Part I
The Nympalid Groundplan (NGP) and Diversification

Chapter 1
The Common Developmental Origin of Eyespots and Parafocal Elements and a New Model Mechanism for Color Pattern Formation

H. Frederik Nijhout

Abstract The border ocelli and adjacent parafocal elements are among the most diverse and finely detailed features of butterfly wing patterns. The border ocelli can be circular, elliptical, and heart-shaped or can develop as dots, arcs, or short lines. Parafocal elements are typically shaped like smooth arcs but are also often "V," "W," and "M" shaped. The fusion of a border ocellus with its adjacent parafocal element is a common response to temperature shock and treatment with chemicals such as heparin and tungstate ions. Here I develop a new mathematical model for the formation of border ocelli and parafocal elements. The models are a reaction-diffusion model based on the well-established gradient-threshold mechanisms in embryonic development. The model uses a simple biochemical reaction sequence that is initiated at the wing veins and from there spreads across the field in the manner of a grass-fire. Unlike Turing-style models, this model is insensitive to the size of the field. Like real developmental systems, the model does not have a steady state, but the pattern is "read out" at a point in development, in response to an independent developmental signal such as a pulse of ecdysone secretion, which is known to regulate color pattern in butterflies. The grass-fire model reproduces the sequence of Distal-less expression that determines the position of eyespot foci and also shows how a border ocellus and its neighboring parafocal element can arise from such a single focus. The grass-fire model shows that the apparent fusion of ocellus and parafocal element is probably due to a premature termination of the normal process that separates the two and supports the hypothesis that the parafocal element is the distal band of the border symmetry system.

Keywords Mathematical model • Eyespot • Parafocal element • Grass-fire model • Temperature shock

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1.1 Introduction

The color patterns of butterflies are extremely diverse, and almost all of the 14,000 or so species can be identified on the basis of their color patterns alone. Adding to this diversity is the fact that dorsal and ventral color patterns are usually entirely different and that many species have polymorphic, sexually dimorphic, and seasonally plastic color patterns. The development and evolution of this diversity of patterns has been of considerable interest, particularly in relation to the genetics and evolution of mimicry (Reed et al. 2011; Nadeau 2016; Baxter et al. 2008; Joron et al. 2006), and the development and evolution of eyespot patterns (Brakefield et al. 1996; Monteiro et al. 1997, 2003; Monteiro 2015; Nijhout 1980).

The organizing principles of color patterns are coming to be increasingly well understood. The diversity of mimicry patterns in *Heliconius* butterflies is due to the variation in only a handful of genes (Nadeau 2016; Kapan et al. 2006), and the specification of color and pattern is now known to be due to a redeployment of many of the genes involved in early embryonic development (Carroll et al. 1994; Martin and Reed 2014; Reed and Serfas 2004; Brunetti et al. 2001).

The developmental mechanism that produces the spatial pattern of pigments that characterizes color patterns is less well understood. It is clear, however, that the wing veins and the wing margin play critical roles in organizing the pattern. This evidence comes, among others, from observations of the color patterns of mutants that lack wing veins and from experimental manipulations that alter the wing margin (e.g., Fig. 1.1 and (Nijhout and Grunert 1988; Koch and Nijhout 2002)).

Fig. 1.1 Color pattern modification in the veinless mutant of *Papilio xuthus* (*right*), compared with the normal pattern (*left*). The longitudinal veins are missing and so are the venous patterns. The submarginal bands are smoothly continuous and parallel to the wing margin, suggesting that the wing margin also plays an important role in color pattern determination

1.2 Eyespots and Parafocal Elements

The color patterns of butterflies are organized as a set of three-symmetry systems (Süffert 1929; Schwanwitsch 1924, 1929; Nijhout 1991). The *basal symmetry system* is often absent or represented only by its distal band. The *central symmetry system* runs in the middle region of the wing and is centered on the discal spot. The *border symmetry system* runs along the distal region of the wing usually paralleling the wing margin (Fig. 1.2). The most complex patterns are typically found in the border symmetry system. The principal elements of the border symmetry system are the border ocelli or eyespots. Although the canonical morphology of an ocellus is a set of concentric circles of contrasting pigments with a well-defined central spot called the focus (Nijhout 1980), circular elements are actually quite uncommon within the larger diversity of butterfly color patterns. More often the shape of the "ocellus" deviates significantly from the circular (heart shaped, dagger shaped, bar shaped) and is often hardly recognizable as homologous to a circular element (Nijhout 1990, 1991).

The proximal and distal bands of the border symmetry system have very different characters. The proximal band, when present, is typically arc shaped, or nearly straight. The distal bands are almost always present and have an exceptionally diverse array of shapes. Because its development and evolution are quite independent of that of the border ocelli, this element has been given a special name: the parafocal element (Nijhout 1990). Süffert (1929) recognized this as the distal band of the border symmetry system but did not give it a special name, and Schwanwitsch (1924) thought it was actually part of the submarginal band system. The results given below in this paper support Süffert's interpretation, as does the recent work of Otaki and colleagues (Dhungel and Otaki 2009; Otaki 2009, 2011).

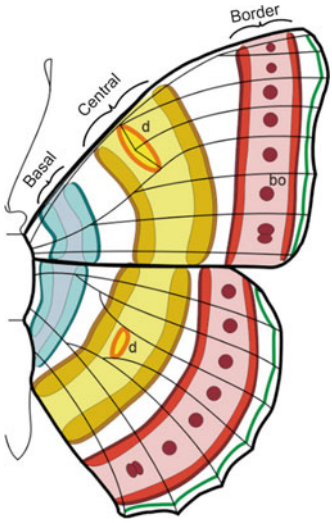

Fig. 1.2 The nymphalid ground plan showing three symmetry systems: basal, central, and border. The border symmetry system has border ocelli (bo) on the compartment midlines. These border ocelli can develop into elaborate eyespots but also into many other shapes. The shape of the distal band of the border symmetry system can also be very diverse, and this band is recognized as the parafocal element

The parafocal elements are developmentally closely related to the border ocelli. Indeed the two are developmentally interdependent in that they appear to arise from a common determination mechanism, although the determinants of their shape are quite different.

1.3 Puzzling Results of Temperature Shock Experiments

A number of investigators have observed that when color pattern aberrations are induced by temperature shock and various chemicals, one of the commonly observed features is a partial or complete fusion of the ocellus and the parafocal element (Otaki 2008; Nijhout 1985, 1991; Nijhout and Grunert 1988). The smooth fusion of these two pattern elements (Fig. 1.3) suggests that that must share a common developmental mechanism. If we interpret the series shown in Fig. 1.3 in reverse order, then it would seem that a single pattern element breaks into two, with the distal one forming the parafocal element and the proximal one the ocellus. None of the current models of color pattern formation can account for this.

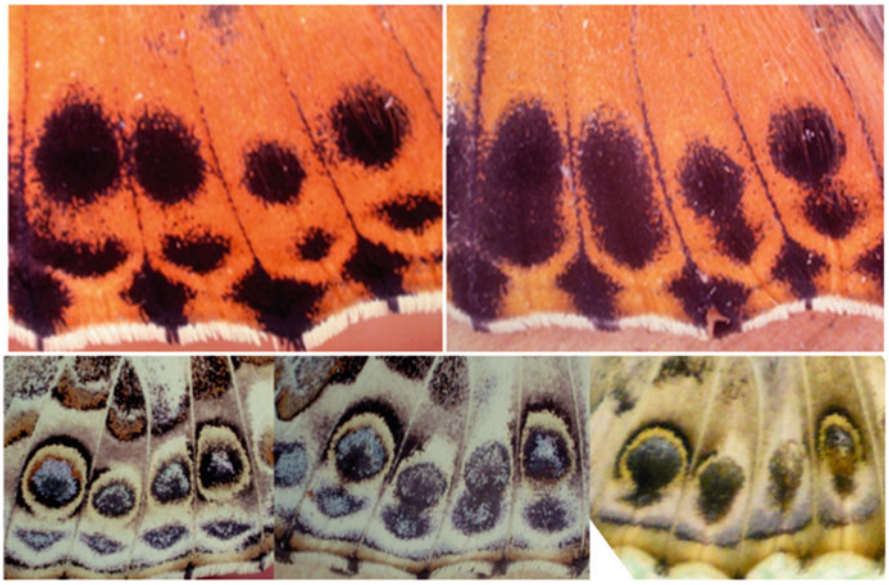

Fig. 1.3 Fusion of ocelli and parafocal elements after temperature shock in *Vanessa cardui*. *Top row*, dorsal surface. *Bottom row*, ventral surface. Normal patterns are on the left in each row. *Bottom row* shows a moderately affected pattern in the middle, and a severely affected pattern in which both pattern elements are completely fused is on the right

1.4 Models of Color Pattern Formation

Previous models for color pattern formation in butterflies have shown that it must be a two-step process. The first step is the establishment of organizing centers, and the second step is the organization of patterns of pigment synthesis by signals produced by these organizing centers. The best known of these organizing centers is the focus, a group of cells that occurs at the center of a canonical eyespot. The foci express both notch and Distal-less, in succession (Carroll et al. 1994; Reed and Serfas 2004), followed by the expression of Spalt and Engrailed in their surrounding, corresponding to the presumptive colored regions of the eyespot (Zhang and Reed 2016; Brunetti et al. 2001).

The mechanism that determines the placement of foci on the wing is still unknown. Foci always occur exactly on the midline of wing compartments delineated by wing veins (i.e., equidistant from the veins). Intervenous stripe patterns (e.g., Fig. 1.6) also occur exactly along the midlines of wing compartments, and in certain papilionids, these stripes break up into spot-like patterns (Nijhout 1991), suggesting a common developmental origin of stripes and spots.

Color pattern determination begins in the wing imaginal disk shortly after the wing venation system is established. The wing imaginal disk is composed of two cell layers, for the dorsal and ventral wing surfaces, respectively. The two cell layers are tightly adhered to each other via a basement membrane. Wing veins develop as tube-like separations between the two layers. The veins are continuous with the hemocoel and allow entry of hemolymph into the developing and growing wing. A special vein called the bordering lacuna (Nijhout 1991) develops around the periphery of the wing imaginal disk and connects the end points of the wing veins (Fig. 1.4).

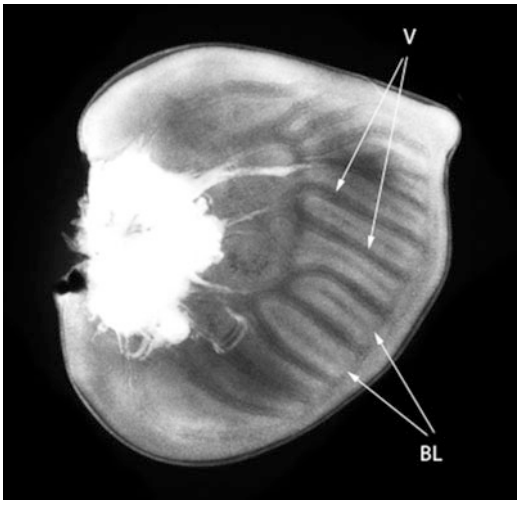

Fig. 1.4 Wing imaginal disk of *Junonia coenia* at the time of color pattern determination. *V* veins, *BL* bordering lacuna. The veins delineate the compartments for pattern formation

The wing veins are bordering lacunae and are the only structural elements in the wing disk when pattern formation begins, and theoretical models of pattern formation assume that these structural elements are the first initiators or organizers for pattern development because they are the only way in which developmental signals can enter the wing (an idea supported by pattern aberrations in veinless mutants (Fig. 1.1). Pattern development including placement of the organizing centers must somehow depend on signals arising from the wing veins and bordering lacuna.

A successful theoretical model for the placement of foci was based on Turing-like reaction diffusion (Turing 1952) using kinetics developed by Meinhardt (1982). The model assumes that a pattern is produced by two chemicals, an autocatalytic activator and an inhibitor, which control each other's synthesis, which can diffuse freely from cell to cell, and in which the inhibitor acts over a larger distance than the activator. Starting with a system at steady state, introducing a small amount of activator from the wing veins, results in a spatial pattern of activator production that rises first as a stripe along the midline between two wing veins in the distal portion of the wing compartment. The end of this midline stripe becomes a particularly strong source of activator production and gradually represses the rest of the stripe, resulting in a stable point-like pattern on the midline resembling the position of a focus. The exact position of the focus as well as the number of foci produced depends on boundary conditions, size of the field, and parameter values of the reaction scheme. This model gained support from the finding that it predicted the spatial sequence of expression of the gene Distal-less, one of the early determinants of color pattern, almost precisely (Fig. 1.5) (Nijhout 1990, 2010).

The spatial pattern of point-like foci and various line-like distributions of the presumptive activator is then used in the second stage of pattern formation to induce the synthesis of specific pigments. A simple diffusion-threshold mechanism using these activator distributions as the origins of new diffusible morphogens proved sufficient to explain almost the entire diversity of color patterns found in the butterflies (Nijhout 1990).

