally interesting to examine how the tempo and direction of evolutionary change in natural populations

are influenced by the genetic and developmental properties of pattern formation.

Genetic variation for wing patterns

The genetic variation that underlies wing-pattern polymorphism has been partly characterized in both natural and laboratory populations of different butterfly species.

Natural populations. Mendelian crossing experiments have shown that the inheritance of pattern polymorphisms in natural populations of the classical examples *B. betularia*, *P. dardanus* and *Heliconius* spp. involves a rather small number of genes. Variation in colour forms of the moth *B. betularia* seems to be controlled primarily by at least five alleles at a single genetic locus²¹. These alleles show a dominance hierarchy, with alleles for darker forms (a larger proportion of black melanized scales) being

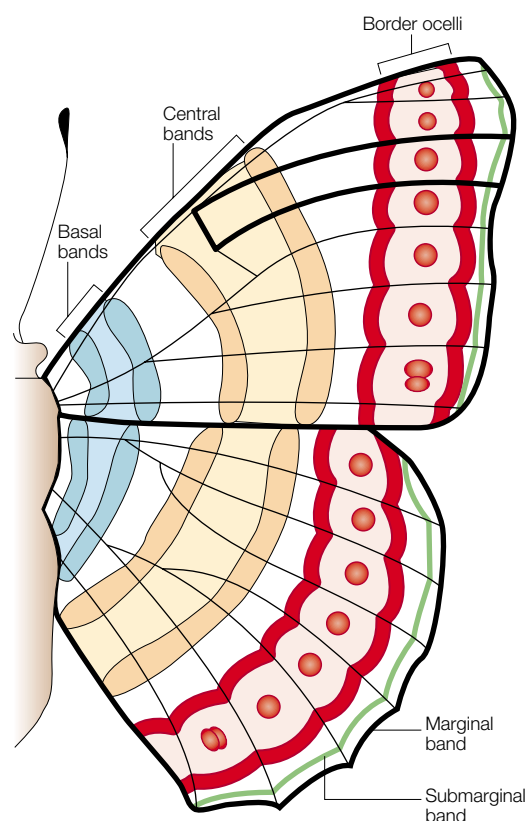


Figure 2 | The 'nymphalid groundplan'. Despite the great diversity in butterfly wing patterns, a recognizable idealized 'groundplan' has been proposed to describe most wing-pattern variants⁶. According to this groundplan, the types of pattern element (for example, eyespots, chevrons and bands) are organized in parallel series: the marginal and submarginal bands (which might form chevron elements), the border ocelli or eyespots, and the central and basal bands. Homologous pattern elements are serially repeated in individual wing compartments that are bordered by veins (thickened wing veins on the forewing illustrate the boundaries of such a compartment, known as a wing cell). Pattern elements in individual wing cells are repeated along the posterior–anterior axis and can, to a large extent, change independently of each other. The figure shows a forewing (top) and a hindwing (bottom), both orientated with the anterior part to the top and distal margin to the right. Reproduced with permission from REF. 33 © (2001) Wiley.

dominant to those producing paler forms. The GENETIC ARCHITECTURE of colour changes in mimetic systems has been studied in species of *Papilio* and *Heliconius* butterflies^{40–42} (reviewed in REF. 43). In *P. dardanus*, alleles of a single gene seem to account for all naturally occurring mimetic forms^{40,43}. The low mapping resolution of Mendelian crossing experiments cannot, however, distinguish between a single locus and several tightly linked loci. The occurrence of such a group of tightly linked loci in a super-gene has been proposed to explain variation in another mimetic swallowtail, *Papilio memnon*⁴¹. For the ecologically distinct mimicry systems of *Heliconius* butterflies, few loci seem to explain most naturally

occurring pattern variation^{9,12,43}. Allelic substitutions at one or two loci account for variation in geographical regions in *H. numata* and *H. cydno*, respectively, whereas variation in *H. erato* and *H. melpomene* seems to be due to five or six unlinked loci (reviewed in REF. 9).