There is, however, a significant problem with this model and, in particular, with the reaction-diffusion mechanism that sets up the initial prepattern of activator distributions. Reaction-diffusion mechanisms are notoriously sensitive to field size and to the exact choice of parameter values and boundary conditions. Even small changes in any of these factors can produce extremely different spatial patterns of activator distribution. Reaction-diffusion mechanisms are particularly sensitive to the size of the field and produce wildly different patterns in fields of different sizes. This seems biologically unrealistic. Biological systems tend to be quite robust to parameter variation and size variation, such as produced by the abundant and often severe genetic and environmental variation to which organisms are subject (Nijhout 2002). In particular, in butterflies, identical patterns often develop in adjoining wing compartments of very different dimensions. Finally, although Turing-style reaction-diffusion mechanisms can be made to produce a wide diversity of realistic patterns, there are, with the possible exception of some fish pigment patterns, no

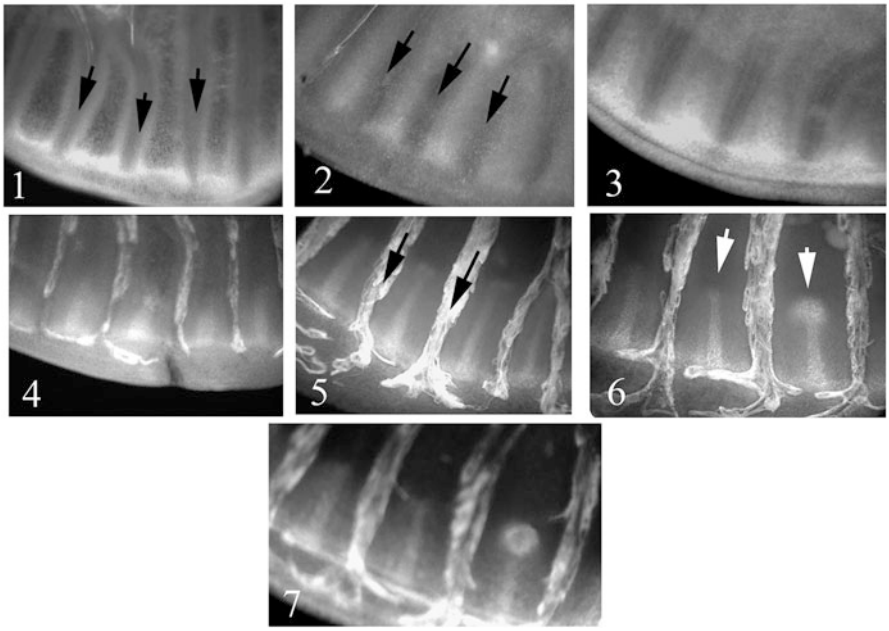

Fig. 1.5 Time series of the development of the expression pattern of Distal-less in the imaginal wing disk of *Junonia coenia*. *Black arrows* indicate the position of wing veins. *White arrows* point to the developing stalks and spots of the Distal-less. Initially Distal-less is expressed along the wing veins and wing margin (*Plate 1*), but then the expression becomes gradually concentrated to the wing compartment midline (*Plates 2–5*). A spot develops at the tip of the midline bar in wing compartment that will develop an ocellus, and the midline bar gradually disappears (*Plates 6–7*)

instances in which they have been experimentally proven to operate during development and in which the activator and inhibitor have been identified (Kondo and Miura 2010).

This has led me to search for a simpler and more robust mechanism that could produce the diversity of color patterns observed. Developmental genetic studies of embryonic development have revealed a broad array of gene regulatory networks that produce dynamically changing spatial patterns of gene expression, in which the product of one gene acts as a transcriptional regulator of one or more other genes. The effect of a gene spreads either by diffusion of the gene product to adjoining cells or by cell-surface signaling interaction among neighboring cells.

These mechanisms for pattern formation are conceptually and physically simple. They are in effect *diffusion-threshold mechanisms*, in which a substance diffuses away from the cells where it is produced and exerts its effect when it rises above a

threshold in surrounding cells. These diffusion-threshold mechanisms can be generalized into what I'll call a *grass-fire model*.

1.5 The Grass-Fire Model

The model consists of the simplest possible set of reactions. A molecule we will call fuel is initially distributed across the field and serves as substrate for the first reaction to produce the product P1. P1 in turn serves as the substrate for the production of P2 and so forth. The model is given by:

$$\partial \mathrm{fuel}/\partial t = -\mathrm{k1} * \mathrm{fuel} * \mathrm{P1} + \mathrm{D}_{\mathrm{fuel}} * \nabla^2 \mathrm{fuel}$$

$$\partial \mathrm{P1}/\partial t = \mathrm{k1} * \mathrm{fuel} * \mathrm{P1} - \mathrm{k2} * \mathrm{P1} + \mathrm{D}_{\mathrm{P1}} * \nabla^2 \mathrm{P1}$$

$$\partial \mathrm{P2}/\partial t = \mathrm{k2} * \mathrm{P1} - \mathrm{k3} * \mathrm{P2} + \mathrm{D}_{\mathrm{P2}} * \nabla^2 \mathrm{P2}$$

Initially there is only fuel, and the patterning mechanism is initiated when P1 is introduced at some point in the field, for instance, along the margins of the field. The model resembles a grass-fire with a fire front, initiated at the ignition point where P1 is introduced, that consumes fuel and leaves combustion products behind, some of which can be used in other reactions. In addition to these reactions, we assume that all chemicals can diffuse from areas of high concentration to low concentration. We assume for the present that all reactions are mass action. Thus we have an exceptionally simple reaction-diffusion system.

In the course of time fuel is depleted, as are all subsequent metabolites. This system does not produce a stable end pattern but rather a slowly changing spatial pattern of values of the three variables. In this respect it resembles the early gene expression patterning events in the *Drosophila* embryo in which a successive series of diffusion gradient-threshold events produce a dynamically progressing spatial pattern of gene expression (Tomancak et al. 2002). We assume that an independent event "reads" the spatial pattern of chemicals at some time point in the development. In butterflies this could be the ecdysone signals that initiate a molt or the wandering stage, both of which occur during the period of color pattern formation and also control growth and morphogenesis of the wing imaginal disk.

The nature of fuel, P1 and P2, is undetermined. Any system with mass action kinetics will do, nor are the kinds of kinetics restricted to mass action. Saturation kinetics like Michaelis-Menten and Hill produce the same patterns as mass-action kinetics over a range of parameter values. The reactions could therefore represent a biochemical reaction sequence, a gene activation sequence, a successive activation of signaling cascades, or a combination of these.

1.6 Basic Patterns

We assume the field is a rectangle that represents a compartment in the wing imaginal disk, where the top and long sides are wing veins and the bottom short side is the bordering lacuna. The reactions can be initiated only along these edges. Variation in pattern can come about by a variation in the position of the initiation points (along the entire margin or only near the proximal, middle, or distal ends), the initial distribution of fuel (homogeneous, proximodistal gradient, vein to midline gradient), and the distribution of the enzymes or rate constants, that run the reactions (homogeneous, proximodistal gradient, vein-to-midline gradient).

1.7 Venous and Intervenous Patterns

Some of the simplest and most widespread patterns are stripes that run along the midline of a compartment and patterns that run parallel to the wing veins. Figure 1.6 illustrates several examples. The patterns show that the veins do not induce pattern along their entire length. In Fig. 1.6a the pattern is only induced in the mid-region of the vein but not near the proximal and distal ends. There is often a proximodistally graded width of the venous bands suggesting (e.g., Fig. 1.6d–e) that the strength of induction, or the propagation rate of the inductive signal, is graded. These patterns are readily produced by the grass-fire model, as illustrated in Fig. 1.7. A proximodistal gradient of reaction rate constants produces venous bands that taper along the length of the vein (Fig. 1.7c). Intervenous stripes (Fig. 1.7a) can be made if the entire wing vein induces the pattern and both the fuel and reaction rates are homogeneously distributed. Reed and Serfas (2004) have shown that in butterflies without eyespots, but with intervenous stripes, there is a long central midline stripe of notch and Distal-less expression. Notch and Distal-less also specify the position of eyespot foci (see below), thus the patterns of P1 and P2 may simulate the expression of these two peptides.

1.8 Simulation of Notch and Distal-Less Progression

The progression of Distal-less expression (Fig. 1.5), beginning with a short midline stripe of the emerging from the margin, followed by the development of a spot at the apex of the stripe, followed by a regression of the stripe, leaving a spot of Distal-less expression behind, was accurately reproduced by a Turing-style reaction diffusion program (Nijhout 1990). Indeed, it provided strong, albeit circumstantial, support for reaction diffusion as the underlying mechanism of focus formation.

Fig. 1.6 Vein-dependent patterns. *Top row* shows venous patterns of *Anaxita decorata* (**a**) and intervenous patterns of *Pseudacraea lucretia* (**b**) and *Eteone eupolis* (**c**). Bottom row (**d**–**f**) shows individual variation in *Danaus affinis*. In *Danaus* the *white venous* pattern varies in the extent to which it expands from the wing veins

Reed and Serfas (2004) and Zhang and Reed (2016) have shown that this pattern of Distal-less expression is preceded by an almost identical pattern of notch expression.

The grass-fire model produces both pattern sequences (Fig. 1.8), simply by assuming that only the distal portion of the wing veins acts as initiation sources and that the fuel is distributed in a shallow gradient that is higher near the midline than near the veins. The shape of the focal spot is slightly elongated across the long axis of the wing compartment, just as the expression of notch and Distal-less described by Reed and Serfas (2004) and Zhang and Reed (2016). The pattern of P2 is identical to that of P1 but lags behind a little, and P2 still has a stalk when P1 is already resolved into a spot (Fig. 1.8). Thus the progression of P1 and P2 resemble those of notch and Distal-less, respectively.

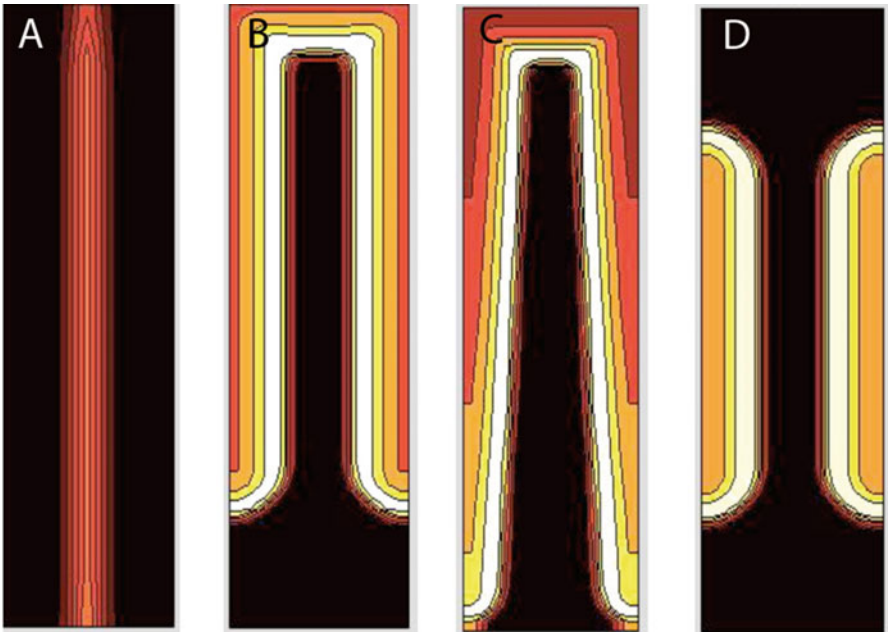

Fig. 1.7 Model simulation for venous and intervenous patterns. In each case the wing veins were used as the initiation points and the "fuel" was either homogeneously distributed or graded slightly from top to bottom (proximal to distal)

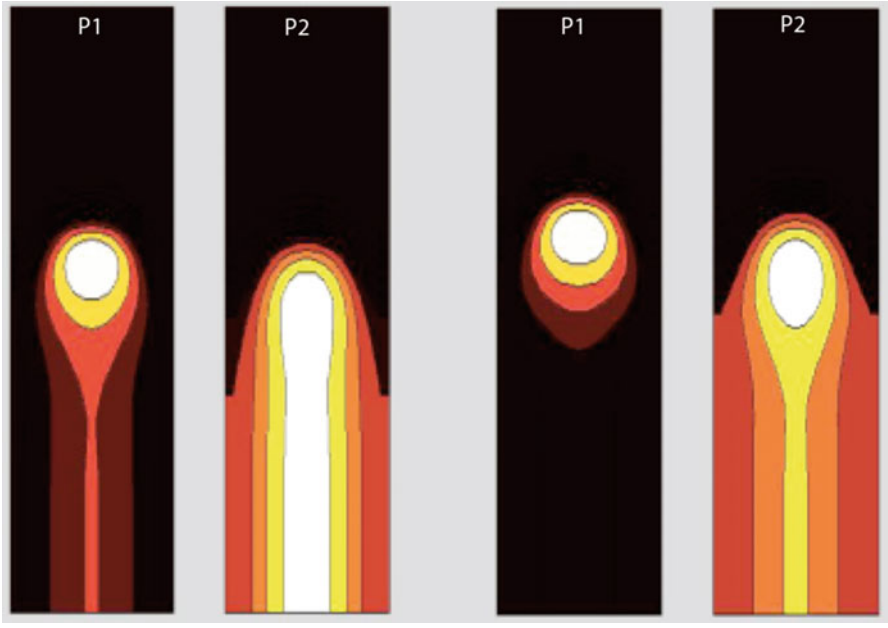

Fig. 1.8 Model simulations of focus formation. Two runs are shown with slightly different initial distributions of "fuel." The distributions of P1 and P2 are shown, which could correspond to the notch and Distal-less, respectively. The two patterns differ in the shape of the lateral gradient of the "stalk," which affects the shape of the parafocal element that will develop

1.9 Shape of the Parafocal Elements

As noted above, once the foci are established, the second step in color pattern formation is a signal that originates from the foci and that specifies a pattern of pigment biosynthesis in their surroundings. We use the grass-fire model for this second step as well, using the focus as the initiation point.

If the grass-fire model is started from a single point source, the pattern produced naturally breaks into two fronts, moving distally and proximally, respectively. If the initial substrate that is used is homogeneously distributed, a circular pattern will form that breaks into two semicircular arcs that move away from the initiation point.

A characteristic feature of the parafocal elements is that they are always symmetrical around the wing compartment midline and are often Λ, V, W, or M shaped (e.g. Fig. 1.9), suggesting a special function of the midline in shaping this element. If the parafocal element is formed by a moving reaction front, then movement near

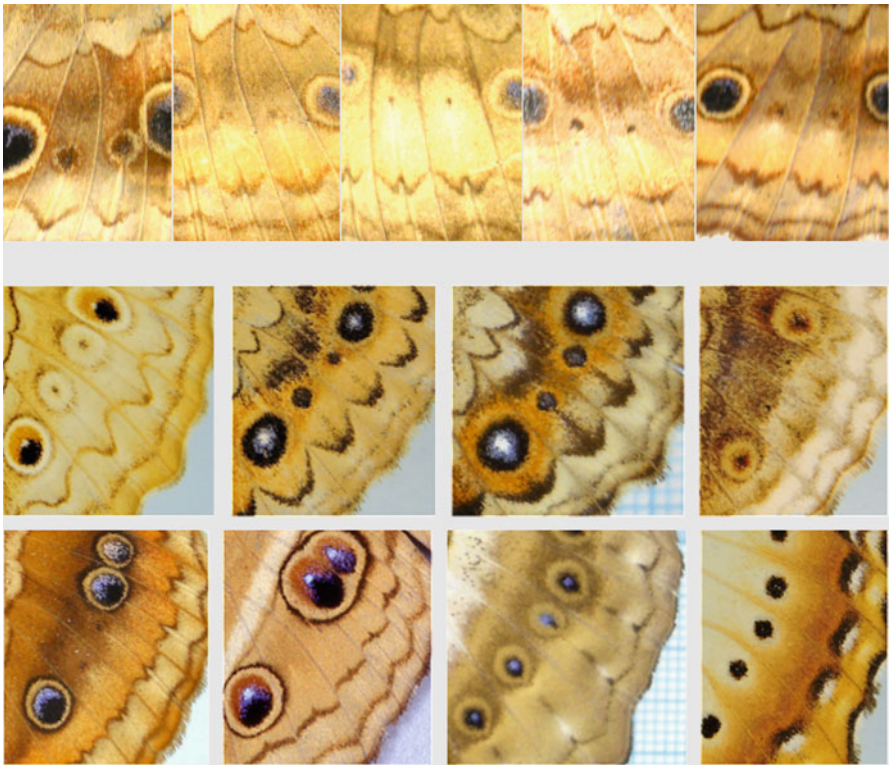

Fig. 1.9 Variation and diversity of parafocal element shapes. *Top row*, individual variation in *Junonia coenia*. *Bottom two rows*, diversity of parafocal elements in selected Junoniini (*middle row J. atlites, J. villida, J. villida, J. oenone*. *Bottom row J. genoveva, J. almana, Yoma algina, Precis ceryne*)

the midline and/or the veins must be either more rapid or slower than movement elsewhere. One way to accomplish this is by having a required metabolite or precursor to the reaction distributed in a pattern that is symmetrical to the midline. A clear candidate for this is the gradient left behind by the midline pattern that preceded the formation of the focal spot (Fig. 1.8). This midline concentration gradient decays only gradually, and its profile depends on the parameter values and initial fuel distribution.

The hypothesis then is that the shape of the parafocal elements is determined by a gradient left behind by the process that formed the focus. This idea can be tested computationally. Figures 1.10a and 1.11 show a sample of the diversity of parafocal element shapes that can be produced by this model. Although these shapes closely mimic those of real parafocal elements (e.g., Fig. 1.9), the shape of the ocellus is not circular, as would typically be the case.

To produce both perfectly circular eyespots and the right diversity of parafocal element shapes, it is necessary to assume that the focus could be the source of two different signals (one perhaps initiated by notch and the other by Distal-less) that use different substrates. If one signal uses a homogeneously distributed substrate, it will produce a circular eyespot (Fig. 1.10b), and if the other uses the gradient left behind by the focus-forming process, it produces the parafocal element. Interestingly, this second source also produces an arc-shaped pattern on the proximal side of the eyespot (Fig. 1.10c–g). This finding is consistent with Süffert's idea that the parafocal element is the distal band of the border symmetry system: the parafocal element and the proximal arc produced by the second source make up paired bands of the border symmetry system. These model results also support the ideas about the nature of parafocal elements and border symmetry systems proposed by Otaki (Dhungel and Otaki 2009; Otaki 2009, 2011).

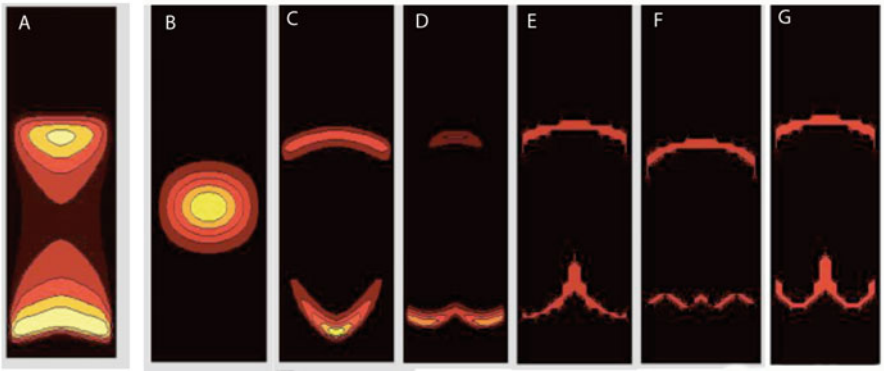

Fig. 1.10 Simulations of pattern generated by focal sources. (**a**) A single source breaks up into an ocellus and a parafocal element, but the ocellus is not circular. (**b**–**g**) A double source at the focus, one producing the eyespot (**b**) and the other producing the parafocal element and the proximal arc-shaped band of the border symmetry system (**c**–**g**)

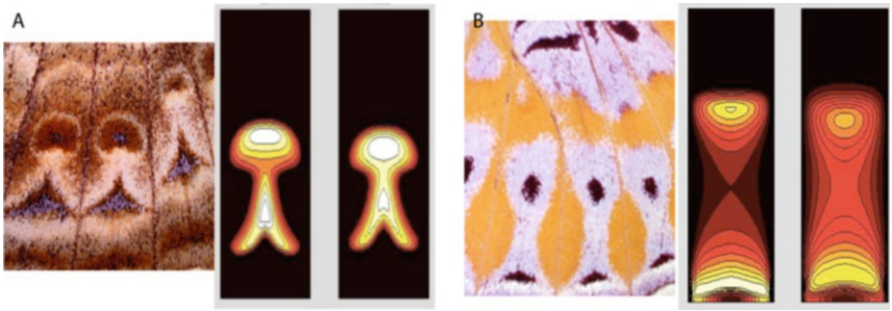

Fig. 1.11 Simulations of patterns that can be generated with a single focal source resembling those of *Vanessa tameamea* (**a**) and *Euryphura concordia* (**b**)

1.10 Fusion and Separation of Ocelli and Parafocal Elements

When the pupae of butterflies are exposed to a temperature shock, many individuals exhibit a fusion between the ocellus and the parafocal element. The degree of fusion is quite variable from individual to individual, and in extreme cases the two fuse into a single pattern element (Fig. 1.3). A possible reason for this effect is that temperature shock freezes the progression of pattern determination, possibly by activating heat shock or stress proteins that stop biosynthetic or transcriptional activity (Mitchell and Lipps 1978; Crews et al. 2016; Welte et al. 1995). The grass-fire model shows that a single pattern element can split into two and that both ocelli and parafocal elements can be produced from a common source.

1.11 Modes of Pattern Evolution

The developing pattern depends on only a few variables: the kinetic parameters of the reactions and the initial gradients of fuel. For all models explored here, these gradients are simple. Beside homogeneous distributions, we used smooth proximodistal gradients or smooth gradients symmetrical to the wing compartment midline, parallel to the wing veins. The latter could be readily set up by diffusion from, or absorption by, the wing veins. Thus the anatomical features of the wing, the wing veins and bordering lacunae, are the only features used to initiate pattern formation.

A significant way in which the proposed patterning mechanism differs from the assumptions of a typical Turing-style reaction-diffusion mechanism is that the system is never at steady state, but the pattern slowly changes over time. The developing pattern becomes fixed, so to speak, by an event such as a pulse of hormone secretion that begins or ends a developmental period, as occurs at several

points during insect metamorphosis (Nijhout 1994, 1999; Nijhout et al. 2014). This property is consistent also with the progressive time-varying patterns of gene expression during embryonic development (Tomancak et al. 2002).

This feature also adds a mode of pattern evolution. Pattern evolution could typically occur due to changes in parameter value reaction rates and gradient shapes. But it is also possible that evolutionary changes in the time when a developing pattern is frozen can lead to changes in the final color pattern. This adds a flexible mode of heterochromic evolution.

Moreover, if, as suggested above, the fixation of pattern depends on the timing of hormone secretion, this mechanism could also account for seasonal polyphenisms of butterfly color patterns. Seasonal polyphenisms in color patterns come about through changes in the timing of ecdysone secretion (Rountree and Nijhout 1995; Brakefield et al. 1998; Koch et al. 1996; Koch and Bückmann 1987) and thus may fix the progression of pattern at different stages. On this view, seasonally polyphenic patterns can be thought of as an expression of plastic heterochrony. Once a plastic pattern switch is established, additional adaptive changes in the patterning system can evolve to refine or further alter the pattern.

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Chapter 2
Exploring Color Pattern Diversification in Early Lineages of Satyrinae (*Nymphalidae*)

Carla M. Penz

Abstract Based on the most recent nymphalid phylogeny, the Satyrinae can be tentatively organized into the species-rich tribe Satyrini plus a clade that includes the Morphini, Brassolini, Haeterini, Elymniini, Melanitini, Dirini, Zetherini, and Amathusiini. Members of the latter eight tribes have the largest body sizes within Satyrinae and also show extraordinary wing pattern variation. Representatives of these tribes are illustrated herein, and pattern elements of the nymphalid ground plan are identified. Five themes are briefly discussed in light of their pattern diversification: (1) central symmetry system dislocations, (2) variation in ventral hind wing ocelli, (3) the color band between elements *f* and *g*, (4) sexual dimorphism and mimicry, and (5) transparency. Within an ecological and evolutionary standpoint, selected genera are provided as examples to explore wing patterns involved in male mating displays, camouflage, and mimicry.

Keywords Pierellization • Ocelli • Sexual dimorphism • Mimicry • Camouflage • Transparency • Mating behavior

2.1 Introduction

The evolution of adult diurnal activity in Lepidoptera paved the way for the widespread use of color for intra- and interspecific signaling (Grimaldi and Engel 2005; Kemp et al. 2015). Following approximately 90 million years of morphological and species diversification (Wahlberg et al. 2009), butterflies in the family Nymphalidae have played an important role in our understanding of how wing color patterns mediate intraspecific interactions and also the evolution of aposematism, mimicry, and camouflage (Vane-Wright and Ackery 1984; Chai 1990; Nijhout 1991; Rutowski 1991). Whether they target conspecifics or other animals, the evolutionary diversification of butterfly color signals involved impressive modifications of wing pattern elements (WPEs hereafter).

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The characterization of a ground plan that identifies individual pattern components across butterfly wings provided a useful framework for research on development, genetics, and evolution (Schwanwitsch 1924; Süffert 1927; Nijhout 1991 and references therein). Border ocelli are the best studied of all individual WPEs possibly because they are conspicuous and ubiquitous in the family Nymphalidae. The Satyrinae constitutes an excellent group to study variation in the border ocelli alone and also how different WPEs can become integrated to produce particular visual effects.

Most Satyrinae species are small bodied and relatively uniform in appearance, such as members of the tribe Satyrini (85% of the species in the subfamily, Peña and Wahlberg 2008). There are, however, noticeable exceptions. Large-bodied species are grouped in a clade that includes the Brassolini (Fig. 2.1), Morphini (Fig. 2.2), Haeterini (Fig. 2.3), Elymniini (Fig. 2.4), Melanitini (Fig. 2.4), Dirini (Fig. 2.5), Zetherini (Fig. 2.6), and Amathusiini (Fig. 2.7; Wahlberg et al. 2009). Exhibiting remarkable color diversification, these butterflies form the focus of this chapter to provide the first detailed comparison among early satyrine tribes. Representatives were selected for an examination of both ventral and dorsal WPEs (see Nijhout 1991 for terminology), and a list of examined species is given in Appendix. Five themes are briefly described and illustrated and, as much as possible, discussed within the context of the natural history and behavior of the butterflies. More detailed accounts will be presented elsewhere (Penz in prep.).

2.2 Central Symmetry System Dislocations in Forewing and Hind Wing

The term pierellization (Schwanwitsch 1925) refers to the dislocation of elements that pertain to the central symmetry system in such a way that distal elements below vein M_3 align themselves with proximal ones located above such vein. This is visible in the ventral forewings of several species in the Brassolini, Morphini, Haeterini, and Dirini (Figs. 2.1c, e, 2.2d–e, 2.3b and 2.5a), and it varies within and between genera. Taking the genus *Pierella* as an example, the anterior dislocation of element *f* below forewing vein M_3 is found in species with rather plain ventral coloration (e.g., *P. lamia* in Fig. 2.3b; also *luna* and *hortona*, not illustrated). Such dislocation disrupts the interplay between *f* and *g*, which seem to serve as boundaries for a light-colored band that occurs in their congenerics (see below). In *Pierella* species that show ventral forewing pierellization of *f*, elements *f* and *g* are also broadly separated on the ventral hind wing (Fig. 2.3b).

Although pierellization seems to be less common on the ventral hind wing, it occurs in some species that display dead leaf camouflage (e.g., *Caerois gerdrudtus*, Fig. 2.2a) or parallel bars (*Morpho marcus*, Fig. 2.2d–e). Some camouflaged species, however, do not show hind wing dislocation of element *f* (e.g., *Amathuxidia amythaon*, Fig. 2.7b), suggesting that ventral camouflage evolved

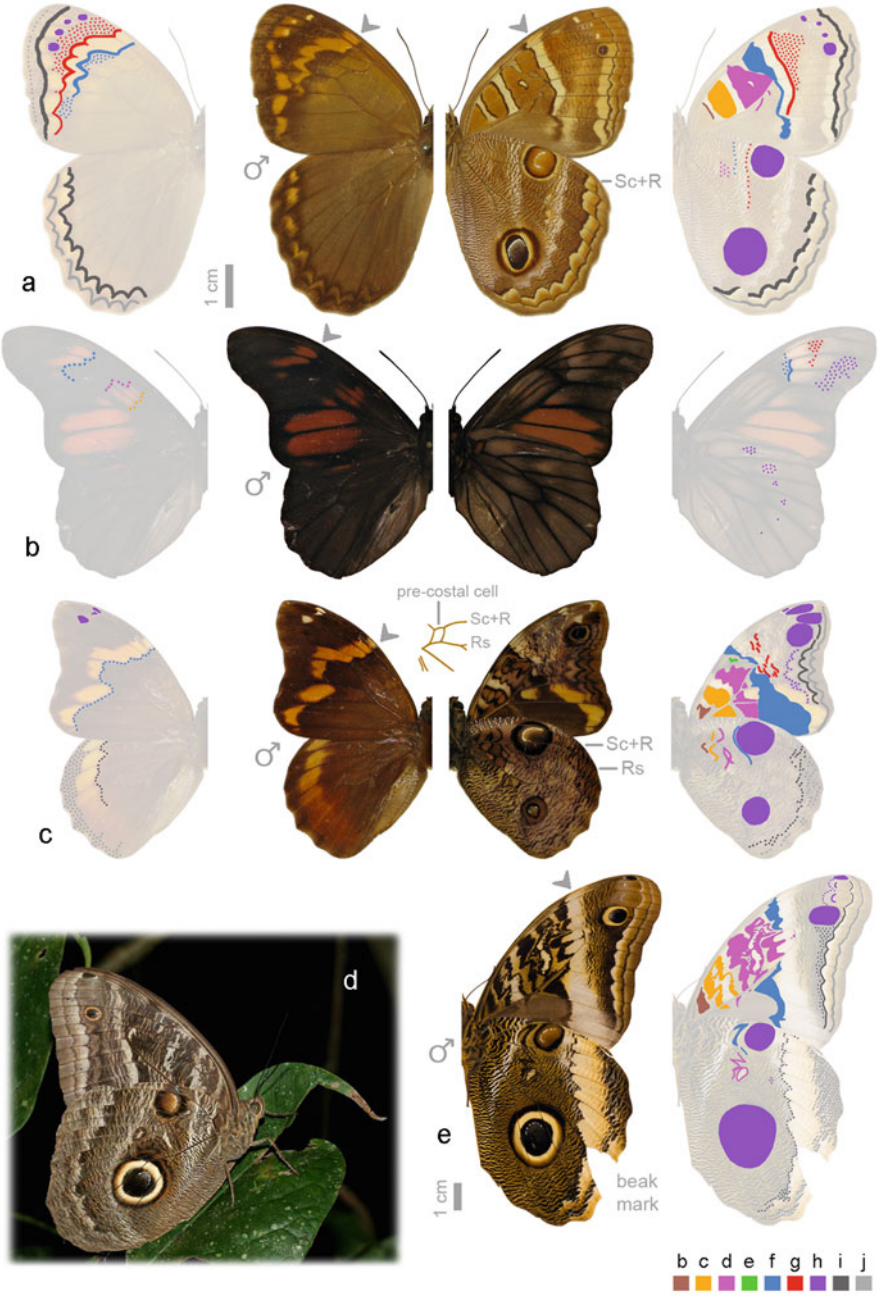

Fig. 2.1 Color-coded wing pattern elements in selected Brassolini. Left side of butterfly image in dorsal view, right side in ventral view. Gray arrows indicate colorful band associated with element *f*. (**a**) *Opoptera syme*. (**b**) *Penetes pamphanis*. (**c**) *Opsiphanes sallei*, note venation detail showing precostal cell present at the base of the hind wing. (**d**) *Caligo illioneus* male perched on leaf (photo by David Powell). (**e**) *Caligo atreus*. All butterflies at the same scale except *C. atreus*