Laboratory studies. The characterization of the genetic basis of variation in colour patterns has greatly benefited from studies in laboratory populations of *B. anynana*. These butterflies have a series of eyespots along the margin of their wings (FIG. 1f) and vary in different aspects of eyespot morphology, including eyespot size, number, colour composition, position and shape. This variation has been revealed both by the gradual responses to artificial selection and by studying single spontaneous mutations that have large effects on eyespot patterns (FIG. 3). Artificial selection experiments have shown that *B. anynana* has substantial additive genetic variance for several aspects of eyespot morphology^{35–38}. The analysis of phenotypic variation across artificially selected lines has been used to estimate the number of genetic factors that are involved in response to selection. For example, 5–10 genes are estimated to be involved in producing lines with divergent eyespot sizes on the ventral wing surface⁴⁴. In this same species, large phenotypic differences result from single mutant alleles that appeared in a single generation in laboratory lines under no artificial selection (for examples, see REFS 38,45,46). Which of the genetic variants that are available in the laboratory contribute to variation in natural populations and to differences across species is an exciting issue that needs to be resolved.

Experimental crosses have been used to estimate the number of genetic factors that are involved in natural and laboratory wing-pattern polymorphisms and their segregation properties. However, the molecular nature and mode of action of such factors remains elusive. Current initiatives in gene mapping should start to unravel the first of these issues; they should also begin to reveal whether the genetic variation that produces small quantitative effects that result in gradual responses to artificial selection maps to the same loci that produce large phenotypic changes.

The making of an eyespot

The one butterfly wing-pattern element about which we know most, in terms of evo–devo, is the eyespot. Eyespots are experimentally manipulable and ecologically relevant pattern elements that appear in different butterfly species, and have been shown to function in interactions with predators^{8,47,48}. They are composed of concentric rings of different colours (BOX 1) and have received much attention from a developmental perspective. Most of our knowledge about eyespot formation has come from experimental manipulation of pupal wings and from the study of gene-expression patterns in larval and pupal wing primordia (reviewed in REFS 9,49).

GENETIC ARCHITECTURE

This term broadly describes the distribution of gene effects that produce a given phenotype. It includes a description of the number of genes that influence the trait, the relative position and magnitude of the effects, and the nature of the interactions between them.

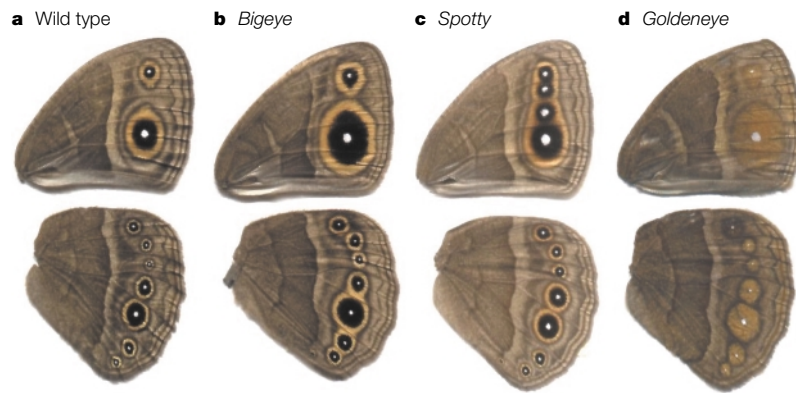


Figure 3 | Wing-pattern mutants. Examples of spontaneous mutants isolated in laboratory populations of *Bicyclus anynana* that show unusual eyespot-pattern phenotypes. All photos are of the ventral surface of the forewing (top) and hindwing (bottom) of *B. anynana* females. **a** | Wild-type phenotype with two eyespots on the forewing and seven on the hindwing, with characteristic relative sizes; **b** | *Bigeye* mutant with overall enlarged eyespots⁴⁶; **c** | *Spotty* mutant with extra eyespots on the forewing⁴⁶; and **d** | *Goldeneye* mutant, in which the characteristically black scales of the central ring of the eyespots are almost completely replaced by gold scales⁵⁶. The individual shown in **d** is, in fact, a *Bigeye*–*Goldeneye* double mutant. Other mutants not illustrated here include *comet* and *cyclops*, which have altered eyespot shape⁴⁶, and mutant 3–4, which has fewer eyespots on its hindwing^{9,38}.