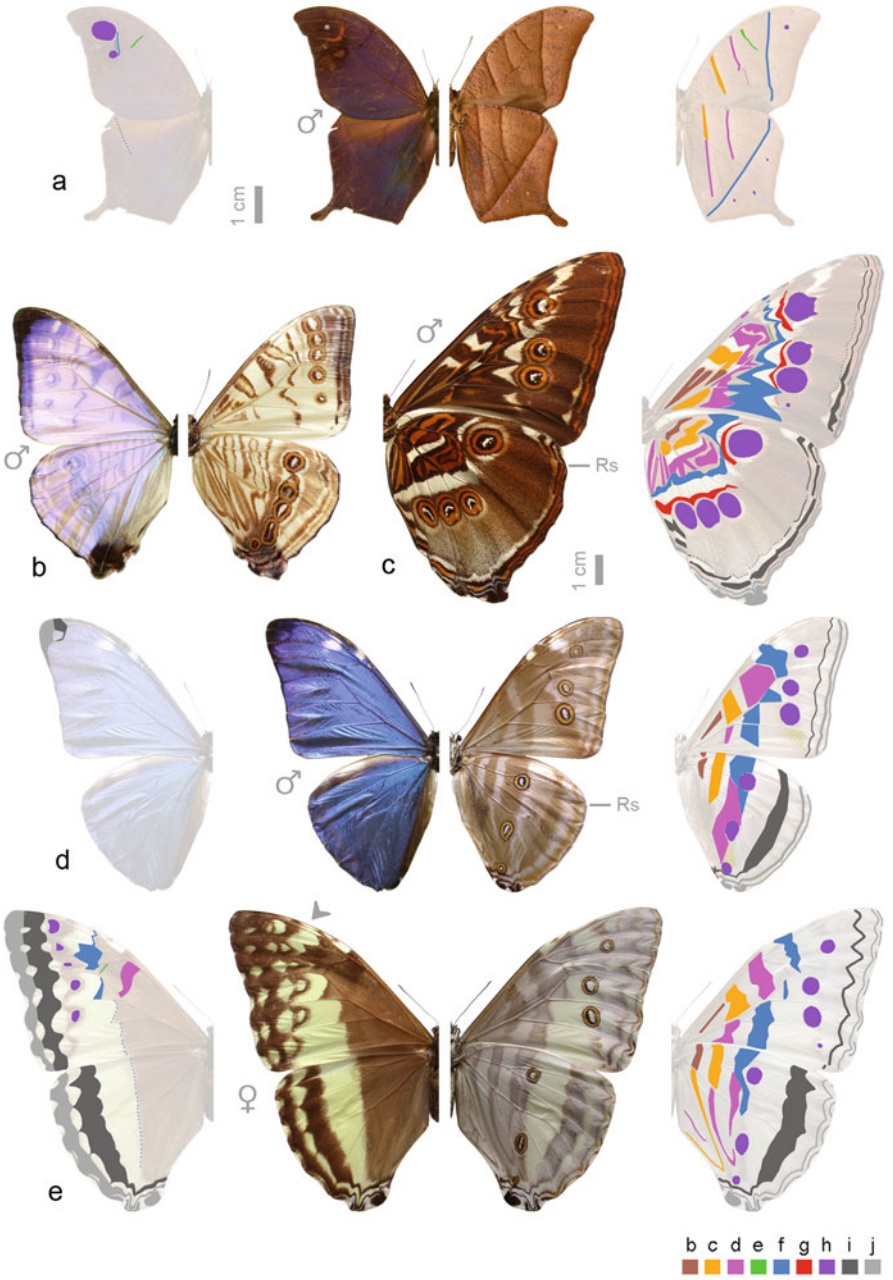

Fig. 2.2 Color-coded wing pattern elements in selected Morphini. Left side of butterfly image in dorsal view, right side in ventral view. Gray arrow indicates colorful band associated with element *f*. (**a**) *Caerois gerdrudtus*. (**b**) *Morpho sulkowskyi*, ventral pattern elements are visible in dorsal view in this semitransparent species. (**c**) *Morpho hecuba*. (**d**) and (**e**) *Morpho marcus*. All butterflies at the same scale except *M. hecuba*

independently multiple times. In the case of *M. marcus*, the comparison of male and female ventral hind wing patterns was helpful to identify the alignment and amalgamation of elements *f* and *d* to produce broad bars (compare Fig. 2.2d–e to *Amathusia phiddippus* in Fig. 2.7e). When *M. marcus* butterflies are at rest, the hind wing bars visibly converge toward an enlarged tornus where the eye-catching parfocal elements seem to be forming a deflection point for predator attack (Fig. 2.2d–e; also present in other species, Fig. 2.2b–c), a pattern that evolved independently in members of the Amathusiini (Fig. 2.7b, e).

2.3 Variation in Ventral Hind Wing Ocelli

Ocelli can take many forms within the Nymphalidae (Nijhout 1991). Species in the eight studied tribes show a broad range of variation, while some species display a complete series at the postmedial area of the wing stereotypical of the nymphalid ground plan (e.g., *Ethope himachala*, Fig. 2.6e; *Faunis eumeus*, Fig. 2.7c); in others the ocelli are markedly reduced (e.g., *Penetes pamphanis*, Fig. 2.1b). Although various types of ocelli are found in members of all tribes, here I limit my discussion to three aspects of the ventral hind wing ocelli: the location of the first ocellus of the series, proximal dislocation of the ocelli, and their use in signaling.

In most members of the eight tribes, the first conspicuous ocellus of the ventral hind wing series is located below vein Rs (Figs. 2.2c, 2.4b–d, 2.5a and 2.7a–b), but there are notable exceptions. In all Brassolini species with well-developed ocelli, the first ocellus is found below Sc + R (Fig. 2.1a, c). All members of Brassolini have a precostal cell (Fig. 2.1c), which increases the distance between Sc + R and Rs, and provides physical space for a well-developed ocellus. Although the function of the precostal cell is unknown, this points to a possible association between wing venation and color pattern in Brassolini. Furthermore, in some Brassolini species this ocellus expands beyond the cell where it originates, suggesting selection for larger size (Fig. 2.1a, c, e). Some members of the Dirini also have a well-developed ocellus below Sc + R, and that of *Paralethe dendrophilus* is particularly large (Fig. 2.5b). In this species the base of Rs is separated from Sc + R, which increases cell height in an analogous way to what is found in Brassolini. Finally, in the transparent Haeterini *Dulcedo*, *Pseudohaetera*, *Haetera*, and *Cithaerias* the first ocellus is located below M_1 (Fig. 2.3e–f), a pattern unique to these taxa.

Border ocelli are usually located in the postmedial area, but dislocations occur in several taxa. Proximal dislocations are more common than distal ones, and the former are associated with a corresponding shift of central symmetry system WPEs. Notable proximal dislocations are found in taxa of Brassolini, Morphini, and Amathusiini (Figs. 2.1, 2.2 and 2.7). In many Brassolini and also *Morpho*, the hind wing ocelli are clearly positioned in the medial area of the wing, which can produce a striking visual effect depending on their size (Penz and Mohammadi 2013; Figs. 2.1d–e and 2.2c). Ocelli dislocations can be uneven with the first, or first and second, ocelli taking a more proximal position than the remaining of the series

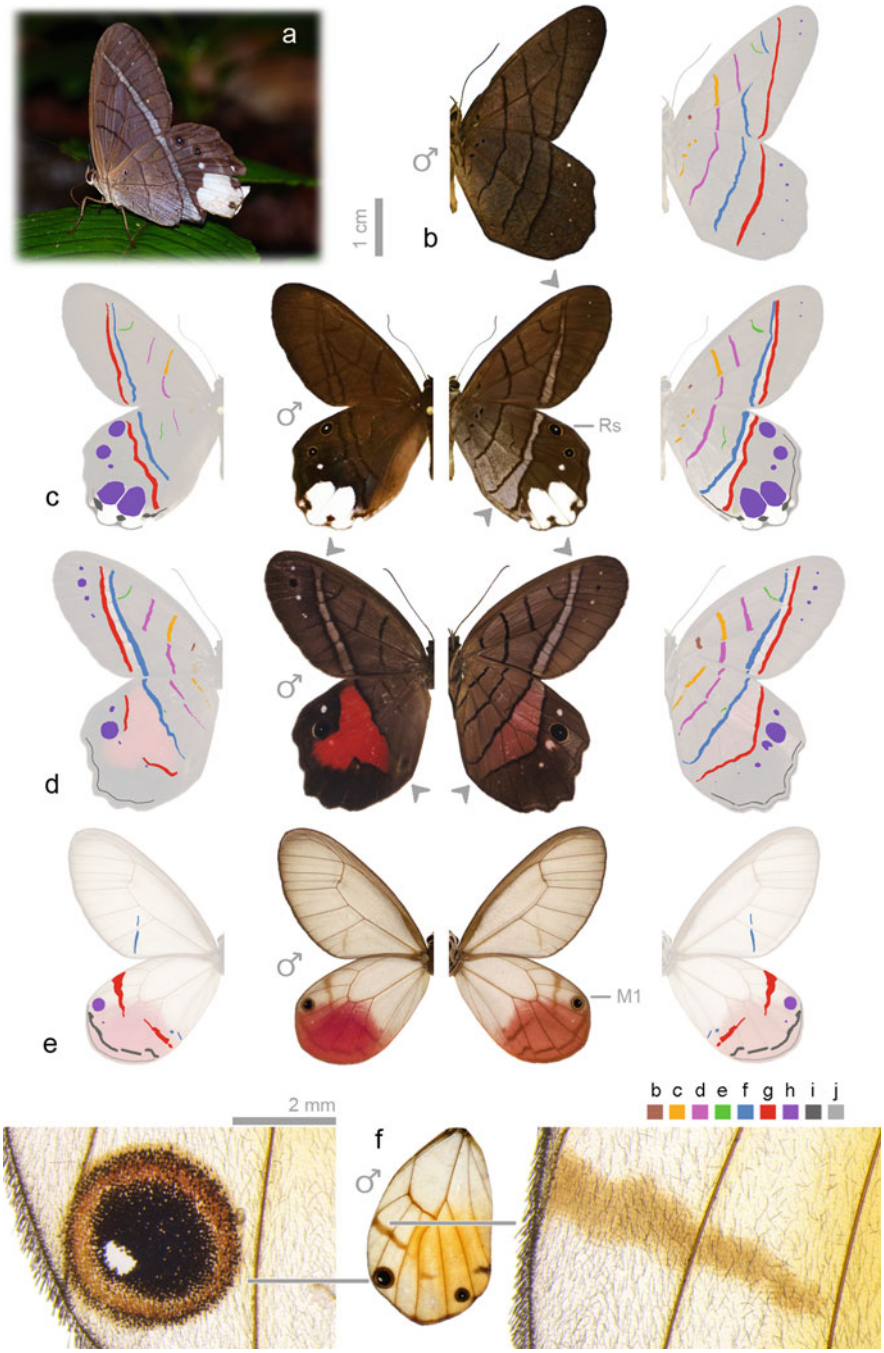

Fig. 2.3 Color-coded wing pattern elements in selected Haeterini. Left side of butterfly image in dorsal view, right side in ventral view. Gray arrows indicate colorful band associated with element

(Figs. 2.6e and 2.5b). Finally, the hind wing ocelli are uniquely dislocated distally in the transparent Haeterini genera by being positioned very near the wing margin (Fig. 2.3e–f). The ocellus below M_1 becomes highly visible when these transparent butterflies alight with their wings closed.

The ventral ocellus located at the hind wing tornus has been hypothesized to function as a defense, either a deflection point in the event of a predator attack or a startle mechanism that prevents or delays attacks (DeVries 2002, 2003; Hill and Vaca 2004; Stevens 2005). Although these hypotheses are compelling, my field observations suggest that in some taxa, ventral hind wing ocelli might have an additional function. Males of some *Caligo* species aggregate at leks along forest edges to wait for virgin females (Freitas et al. 1997, Srygley and Penz 1999; Fig. 2.1d). As they fly into the lek, the large ventral ocelli appear to help airborne females locate perched males (pers. obs.), suggesting a potential function in male-female interactions. *Pierella lucia* has two large white ocelli at the hind wing tornus that show perfect dorsoventral correspondence, likely enhancing light reflection (Fig. 2.3a, c). Hill and Vaca (2004) demonstrated that the hind wing tornus of *Pierella lucia* is weaker than surrounding wing areas, thus supporting the deflection hypothesis (see beak marks in Fig. 2.3a). Nonetheless I once observed the complex courtship behavior of this species. While a female was perched on a leaf, a male hovered in her view, beating the forewings only and keeping the hind wings open and motionless. The male clearly displayed the ventral hind wing ocelli to the female as he repeatedly dipped closer and closer to her. Dorsal ocelli have been considered more important during mating displays (e.g., Oliver et al. 2009), but my observations suggest that ventral ocelli may also be used in this context. In the case of both *Caligo* and *Pierella lucia*, it is possible that both natural and sexual selection could be operating concomitantly on the ventral hind wing ocelli. This is perhaps the case in other species as, for example, male *Faunis phaon leucis* that has larger ventral ocelli than the female (Fig. 2.7d; note that dorsal ocelli are absent in *Faunis*).