Focal signal and epidermal response. Surgical manipulations have been used traditionally to examine the cellular interactions that underlie eyespot formation in different species. Landmarks on the pupal cuticle show the location of the centres of the presumptive eyespots on the dorsal surface of the adult forewing, thereby making them accessible to surgical manipulation. Each eyespot is produced around a small central organizer, or focus, the ablation of which in early pupae reduces or eliminates the eyespot. The focus also induces the formation of an ectopic eyespot when transplanted into a normally eyespot-less wing region^{46,50,51}. It has been suggested that these central focal cells signal to the neighbouring cells by producing (or perhaps degrading⁵²) a diffusible morphogen that forms a concentration gradient in the surrounding pupal epidermis. The gradient contours then define the rings of colour that make up an eyespot by determining which particular pigment the neighbouring cells will synthesize shortly before adult emergence⁶. The nature of the pigments and the timing of their synthesis have partly been characterized⁵³; however, little is known about the identity of the focal signal and how the focal sources are established. Theoretical models predict the involvement of substances diffusing away from the wing vein primordia⁶, but there is, as yet, no experimental support for such ideas.

Developmental pathways. More recently, the study of gene expression in larval and pupal wing primordia has greatly advanced our understanding of the developmental genetic pathways that are involved in eyespot formation. Through such surveys, a series of wing-patterning genes in *D. melanogaster* have been implicated in butterfly wing development. These include the transcription-factor-encoding genes:

apterous, which is expressed on dorsal but not ventral cells⁵⁴; *wingless* and *Distal-less*, which are expressed along the wing margin^{46,54}; *engrailed*, which is restricted to the posterior part of the wing⁵⁵; and *cubitus interruptus*, which is restricted to the anterior part of the wing⁵⁵. It is fascinating that, in butterflies, many of these genes are redeployed in eyespot formation within specific wing regions. *Distal-less*, *engrailed* and *spalt* are expressed together in the organizing foci of the eyespots and, separately, in each of the colour rings around the focus⁵⁶ (FIG. 4). Other genes that are expressed at specific times in or around the focal area include *cubitus interruptus*, *patched* and *hedgehog*^{54,55}. Wing-patterning genes that are not expressed around foci, but which might be influencing their activity, include *decapentaplegic* and *wingless*, which could be involved in positioning the eyespot^{54,57}, and *Ultrabithorax*, which might be involved in specifying eyespot size⁵⁸. Even though the patterns of gene expression implicate the protein products of these genes in eyespot formation, the development of gene-manipulation techniques in butterflies is essential to unravel gene function and to make the necessary step from expression data to the direct involvement of the genes in pattern formation.

The necessary integration. Although the experimental manipulation of pupal wings and the study of gene expression in larval and pupal wing primordia have partly characterized the mechanisms that underlie eyespot formation, the results of such studies remain as separate components of the overall mechanism. These need to be connected directly in the future and to be integrated with other levels of pattern formation, such as the pigment biosynthetic pathways.

The developmental characterization of wing-pattern formation has been advancing, but little is known about how the mechanisms that underlie this process interact to produce the adult phenotypes. The expression of some wing-patterning genes has been associated with particular cellular organizers of pattern formation (discussed above), but it is not known whether or how such genes determine the properties of these organizers. Also, nothing is known about the molecular nature of the presumed signal they produce. Associations between the biochemical and cellular components of pattern formation are also only starting to be unravelled. The synthesis of different pigments is correlated with differences in the timing of scale-cell maturation⁵³ and can explain changes in wing pattern in response to experimental damage of pupal wings^{59–61}. However, it is not known which and how the genes from the developmental pathways involved in pattern formation lead to pigment synthesis and are affected by hormone titres that regulate plasticity in phenotype (BOX 3). In the future, we will also need to understand more details of how the mechanisms that underlie pattern formation can be altered to produce different phenotypes and at expanding research beyond eyespots. These points are discussed in the following sections.

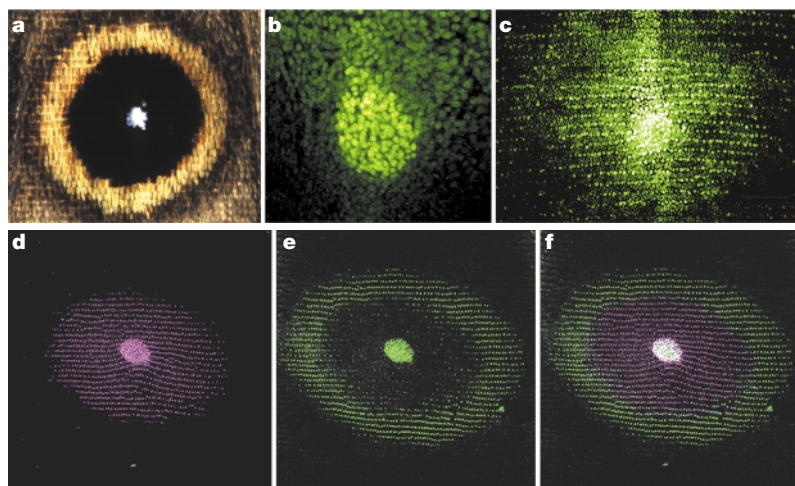


Figure 4 | Gene expression in butterfly wing primordia. Several genes are expressed in the eyespot field of pupal wing primordia in *Bicyclus anynana*. **a** | Typical adult eyespot morphology with a central white pupil, a black ring and an outer gold ring. **b, c** | Expression pattern of *Distal-less* in the centres of the presumptive eyespots in larval (**b**) and pupal (**c**) wings⁴⁶. **d–f** | Expression of *spalt* (purple, **d**) and *engrailed* (green, **e**) in pupal wings in the rings of colour of an adult eyespot (**a**). Panel **f** shows the co-expression of these two genes⁵⁶. **a–c** reproduced with permission from REF. 46 *Nature* © (1996) Macmillan Magazines Ltd. **d–f** reproduced with permission from REF. 56 © (2001) Elsevier Science. Images courtesy of Craig Brunetti, Steve Paddock and Sean Carroll, University of Wisconsin, Madison, USA.

Development and the generation of variation

We have some understanding of eyespot formation but, as yet, little knowledge of how this process is regulated to generate the phenotypic variation that can be sorted by natural selection. Several experimental studies in *B. anynana* have uncovered inter-individual variation in the cellular components and in the patterns of gene expression that are associated with differences in adult eyespot phenotypes.

Cellular basis of variation. How are the cellular interactions that underlie eyespot formation modulated to produce changes in adult patterns? Theoretical models have been made to examine how changes in the components that underlie wing-pattern formation can lead to wing-pattern evolution⁶². It has also been shown experimentally that these components can vary among individuals and, therefore, provide a basis for evolutionary changes in phenotype. Artificial selection in *B. anynana* has produced divergent phenotypes for various features of eyespot morphology, including size (lines with larger or smaller eyespots³⁵) and colour composition (lines with more black or more gold eyespots³⁶). Reciprocal transplant experiments of the eyespot focus between pupae of phenotypically divergent lines examined how genetic differentiation led to changes in components of eyespot development^{35,36}. Transplants showed that eyespot size was primarily dependent on properties of the focal tissue, whereas colour composition was entirely accounted for by properties of the host tissue that surrounded the focal graft and not the signal itself (FIG. 5). The inference from such results is that genetic changes for eyespot size can be traced primarily to effects of signal strength, whereas

those for colour composition involve changes in the response threshold to the signal. So, changes in various features of eyespot morphology are achieved through changes in different components of the eyespot-forming process. This finding is further illustrated by changes in eyespot shape; lines with ‘fat’ and ‘thin’ eyespots differ in the arrangement of scale cells^{63,64}. Are the genes that affect these eyespot features also different? The fact that eyespot features can respond independently in artificial selection experiments indicates that they might^{35,36}, but gene-mapping studies are needed to resolve the issue.