2.4 The Color Band Between Elements *f* and *g*

Many nymphalid butterflies have a conspicuous, forewing band that constitutes a highly visible component of the dorsal, and sometimes ventral, coloration (e.g., *Melanitis amabilis*, Fig. 2.4d). This band is common among the species studied here

Fig. 2.3 (continued) f. (**a**) *Pierella lucia*, note multiple beak marks on the hind wing tornus (photo by Andrew Neild). (**b**) *Pierella lamia*. (**c**) *Pierella lucia*. (**d**) *Pierella helvina*. (**e**) *Cithaerias aurora*. (**f**) details of the dorsal hind wing of *Haetera piera*: the ventral orange scales in the ocellus are visible dorsally through transparency; element *g* is expressed on the wing membrane. All butterflies are at the same scale

Fig. 2.4 Color-coded wing pattern elements in selected Elymniini and Melanitini. Left side of butterfly image in dorsal view, right side in ventral view. Gray arrows indicate colorful band associated with element *f*. (**a**) and (**b**) *Elymnias hypermnestra*. (**c**) *Elymnias patna*. (**d**) *Melanitis amabilis*. All butterflies are at the same scale

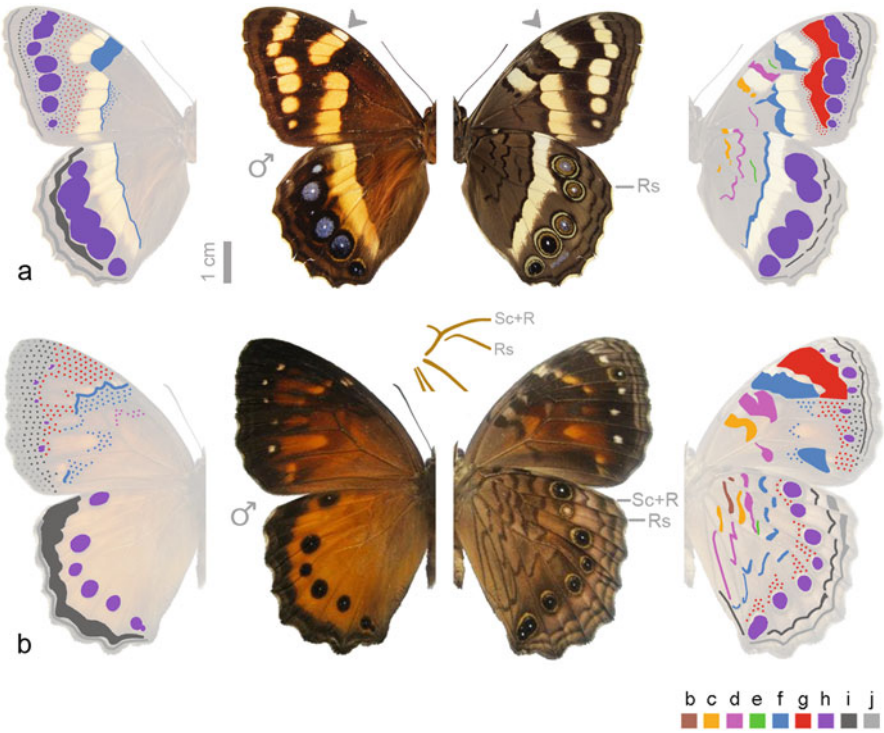

Fig. 2.5 Color-coded wing pattern elements in selected Dirini. Left side of butterfly image in dorsal view, right side in ventral view. Gray arrows indicate colorful band associated with element *f*. (**a**) *Aeropetes tulbaghia*. (**b**) *Paralethe dendrophilus*, note venation detail showing separation of Rs from Sc + R at the base of the hind wing. All butterflies are at the same scale

(see gray arrows in Figs. 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7, and 2.8). It appears to be associated with element *f* (or bounded between *f* and *g*) and varies between and within the studied tribes. For instance, this band differs noticeably in color, width, and extent of fragmentation between the closely related *Aeropetes tulbaghia* and *Paralethe dendrophilus* (Fig. 2.5a–b). The dorsal forewing band can also vary in orientation (vertical or transverse). A vertical band is found in species where *f* is positioned straight across the medial area of the wing (e.g., Fig. 2.2e). In contrast, a transverse band results from element *f* being slightly diagonal (displaced distally toward the wing tornus, e.g., Fig. 2.7b). Members of the Brassolini, for example, vary in the orientation of this band (compare *Catoblepia* and *Caligo*; Fig. 2.8a–b).

Within the same species and sex, the expression of the band associated with *f* usually differs between the forewing and hind wing and may also show dorso-ventral variation. This is readily apparent in *Pierella helvina* (Fig. 2.3d), where elements *f* and *g* are clearly visible and appear to function as developmental boundaries. Ventrally, the pale-colored band of *P. helvina* is much narrower on the forewing than on the hind wing. Although element *g* forms a continuous line in

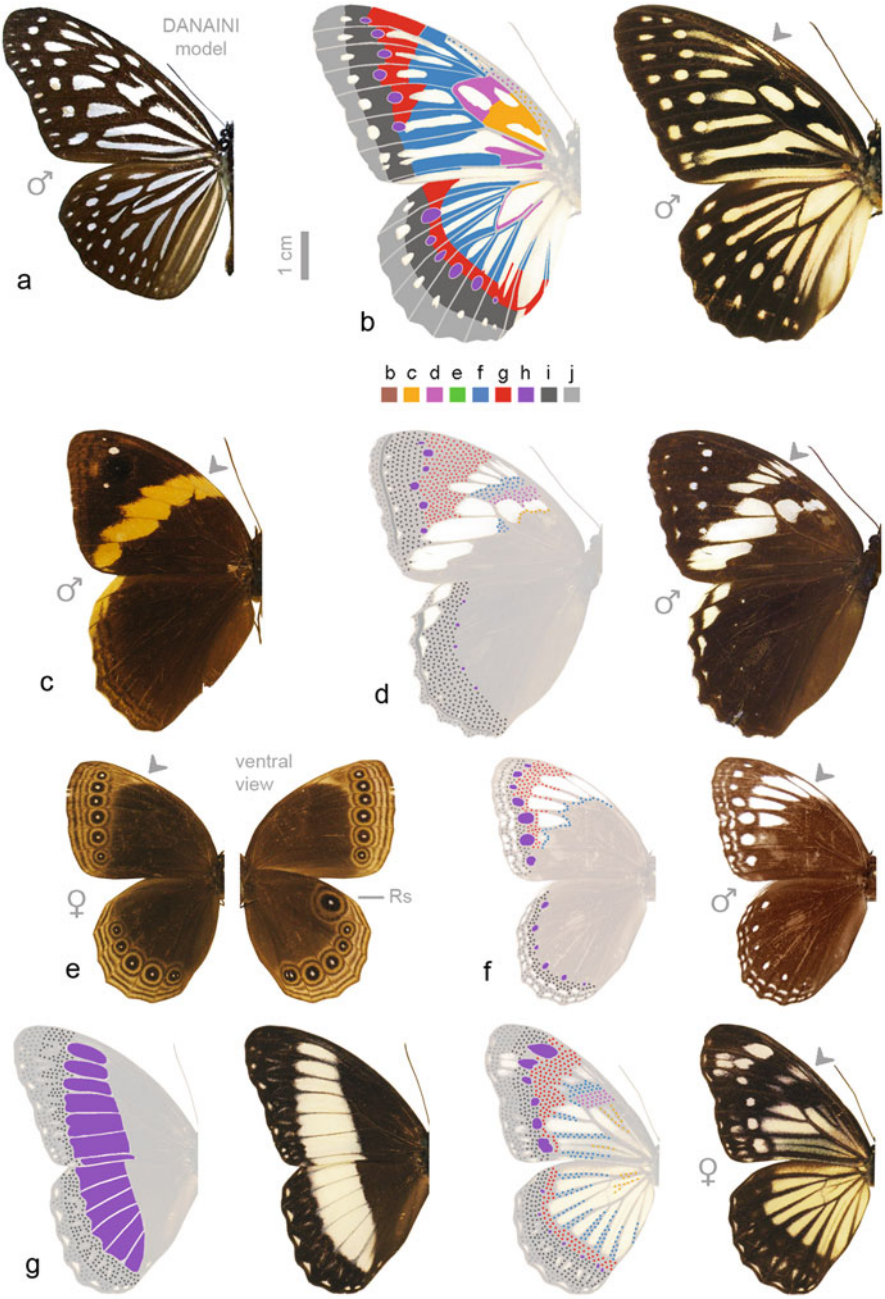

Fig. 2.6 Color-coded wing pattern elements in selected Zetherini. Left side of butterfly image in dorsal view, right side in ventral view. Gray arrows indicate colorful band associated with element *f*. (**a**) *Ideopsis vulgaris* (Danaini) model. (**b**) *Penthema lisarda*, hypothesized delimitation of pattern elements based on Nijhout (1991) plus tentative identification of pattern elements (dotted) for species of nonmimetic or intermediate patterns. (**c**) *Neorina hilda*. (**d**) *Penthema adelma*.

the ventral hind wing, it is not expressed dorsally between M_2 and CuA_1, allowing the bright red band to expand distally. For comparison, note that *f* and *g* are also clearly visible on the hind wing of *Pierella lucia* (Fig. 2.3c), where a pale band is expressed ventrally only. The genus *Pierella* constitutes an excellent example of how different WPEs and associated bands can be modified by evolution to give rise to broadly distinctive species-specific patterns (Fig. 2.3b–d).

2.5 Sexual Dimorphism and Mimicry

The species studied here range from sexually monomorphic to slightly or strongly dimorphic, and color pattern divergence implies that selection can operate independently on males and females. When there is little divergence between sexes, both dorsal and ventral WPEs are more conserved in females (Figs. 2.2d–e and 2.4c). In contrast, strong sexual dimorphism can result from simple modifications in few WPEs and the colorful bands associated with them (e.g., *Mielkella singularis*, Penz and Mohammadi 2013) or more complex changes involving a larger number of WPEs (Fig. 2.2d–e).

Strong sexual dimorphism can arise through sexual selection operating on male pattern or natural selection on female pattern (see Kunte 2008 and Oliver and Monteiro 2010 for reviews). Here I confine my discussion to potential natural selection on female pattern. Females could diverge from males to become less conspicuous to potential predators, as might have been the case in five species of *Morpho* (see example in Fig. 2.2e). Furthermore, the evolution of mimetic convergence can be limited to the female sex, although not always the case. Female-limited mimicry has evolved independently in members of various tribes (e.g., Fig. 2.4a–b), and depending on the model, it required simple or complex changes in WPEs. For instance, the convergence of female *Catoblepia orgetorix* with monomorphic *Caligo atreus* (Fig. 2.8a–b) involved a relatively simple set of color pattern modifications. When compared to other species of *Catoblepia*, the band associated with element *f* is dislocated proximally on the dorsal forewing of *C. orgetorix*, its color changed from orange to white, and it acquired purple iridescence. On the dorsal hind wing, the band associated with *i* became wider and changed color from orange to yellow. Mimicry is rare in neotropical Satyrinae, and this example is peculiar as neither *Caligo* nor *Catoblepia* are known to possess chemical defenses.

In contrast, mimicry (female-limited or both sexes) is common in the old-world tribes Zetherini and Elymniini and the Amathusiini genus *Taenaris*. In their case, evolution took two distinctive paths. Figure 2.8c–e shows cross-tribal convergence that resulted from an extreme reduction in the expression of most WPEs plus the

Fig. 2.6 (continued) (**e**) *Ethope himachala*. (**f**) *Ethope noirei*. (**g**) *Zethera pimplea*, note that males of other *Zethera* species have small, dorsal ocelli on both wings. All butterflies are at the same scale

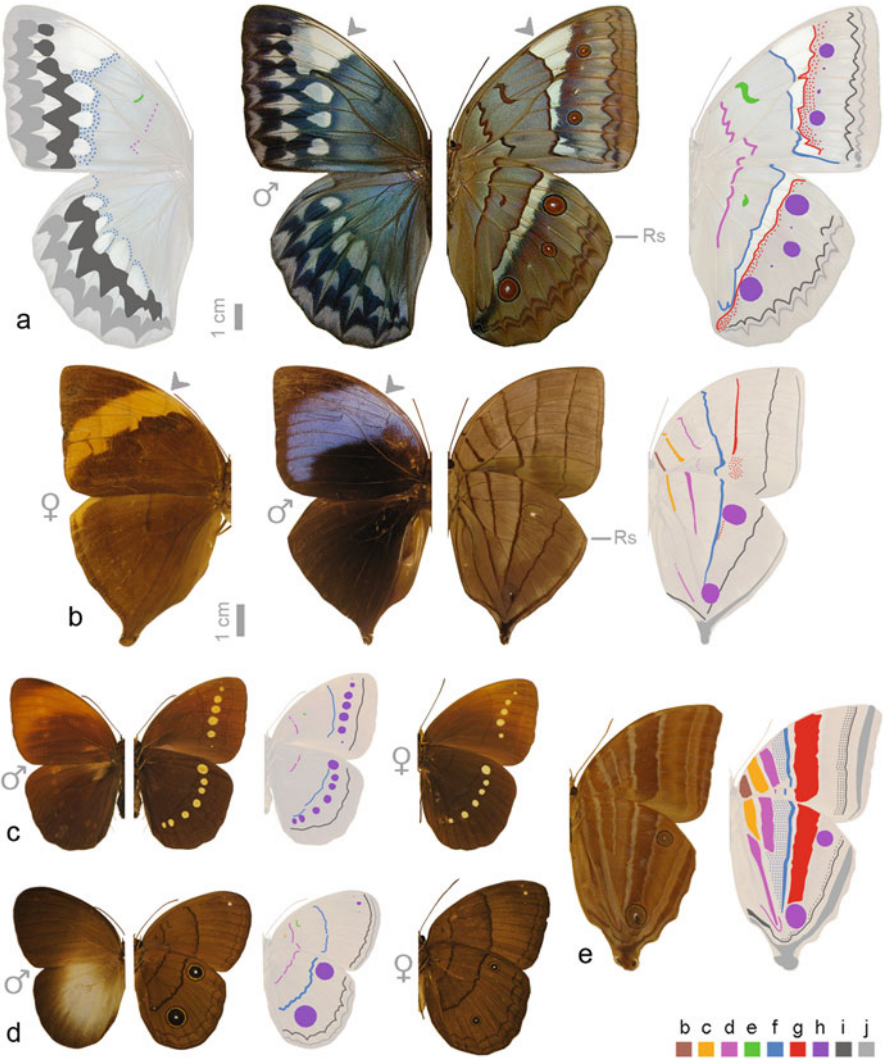

Fig. 2.7 Color-coded wing pattern elements in selected Amathusiini. Left side of butterfly image in dorsal view, right side in ventral view. Gray arrows indicate colorful band associated with element *f*. (**a**) *Stichopthalma godfreyi* (photo by Saito Motoki). (**b**) *Amathuxidia amythaon*. (**c**) *Faunis eumeus*. (**d**) *Faunis phaon leucis*. (**e**) *Amathusia phidippus*. All butterflies are at the same scale except *S. godfreyi*

increase in size of some ocelli to create a similar visual appearance. In other taxa, mimicry involved complex modifications of most WPEs. Nonmimetic and intermediate patterns can help interpret WPE modifications that lead to mimetic convergence of zetherines onto chemically protected danaines (e.g., *Ideopsis vulgaris*, Fig. 2.6a). Figure 2.6c, d, f, and g exemplify a series of such modifications, which