Molecular basis of variation. Changes in the expression of patterning genes have been shown to parallel changes in adult eyespot morphology^{46,56}. How these might contribute to the phenotypic variation that is accessible to selection is a question that has only recently begun to be explored. The study of gene-expression patterns in butterfly wing primordia has identified some developmental pathways that are involved in eyespot formation but not the actual genes that contribute to variation in eyespot patterns. A recent study has begun to establish the link between developmental pathways and the nature of the molecular variation that underlies quantitative differences in wing-pattern phenotype. *Distal-less* expression in wing primordia of *B. anynana* butterflies is associated with eyespot-organizing centres and changes in parallel with adult changes in eyespot morphology⁴⁶. Quantitative changes in *Distal-less* expression between phenotypically divergent lines, coupled with the segregation of DNA polymorphisms in this gene with eyespot variation in crosses between those lines, have indicated that molecular variants at *Distal-less* itself might contribute to within-species variation in eyespot patterns⁶⁵. This study has shown that, even though *Distal-less* encodes an evolutionarily conserved transcription factor, it contributes to inter-individual variation in eyespot size in *B. anynana*. The contribution of important developmental genes to quantitative morphological variation has been shown in other systems (for example, the *Achaete-scute Complex* and *Delta* genes for bristle number in *D. melanogaster*; reviewed in REF. 66), and presumably is possible because of the high spatial and temporal specificity of gene regulation. Studies in different species are providing accumulating evidence that developmental systems have evolved largely by changing the regulation of a conserved set of patterning genes (for examples, see REF. 67). The evolution of cis-regulatory regions seems to be involved in morphological differences between closely related species, as well as in major transitions in body plan⁶⁸.

Beyond eyespots

Although we have reviewed research on the adult phenotype of several species of butterfly with diverse types of pattern, we have focused on the development of eyespots of *B. anynana*. We know about the ecological relevance of patterns in other species and even their

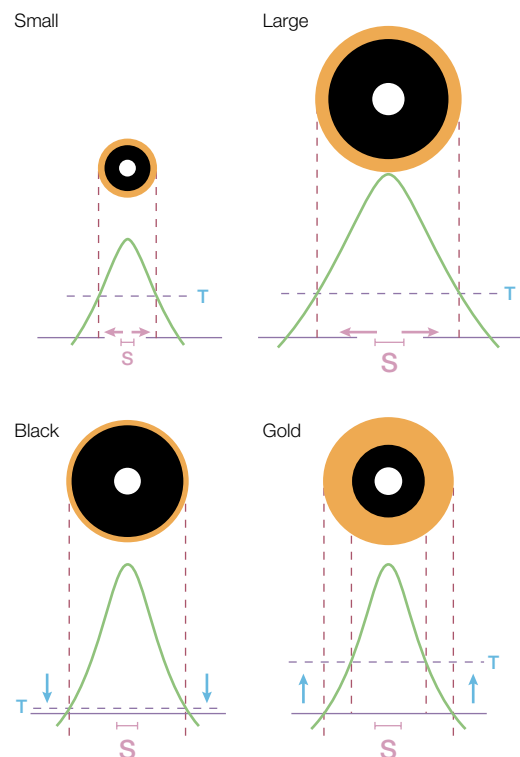


Figure 5 | Cellular basis of variation in eyespot morphology. The formation of an eyespot is thought to involve the production of a signal (S) that diffuses away from a central group of signal-producing cells (the focus) to form a concentration gradient (green line in diagram). The neighbouring cells experience a particular concentration of this signal and, depending on their response thresholds (T), become fated to synthesize a particular pigment in rings around the central focus. Changes in eyespot size (small versus large) have been attributed mostly to changes in focal signal strength³⁵, whereas changes in eyespot colour composition (black versus gold) seem to depend on the response sensitivity thresholds³⁶ (see text for details).

underlying genetics, but we know little about their development. Can the knowledge about eyespot formation in *B. anynana* help in understanding the development of the sometimes completely different patterns that decorate the wings of other butterfly species? Eyespot formation involves the diffusion of a signal from a central focus. What about bands, chevrons or large patches of colour? Theoretical models propose that similar mechanisms might operate in producing all types of pattern⁶, but there is, as yet, no direct evidence that any corresponding developmental organizers exist in genera such as *Heliconius* or *Papilio*. It will also be exciting to determine whether the genetic pathways that are involved in eyespot formation overlap with those underlying the colour bands and patches that are characteristic of *Papilio* and *Heliconius* or even other types of pattern element in the eyespot-bearing *B. anynana* and *Precis coenia*. Several genetic pathways have been implicated in eyespot formation, but, of all the genes tested, only *wingless* seems to be associated with other types of pattern

element, namely, the orange forewing bands in *P. coenia*⁵⁴ (FIG. 1). These results indicate that different pathways might be implicated in the development of different types of pattern. However, more thorough studies of the developmental genetics of pattern formation in different species are needed, but will hopefully be forthcoming through a combination of further developmental studies and gene-mapping approaches. For now, the apparent lack of localized signal sources, and of the expression of *Distal-less* and other genes in association with pattern elements other than eyespots, indicates that the early, attractive idea of a unitary underlying process of pattern determination coupled with the groundplan organization was too simplistic.