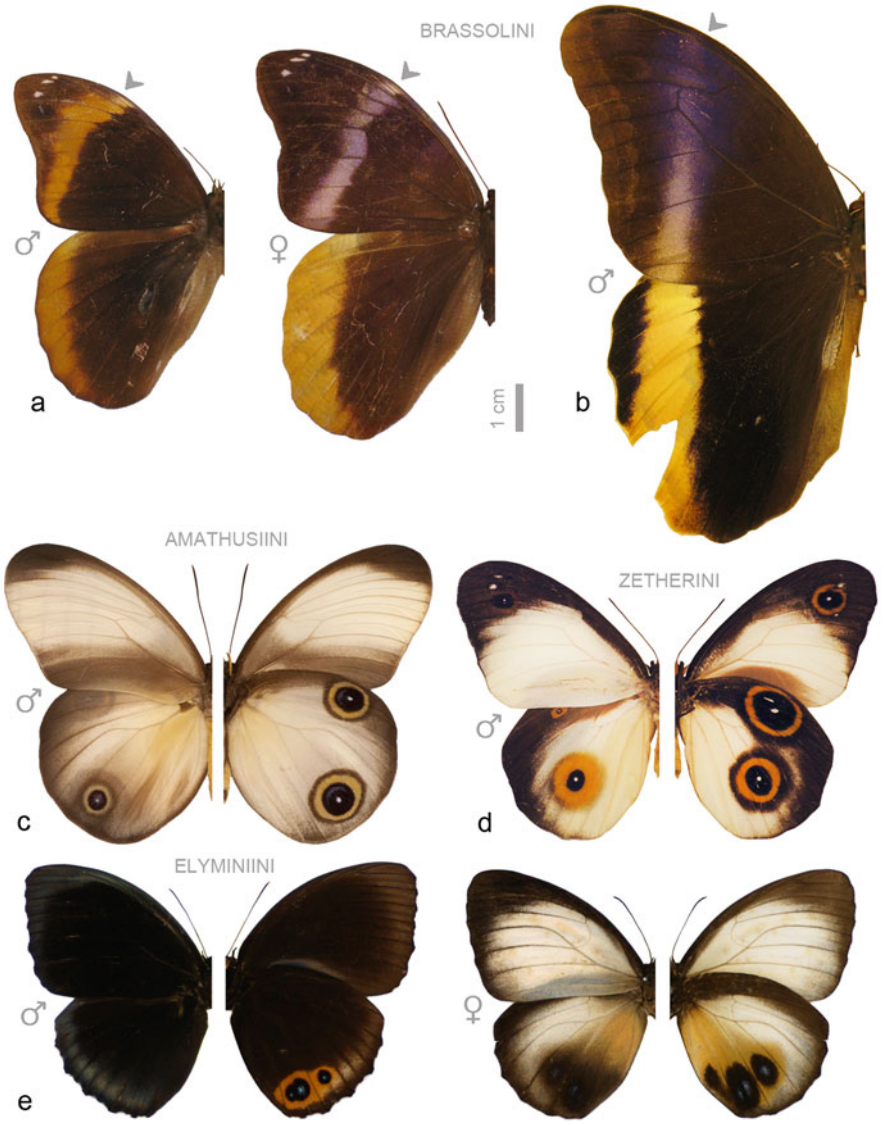

Fig. 2.8 Examples of mimetic convergence. Gray arrows indicate colorful band associated with element *f*. (**a**) nonmimetic male and mimetic female of *Catoblepia orgetorix*. (**b**) *Caligo atreus* model. (**c**) *Taenaris artemis*. (**d**) *Hyanthis hodeva*. (**e**) nonmimetic male and mimetic female of *Elymnias agondas*. All butterflies are at the same scale

can be used to hypothesize the WPE configuration of *Penthema* (Fig. 2.6b; see also Nijhout 1991). Notably, male and female of the sexually dimorphic *Zethera pimplea* have brown and off-white dorsal coloration, but the female pattern is

more intricate and Danaini-like than the male (Fig. 2.6g). Although some WPEs can be identified in *Elymnias* species that have complex Danaini-like dorsal patterns, they are generally difficult to interpret (Fig. 2.4a–c).

2.6 Transparency

Layers of scales make butterfly wings generally impenetrable to light. Nevertheless, some members of Satyrinae have evolved partial or complete transparency. In *Morpho sulkowskyi*, the dorsal scale size and pigmentation are reduced to such a degree that the ventral WPEs are visible through the wing (Fig. 2.2b). Partial transparency has evolved in more than one species of *Morpho*, but its function within the context of their natural history is unknown.

Scale cover is dramatically reduced in *Dulcedo*, *Pseudohaetera*, *Haetera*, and *Cithaerias* (Haeterini; Fig. 2.3e–f), and this possibly evolved ca. 29 million years ago (Cespedes et al. 2015). Transparency makes these butterflies nearly invisible in the forest understory and can be considered a defense against predation. Despite the extensive absence of scales, some WPEs are conserved, and this suggests they serve a function in the behavior of these butterflies. For example, their hind wing ocellus below M_1 is highly visible (Fig. 2.3e–f), and it might be involved in signaling. In the forest, male *Cithaerias* that are perched on the ground repeatedly flash their vivid dorsal hind wing colors (pers. obs.), which can likely be seen by other males or potential mates flying nearby.

The interplay between lost versus conserved wing color patterns is an interesting attribute of transparent Haeterini for two reasons. First, some pattern elements are expressed directly onto the wing membrane to form scale-less bands (Fig. 2.3e–f). This shows that the loss of scales does not necessarily lead to a loss of pattern. Membrane-level expression of WPEs can also be seen in areas that have scales, for example, the ocellus in Fig. 2.3f. To my knowledge, *Dulcedo*, *Pseudohaetera*, *Haetera*, and *Cithaerias* are the only butterflies in which WPEs are expressed on the wing membrane. Second, these butterflies show differential dorsoventral regulation of scale formation. For instance, in most transparent Haeterini, the ocellus below M_1 has a complete set of rings on the ventral hind wing surface, but the dorsal one lacks the orange ring (Fig. 2.3f). In *Cithaerias*, colorful scales are present on the dorsal hind wing only, and WPEs expressed at the wing membrane are thus more visible on the ventral surface (Fig. 2.3e). The colorful dorsal vestiture does not seem to correspond to a given WPE, and it spreads across the hind wing surface unaffected by elements *g*, *i*, and *j*. This begs the question of whether these WPEs are expressed on the ventral surface only (C. M. Penz, work in progress).

2.7 Concluding Remarks

The butterflies that form the focus of this chapter provide remarkable examples of color pattern variation. The significant changes in ocelli size and shape observed in *Bicyclus* selection experiments (e.g., Monteiro et al. 1997) suggest that butterflies can undergo rapid adaptive evolution. As a result, lineages might accumulate substantial wing pattern element modifications in relatively short evolutionary time scales. This is consonant with the observation that every tribe studied here includes species with nearly complete to highly reduced wing pattern elements—evolution repeats itself. Convergent appearance resulting from different pattern element modifications could reflect similarities in natural history or microhabitat use, e.g., ventral stripes in species of neotropical *Caerois* and old-world *Amathuxidia* (Figs. 2.2a and 2.7b). Field observations on mating behavior suggest the ventral hind wing ocelli may be used in male-female interactions in species of *Caligo* and *Pierella* (Figs. 2.1d and 2.3a), and this adds a new dimension to previous work. In the tribes studied here, pattern reduction is intriguing because it is accomplished in exceptionally different ways—pattern elements might not be expressed, or the scale vestiture may disappear almost completely (Figs. 2.8c–e and 2.3e–f). Transparency evolved independently in various ecologically and behaviorally distinct groups of Lepidoptera, the Haeterini being an example. How is scale loss adaptive in different taxa, what are the developmental mechanisms involved, and is it reversible? To further our understanding of the role wing coloration plays within the Satyrinae, the work presented here advocates baseline research on two fronts: documentation of pattern variation and field studies aimed at placing wing color diversification in a behavioral and evolutionary context.

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Appendix: List of Examined Taxa

Note that most, but not all, tribes within the focal clade are monophyletic (Wahlberg et al. 2009), and the classification used here is therefore tentative and expected to change (e.g., Zetherini). Genera and species are listed in alphabetic order, and those marked with an asterisk were examined from images only.

Brassolini: *Aponarope sutor*; *Bia actorion*, *B. peruana*; *Blepolenis batea*, *B. bassus*; *Brassolis dinizi*, *B. sophorae*; *Caligo atreus*, *C. idomeneus*, *C. martia*,

C. oberthuri; *Caligopsis seleucida*; *Catoblepia berecynthia*, *C. orgetorix*, *C. xanthus*; *Dasyophthalma creusa*, *D. rusina*; *Dynastor darius*; *Eryphanis aesacus*, *E. automedon*, *E. bubocula*; *Mielkella singularis*; *Narope cyllastros*, *N. panniculus*; *Opoptera aorsa*, *O. fruhstorferi*, *O. syme*; *Opsiphanes cassiae*, *O. invirae*, *O. sallei*; *Orobrassolis ornamentalis*; *Penetes pamphanis*; *Selenophanes cassiope*, *S. josephus*, *S. supremus*. See Penz and Mohammadi (2013) for additional species. Morphini: *Antirrhea archaea*, *A. avernus*, *A. philoctetes*; *Caerois chorineus*, *C. gerdrudtus*; *Morpho aega*, *M. anaxibia*, *M. aurora*, *M. catenarius*, *M. cypris*, *M. hecuba*, *M. helenor*, *marcus*, *M. menelaus*, *M. rhetenor*, *M. theseus*. Haeterini: *Cithaerias andromeda*, *C. aurora*, *C. aurorina*, *C. bandusia*, *C. pireta*, *C. pyritosa*, *C. pyropina*; *Dulcedo polita*; *Haetera piera*; *Pierella helvina*, *P. hortona*, *P. hyalinus**, *P. lamia*, *P. lena*, *P. lucia*, *P. luna*, *P. nereis*; *Pseudohaetera mimica*. Elymniini: *Elymnias agondas*, *E. cumaea*, *E. hypermnestra*, *E. nessaea*, *E. patna*; *Elymniopsis bammakoo*. Melanitini: *Melanitis amabilis*, *M. constantia*, *M. leda*. Dirini + *Manataria*: *Aeropetes tulbaghia*; *Dingana dingana**; *Dira clytus**; *Paralethe dendrophilus*; *Torynesis mintha**; *Manataria maculata*. Zetherini: *Ethope diademoides*, *E. himachala*, *E. noirei**; *Hyantis hodeva*; *Morphopsis albertisi*, *M. biakensis*, *M. meeki*, *M. ula*; *Neorina crishna*, *N. hilda*, *N. lowi*, *N. patria*; *Penthema adelma*, *P. darlisa*, *P. formosanum*; *Xanthotaenia busiris*; *Zethera incerta*, *Z. musa*, *Z. musides*, *Z. pimplea*. Amathusiini: *Amathusia binghami*, *A. phidippus*, *A. plateni*; *Amathuxidia amythaon*; *Discophora bambusae*, *D. sondaica*, *D. timora*; *Ensipe cycnus*, *E. euthymius*; *Faunis canens*, *F. eumeus*, *F. menado*, *F. stomphax*, *F. phaon leucis*; *Melanocyma faunula*; *Morphotenaris schoenbergi*; *Stichophthalma camadeva*, *S. godfreyi**, *S. howqua*, *S. louisa*, *S. nourmahal*, *S. sparta*; *Taenaris artemis*, *T. butleri*, *T. catops*, *T. myops*, *T. onolaus*; *Thaumantis diores*, *T. noureddin*, *T. odana*; *Thauria aliris*; *Zeuxidia amethystus*, *Z. aurelius*, *Z. doubledayi*.

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Chapter 3
Camouflage Variations on a Theme of the Nymphalid Ground Plan

Takao K. Suzuki

Abstract Lepidopteran camouflage patterns offer sophisticated and captivated examples of morphological evolution. Previous studies focused on how and why camouflage patterns are modulated at the microevolutionary level and determined, for instance, the adaptive role of camouflage patterns in avoiding predator attacks. However, less attention has been paid to the macroevolution of camouflage, including the evolutionary paths leading to the origination of leaf mimicry patterns. To understand the deep origins and evolvability of camouflage patterns, a key principle comes from a highly conserved ground plan (termed the nymphalid ground plan; NGP). The ground plan generates a variety of morphological forms, while it maintains its own type. This review introduces several seminal studies that used NGP-known features to reveal the macroevolutionary aspects of lepidopteran camouflage patterns, providing a roadmap for further understanding this biological phenomenon. The following core themes are discussed: (1) how complex camouflage patterns evolved (macroevolutionary pathways), (2) what kind of flexible mechanisms facilitate the origin of such complex patterns (macro-evolvability), and (3) how such complex patterns are tightly integrated through the coupling and uncoupling of ancestral developmental mechanisms (body plan character map). These approaches will provide new research lines for studying the evolution of camouflage patterns and the underlying flexibility of the NGP.