From evo to devo and back again

Initial interest in butterfly wing patterns focused mainly on the ecological relevance of natural polymorphisms and on comparative studies of patterns among species. More recently, the study of development has opened up some of the processes that are involved in pattern formation but concentrated almost exclusively on eyespots. We now need to combine different levels of developmental descriptions and move on to other types of pattern. In the short term, research will be focused on fully understanding the development and genetics that underlie pattern polymorphism, which is essential to study the evolutionary diversification of butterfly wing patterns. We need a more complete understanding of the genetic and developmental mechanisms that produce wing-pattern variation in several model species. The challenge, then, will be to integrate the knowledge about different patterns in different species (a return to the comparative method) and the insights about the ecological factors that shape pattern variation in natural populations (a return to ecological studies).

Comparative studies of adult patterns can now be complemented by information on their underlying genetic basis. Studies on *B. anynana* eyespot patterns have uncovered genes of both small and large phenotypic effect³⁸, have traced genetic and phenotypic effects to different components of development^{35,36} and have surveyed associations with gene-expression patterns^{46,56}. Combining artificial selection with a candidate-gene approach has, recently, also started to identify specific genes that contribute to inter-individual differences in eyespot patterns and therefore might contribute to differences among species⁶⁵. It is likely that such genes are highly relevant to understanding how patterns of morphological diversity have arisen among related species of butterfly. Their contribution to the different patterns of more distantly related species, including the bands and fields of colour in the classical systems of *Papilio* and *Heliconius* butterflies, is unclear. Studies of colour polymorphism in species such as these have the great advantage, from an evolutionary perspective, of focusing on phenotypic variants that are influenced by known modes of natural selection^{17,23,69}. Classical genetic crosses indicate that genes of large effect might

be important contributors to these polymorphisms, but future gene-mapping experiments are required to unravel the genetic variation that is available for their evolutionary divergence. Once this has been achieved, an integration of knowledge from different species, each emphasizing different pattern elements and modes of evolution, will show how uniform the developmental and genetic mechanisms that are involved in morphological divergence really are and give an integrated view of the evolutionary diversification of wing patterns.

Looking further ahead, we can foresee the time when there is such a refined understanding of gene–phenotype mapping for eyespots and other pattern elements on butterfly wings that the studies of natural selection will again become the principal challenge. We will know how subtle and specific changes in phenotype are, or can be, generated, but it will become much more difficult to determine exactly how such differences are sorted by natural selection in the wild. Once again, the comparative studies that are based on evo–devo research among related species, in combination with inferences from phylogenetic studies, are likely to be a way forward. With the advent of a highly sophisticated understanding of the generation of evolutionarily relevant phenotypes in butterflies investigated in such an integrated manner, the study of their evolution will have turned full circle.

Conclusion

In only a few experimental systems, we can readily combine studies of the ecological relevance of morphological patterns with an expanding understanding of their development. The classical model systems of developmental biology provide limited opportunities for studying the processes of adaptive evolution in morphology among closely related taxa. For example, we have some exciting insights into the molecular nature of the genes that contribute to variation in *Drosophila* bristle patterns, both for the number of bristles in *D. melanogaster*⁶⁶ and for the divergent phenotypes of trichome patterning on the legs of *Drosophila* sister species⁷⁰. However, the adaptive value of such variation in natural populations is likely to remain elusive. By contrast, it is difficult to envisage that deep developmental insights about gene–phenotype mapping will be forthcoming for some classical study systems of evolutionary genetics in natural populations of animals, such as colour pattern in guppy fish and bill morphology in the Galapagos finches^{71–73}. This is where evo–devo studies of butterfly wing patterns, as well as certain other morphologies, such as spines and lateral plates in stickleback fish^{74,75} and the melanization patterns in *Drosophila* wings^{76,77} can contribute to integrating effectively diverse evolutionary, genetic and developmental studies of adaptive traits at all levels of biological organization.

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Acknowledgements
We thank A. Monteiro, F. Nijhout and M. Serfas for comments on this manuscript, and M. Brittin for figure 5. This work was supported by a grant from the Human Frontier Science Program.

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