Keywords Crypsis and masquerade • Butterfly and moth • Comparative morphology • Macroevolution • Evolutionary path • Phylogenetic comparative methods • Tinkering • Morphological integration and modules • Morphometrics • Genotype-phenotype map

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3.1 Introduction

Complex and sophisticated camouflage patterns have fascinated many biologists (Poulton 1890; Cott 1940; Edmunds 1974; Ruxton et al. 2004; Stevens 2016). Recently, camouflage has been classified into two major types: crypsis (blended into environmental backgrounds to avoid detection by potential predators) and masquerade (special resemblance to natural objects to avoid recognition by potential predators) (Stevens and Merilaita 2009; Merilaita and Stevens 2011; Skelhorn et al. 2010a, b; Skelhorn 2015). Prominent cases of camouflage are found in butterfly and moth wing patterns, including tree bark crypsis in *Biston betularia* (van't Hof et al. 2016), lichen crypsis in *Agriopodes fallax* (Schmidt et al. 2014), leaf vein masquerade in the noctuid moth *Oraesia excavata* (Fig. 3.1a; Suzuki 2013) or in the nymphalid butterflies *Kallima inachus* and *K. paralekta* (Fig. 3.1b; Suzuki et al. 2014), and dried leaf masquerade in *Polygonia c-album* (Wiklund and Tullberg 2004). Most studies focused on the microevolutionary aspects of camouflage generation. For example, research on the industrial melanism shown in peppered moths deciphered both the adaptive significance (Cook et al. 2012) and the genetic basis of cryptic color variation (Cook and Saccheri 2013; van't Hof et al. 2016). Studies on the seasonal polyphenism of the butterflies *Araschnia levana* (Koch and Bückmann 1985), *Bicyclus anynana* (Brakefield and Larsen 1984; Monteiro et al. 2015), and *Polygonia c-aureum* (Fukada and Endo 1966; Endo 1984; Endo et al. 1988) have also uncovered hormonal switches in the generation of the cryptic patterns matching dry or autumnal color environments. In contrast, the macroevolution of camouflage has received little attention. The present review focuses on the comparative morphology of camouflage patterns in butterfly and moth wings and proposes a research roadmap for further advancing our understanding of the generative mechanisms underlying camouflage evolution.

For addressing the macroevolutionary aspects of lepidopteran camouflage, a key principle is that comparison of the anatomy of many species allows the extraction of

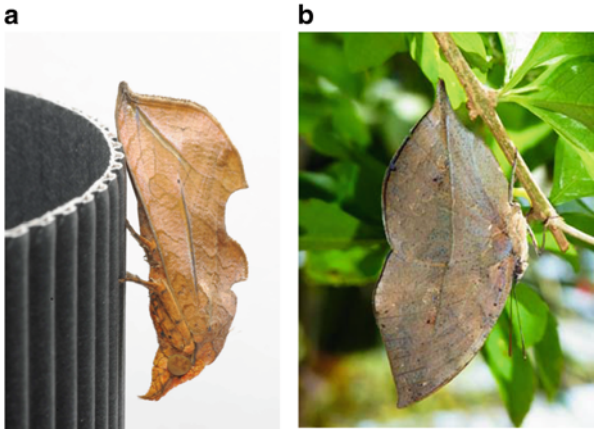

Fig. 3.1 Camouflage of moth and butterfly wing patterns. (**a**) *Oraesia excavata*. (**b**) *Kallima inachus* (Figure panel **a** is reproduced with modification from Suzuki (2013). Figure panel **b** is reproduced with modification from Suzuki et al. (2014))

a common theme behind diversity, termed the "body plan" or "ground plan," which refers to the structural composition of organisms based on homologous elements shared among species (Wagner 2014). To date, butterfly and moth (at least within Macrolepidoptera) wing patterns are thought to be based on a highly conserved ground plan (termed the nymphalid ground plan, NGP; Fig. 3.2a; Schwanwitsch 1924; Süffert 1927; Nijhout 1991). The NGP describes the diversification of wing patterns as modifications of an assembly of discrete pattern elements shared among species (Schwanwitsch 1956; Nijhout 1991) and is suggested to be homologous and inherited across species. From the comparative morphology point of view, the essential question is how effective is the NGP scheme in understanding lepidopteran camouflage patterns? Moreover, if certain camouflage patterns are illustrated by the NGP, what information can this scheme provide for understanding

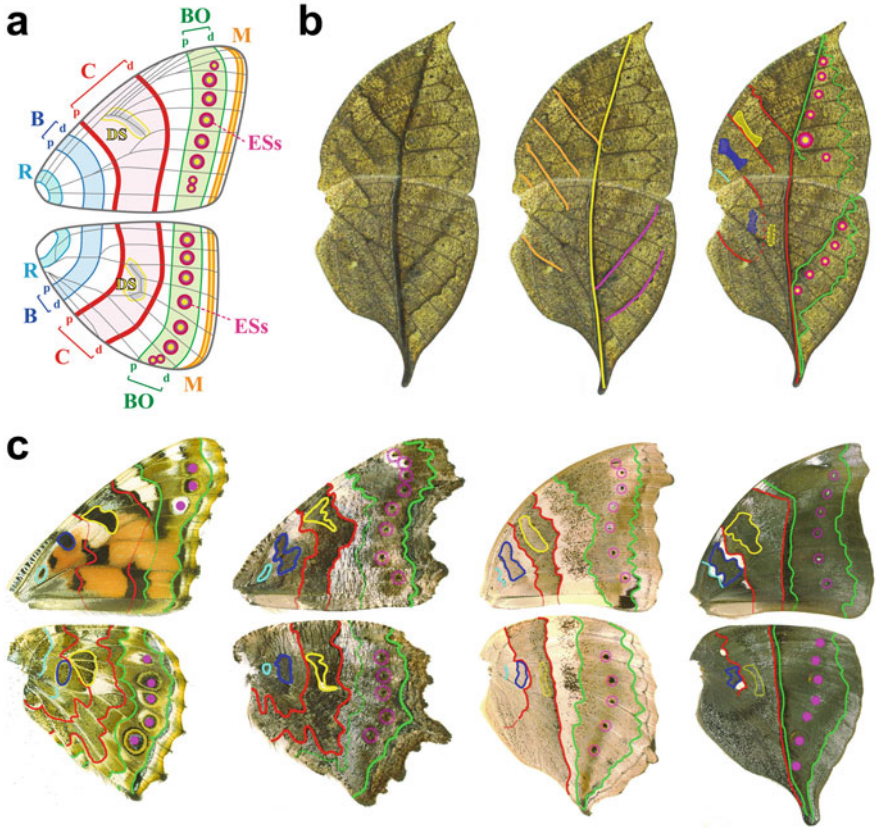

Fig. 3.2 Nymphalid ground plan and the variations generating diversified wing patterns. (**a**) Nymphalid ground plan (NGP). (**b**) Leaf vein-like pattern and the NGP of *Kallima inachus*. (**c**) NGP of *Vanessa cardui*, *Nymphalis vaualbum*, *Yoma sabina*, *Doleschallia bisaltide* (This figure is reproduced with modification from Suzuki et al. (2014))

lepidopteran camouflage patterns and can it contribute to the morphological evolution and organization of such spectacular examples of adaptation to the environment?

The present review introduces several NGP studies that are crucial for revealing the macroevolutionary aspects of lepidopteran camouflage patterns and provide a basis for further understanding this biological phenomenon. First, the foundations for using comparative morphology to identify homologous elements across species are described along with how NGP has led the way to the elaboration of diverse wing pattern configurations. Next, the potential of phylogenetic comparative methods to reveal the sequential evolutionary steps that built up leaflike patterns from nonmimetic ones is discussed. Third, the scheme of the NGP is used for discussing a flexible building logic of leaf mimicry patterns. Fourth, a methodological framework for analyzing the degree of integration and modularity in leaf vein-like pattern is proposed, and arguments favoring the evolutionary origin of *de novo* functional modules are presented. Finally, a research roadmap for further macroevolutionary studies on the origin and diversification of camouflage patterns is proposed.

3.2 Morphological Foundations of the Nymphalid Ground Plan

The concepts of body plan and ground plan are traditionally rooted in comparative morphology (Rieppel 1988). The criteria for identifying structural or positional homologs across different species were summarized by Remane (1952) and are considered a validated procedure in systematic and comparative morphology studies (Williams and Ebach 2008). These criteria consist of three principal rules: (1) similarity of topographical relationships, (2) similarity of special features, and (3) transformational continuity through intermediate ontogeny or phylogeny. The first criterion is logically consistent with Geoffroy St. Hilaire's "*principe des connexions*" (Saint-Hilaire 1818), the second is based on the specific properties of a character of interest, and the third is based on the evolutionary continuity of developmental genetic mechanisms underlying the character of interest. Although the concept of homology is still widely discussed (Patterson 1982; Roth 1988; Wagner 1989, 2007; Brower and Schawaroch 1996; Hall 2000), Remane's criteria remain valuable consensuses that crystallize empirical facts through numerous careful observations of morphological structures. Currently, these criteria provide a powerful tool to decipher the homology of anatomical structures in a broad spectrum of animals and plants (for animals: Nagashima et al. 2009; Hutchinson et al. 2011; Luo 2011; Holland et al. 2013; for plants: Sattler 1984; Buzgo et al. 2004).

The NGP is a scheme for describing homologous elements shared across species and thus should be evaluated within the logical framework of Remane's criteria.

Although Remane's criteria were inherent to NGP studies by Schwanwitsch (1956) and Nijhout and Wray (1986), to my knowledge, there is no explicit citation to Remane's work in NGP studies. Recently, I tackled to apply Remane's criteria to analyze the NGP of *Kallima inachus* and *K. paralekta* leaf vein-like patterns and succeeded in demonstrating that these can be explained by the NGP (Fig. 3.2b; Suzuki et al. 2014), and the results were consistent with Schwanwitsch (1956) analysis and validated the empirical inference proposed by Süffert (1927). The wing patterns of species closely related to *Kallima* spp. can also be explained by the NGP, although these patterns differ from that found in *Kallima* spp. (Fig. 3.2c, only four species were selected; for further details, see Suzuki et al. 2014). Interestingly, these analyses revealed that the differences between the leaf vein-like pattern and the other non-leaf patterns resulted only from differences in the character states of NGP elements. Thus, comparative morphology provides in-depth information about the way of diversification of lepidopteran wing patterns, even in extreme cases such as leaf mimicry.

It is important to mention that the NGP framework has limitations, which are most evident when lepidopteran wing patterns have so dramatically deviated from a stereotypical pattern that they challenge reasonable homology assignments. For example, the wing patterns of some papilionids are intensively fragmented through dislocation and thus difficult to connect to the NGP (Mallet 1991). In the nymphalid butterflies *Heliconius* sp., the NGP has undergone complex rearrangements that culminated in a highly modified state (Mallet 1991), although NGP was previously reported for this genus (Nijhout and Wray 1988). In such cases, less derived species can provide clues on intermediate states and clarify the nature of homologous characters but are prone to misidentifications without a more mechanistic understanding of wing pattern architecture. To further understand the evolutionary trajectories of the NGP, it is necessary to investigate the molecular mechanisms underlying NGP. Previous studies revealed the molecular mechanisms underlying eyespots (*ocelli*), one of the NGP elements in butterfly wings (Carroll et al. 1994; Brakefield et al. 1996; Keys et al. 1999; Brunetti et al. 2001; Beldade and Brakefield 2002; Monteiro et al. 2006; Oliver et al. 2012; Monteiro et al. 2013; Monteiro 2015; Zhang and Reed 2016; Beldade and Peralta 2017). Molecular studies have also uncovered several morphogens (e.g., *Wnt1*/*wingless*, *WntA*) and transcription factors (e.g., *aristaless2*, *engrailed*) associated with other elements of NGP (Brunetti et al. 2001; Monteiro et al. 2006; Martin and Reed 2010, 2014).

3.3 Evolutionary Path: Gradual Evolutionary Steps Toward Leaf Vein-Like Patterns

The ground plan architecture of lepidopteran wing patterns provides a starting point to investigate the evolutionary paths leading to complex camouflage patterns, but how can these trajectories be analyzed in exquisitely detailed phenotypes?

Character polarity has been used in most studies investigating the evolutionary processes that generate traits (Donoghue 1989; Swofford and Maddison 1992; Wiley and Lieberman 2011), and it refers to the biased phylogenetic placement of certain states of a character of interest (Fig. 3.3a). Clear detection of character polarity indicates a nested hierarchical relationship between traits, whose character states are evolutionarily transformed from ancestral to derived states in a specific temporal order. As shown in Fig. 3.3a, the evolution of trait A follows that of the trait B. However, this approach has a crucial practical limitation: traits of interest often lack a clear character polarity. To cope with this limitation, some statistical methods, collectively termed phylogenetic comparative methods (PCMs), were developed for analyzing traits' evolution (Fig. 3.3b; Harvey and Pagel 1991; Losos and Miles 1994; Garamszegi 2014). In PCMs, statistical testing is incorporated into the examination of phylogenetic information and character states to analyze the evolution of traits (Pagel 1999a). Accordingly, these methods can be used to detect subtle nuances of trait evolution that lack a clear signature of character polarity and thus can be applied in a broad spectrum of scenarios featuring a complex distribution of character states. In such scenarios, PCMs can be used in the reconstruction of traits' ancestral states (Schluter et al. 1997; Pagel 1999b; Pagel et al. 2004) or to infer the temporal order in which traits evolved, within a phylogenetic framework (Pagel 1994; Pagel and Meade 2006).

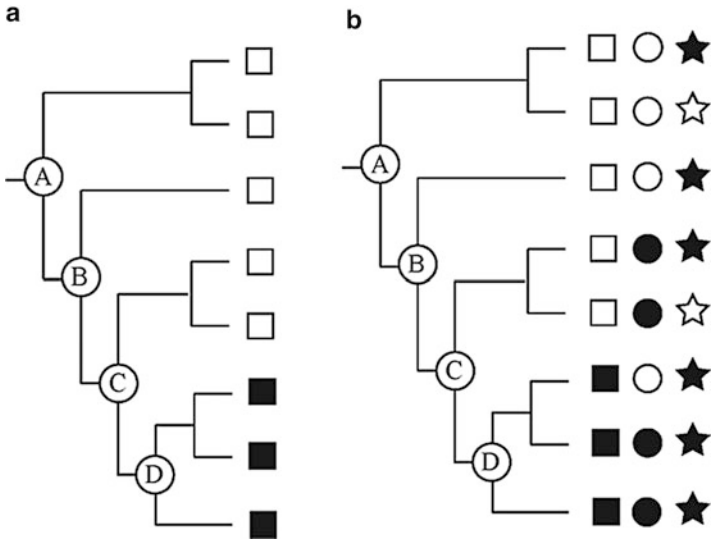

Fig. 3.3 How to infer macroevolutionary paths toward complex traits. (**a**) Simple case of character polarity, in which a trait (*square*) evolved from state 0 (*open square*) to state 1 (*close square*) at the node D of the phylogeny. (**b**) Complex case of character polarity, in which phylogenetic comparative methods were used to estimate the ancestral states of the traits (*squares*, *circles*, and *stars*)

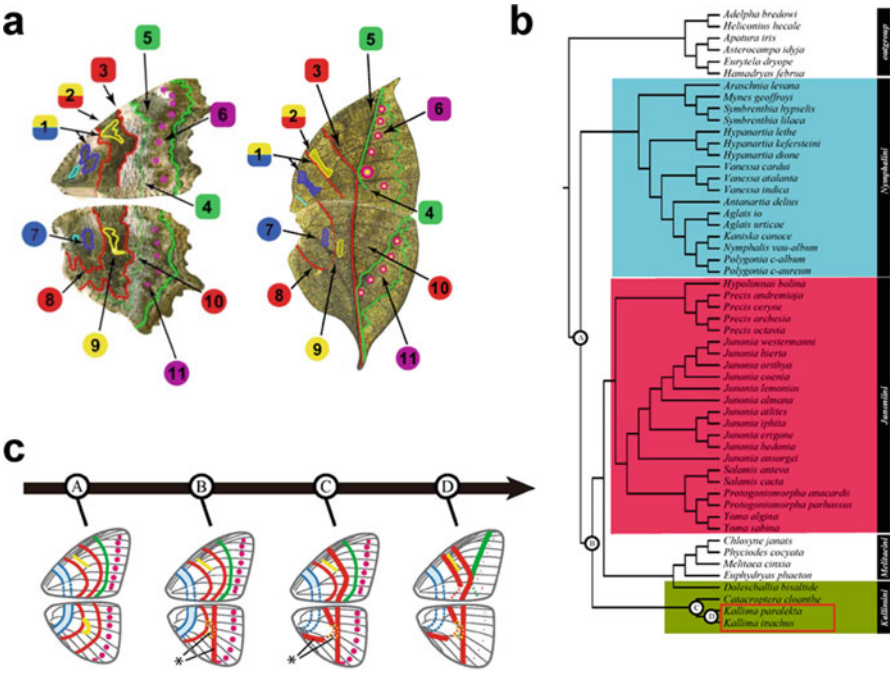

Fig. 3.4 Evolutionary steps that generated Kallima sp. leaf vein-like patterns. (**a**) Decomposition of wing patterns into 11 character states. (**b**, **c**) Reconstructed ancestral character states are represented at four selected nodes (*A, B, C*, and *D*), which are illustrated as the time required for the evolutionary transformation of wing patterns (from *A* to *D*). In the molecular phylogeny, the genus *Kallima* is evidenced using a *red box* (This figure is reproduced with modification from Suzuki et al. (2014))

The evolution of leaf resemblance in *Kallima* spp. has been a long-term conundrum and remains unresolved. Under a gradualistic view (Darwin 1871; Wallace 1889; Poulton 1890; Weissman 1902; Watson et al. 1936), the leaf mimicry pattern is a product of slow gradual evolution, with natural selection progressively perfecting masquerade forms; under the alternative saltationist view, leaf mimicry pattern evolved via relatively sudden leaps in the morphospace without intermediate forms (Mivart St 1871; Goldschmidt 1945). Despite the enthusiastic debate, no formal assessment of the tempo and mode of evolution in leaf mimicking has been provided so far. Recently, I applied PCMs to gain insight on the evolutionary paths that led to the leaf vein-like pattern in *Kallima* spp. (Fig. 3.4; Suzuki et al. 2014). If overall phenotypes are treated as integrated units, PCM analyses cannot reconstruct the evolutionary history of complex traits, simply by informing how many times the traits evolved (e.g., Mugleston et al. 2013). To avoid this, the butterfly wing patterns including the leaf patterns were decomposed into a set of several subcomponents using the NGP (Fig. 3.4a), which allowed inferring the ancestral states of each component and reconstructing the evolutionary process as the sum of the changes occurring in all

components (Suzuki 2017). Thus, tracing ancestral states at various phylogenetic nodes illustrates the sequential transformation of the character states of multiple components that led to the complex traits (Fig. 3.4b). This analysis revealed the successive steps in the evolution of leaf masquerade patterns from nonmimetic wing patterns within a phylogenetic framework (Fig. 3.4c; Suzuki et al. 2014) and provided the first evidence for gradual evolutionary origin of leaf mimicry (Skelhorn 2015). Thus, combining NGP and PCMs information provides an insight into the structural complexity of lepidopteran wing patterns and the possibility to depict the evolutionary paths leading to the formation of complex and detailed patterns (Suzuki 2017).

3.4 Tinkering: The Flexible Building Logic of Leaf Vein-Like Patterns

In addition to the reconstruction of evolutionary paths described above, identifying the NGP of lepidopteran wing patterns will provide resources to assess the different ways to produce leaf vein-like patterns. Regarding this issue, Schwanwitsch (1956) described the NGP of several species presenting leaf patterns such as *Siderone marthesia* (Fig. 3.5a), *Zaretis isidora* (Fig. 3.5b), and *K. inachus* (Fig. 3.2b). According to his scheme, the mode of derivation from the NGP is in most part repeated in these three species. Interestingly, the genera *Siderone* and *Zaretis* (Charaxinae, a subfamily of Nymphalidae) are taxonomically distant from the genus *Kallima* (Nymphalinae), which is also supported by Wahlberg et al. (2009) molecular phylogeny. Because convergence is considered to represent independently evolved features that are both structurally and superficially similar (Stayton

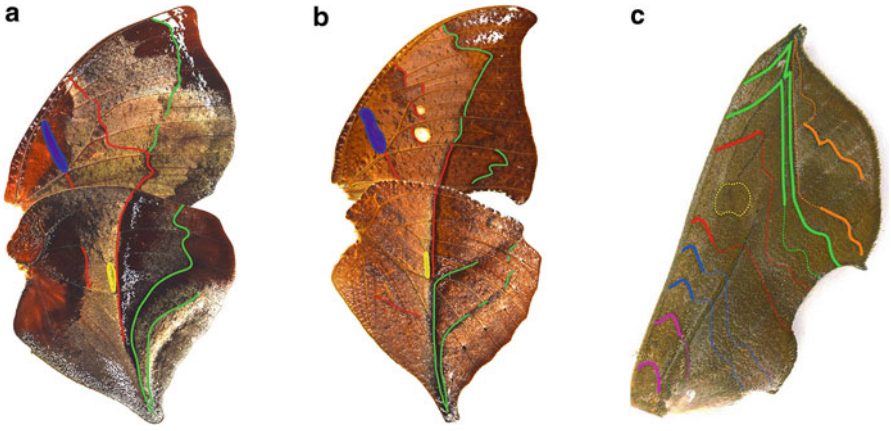

Fig. 3.5 Leaf vein-like variations on the same NGP theme. (**a**) *Siderone marthesia*. (**b**) *Zaretis isidora*. (**c**) *Oraesia excavata*. The NGP of *S. marthesia* and *Z. isidora* is based on Schwanwitsch (1956) (Figure panel c is reproduced with modification from Suzuki (2013))

2015), the similar mode of derivation from the NGP found in Charaxinae and Nymphalinae probably resulted from independent events of convergent evolution.

Does this similar mode of NGP-derived patterns, which seems to indicate that leaf pattern construction modes are quite constrained in butterflies, hold true for more distantly related taxa than Nymphalinae and Charaxinae? To address this question, I here compare the NGP of the leaf vein-like pattern found in the noctuid moth *O. excavata*, one of the most abundant moths in Northeast Asia (Fig. 3.1a), to that of *K. inachus*. Although these leaf vein-like patterns look similar, both consisting of a main vein and two sets of lateral veins, the way in which these two leaf patterns were built from the NGP is quite different (Figs. 3.2b and 3.5c). For example, in *K. inachus* butterflies, the main vein of the leaf pattern is derived from a green element (the proximal band of the border symmetry system) and a red element (the distal band of the central symmetry system), whereas in *O. excavata*, the main leaf vein is derived only from green elements (the border symmetry system). These observations showed that Lepidoptera leaf patterns can evolve through different paths, revealing a higher flexibility than that suggested from the analysis centered on nymphalid butterflies only.

This flexibility in leaf pattern building could be discussed within the concept of tinkering, which was in biology proposed by François Jacob (1977). This concept was described as "a tinkerer who does not know exactly what he is going to produce, but uses whatever he finds around him, whether it be pieces of string, fragments of wood, or old cardboards; in short it works like a tinkerer who uses everything at his disposal to produce some kind of workable object." Based on this statement, the leaf patterns of *Kallima* spp. and *Oraesia* spp. evolved in a tinkering mode of innovation, managing with odds and ends. Additionally, and although it might seem unexpected, the dead leaves of Charaxinae might have achieved the same construction style observed in *Kallima* as a result of tinkering evolution. Strictly speaking, tinkering likely refers to the evolutionary process of building up traits and not just to the traits. Thus, the flexible building logic of Lepidoptera leaf patterns might reflect the tinkering logic of the evolutionary processes behind them.

3.5 Modularity: Developmental Modules of the NGP and a Simple Cryptic Pattern

How a morphological structure is integrated is crucial to understand the genetic and developmental architecture of trait adaptation (Olson and Miller 1958; Cheverud 1996; Klingenberg 2008). The concept of morphological integration postulates that functionally related elements are tightly coupled (Olson and Miller 1958; Cheverud 1996). A special form of integration is modularity, in which units are tightly coupled but can be individually decoupled (Wagner and Altenberg 1996). Modularity results from the regulatory interactions of developmental mechanisms (Klingenberg 2008) and/or from accumulated structural changes shaped by natural

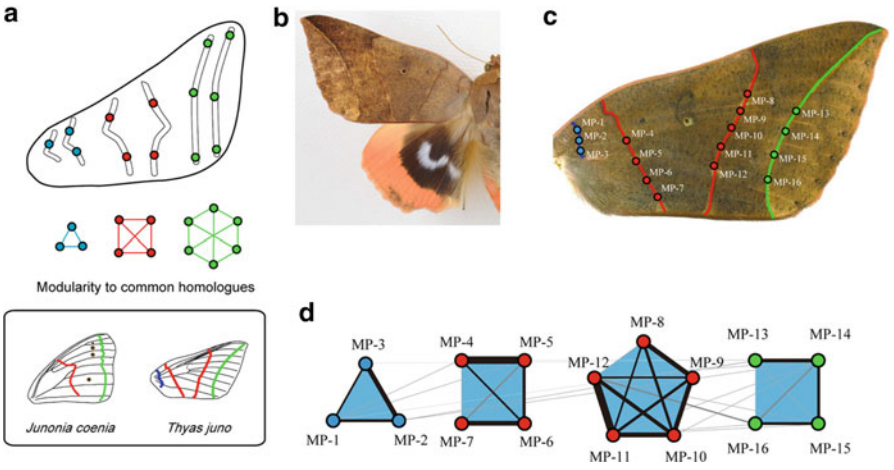

Fig. 3.6 Modularity of the simple cryptic pattern of *Thyas juno*. (**a**) Schematic illustration of divergence strategy in moth and butterfly wing patterns. The modularity of simple patterns corresponds to the original developmental modules of the NGP. (**b**) Forewings and hind wings of *T. juno*. (**c**) Forewings comprise four elements, each corresponding to an NGP element. (**d**) Morphological correlation network of the *T. juno* forewing pattern. In this correlation network, nodes represent measurement points and lines represent the correlations between measurement points (larger correlation coefficients are indicated by *thicker arrow edges* and *darker lines*). The modules detected are illustrated as *light-blue areas* (This figure is reproduced with modification from Suzuki (2013))

selection (Lande 1979; Arnold 1983; Wagner and Altenberg 1996). Previous studies suggested that the NGP is the sum of several developmental modules where each NGP element is genetically and/or developmentally autonomous (Fig. 3.6a; Nijhout 1991, 1994, 2001; Beldade and Brakefield 2002). In fact, the central symmetry system of the NGP appears to be a genetically and phenotypically independent unit (Brakefield 1984; Paulsen and Nijhout 1993; Paulsen 1994, 1996), and eyespots are developmental units formed by factors diffused from foci (Nijhout 1980; French and Brakefield 1995). These considerations strongly suggest that butterfly and moth wing patterns, including camouflage patterns, obey to NGP's rule of modularity.

How are lepidopteran camouflage patterns integrated and modularized? To address this issue, the relatively simple camouflage pattern of the noctuid moth *Thyas juno* was examined (Fig. 3.6b; Suzuki 2013). At rest, this species displays only the cryptic forewings covering the conspicuous hind wings, but, once it detects a potential enemy, the forewings are unfolded and display the warning-colored hind wings. The forewing pattern consists of four elements, each corresponding to an NGP element (Fig. 3.6c). To detect the modules involved in an overall wing pattern, I developed a new analytical method (termed morphological correlation network), which allows analyzing geometric morphometric data by combining graph theory and the statistical physics of spin glass (Suzuki 2013; Esteve-Altava 2016). This approach revealed that the modules involved in *T. juno* wing pattern corresponded to individual NGP symmetry elements, which might reflect the original modular

architecture of the NGP (Fig. 3.6d) as supported by previous considerations regarding NGP organization (Nijhout 1991, 1994, 2001; Beldade and Brakefield 2002). Although studying a practical case is limited, at least in relatively simple camouflage patterns, these results supported the hypothesis that the genetic and developmental architectures underlying camouflage patterns reflect the original developmental modules of the NGP (Fig. 3.6a).

3.6 Evolutionary Origin of *De Novo* Modules: Rewiring of the NGP Developmental Modules to Generate Functional Modules

How modules of morphological structures originated is an important question to understand the complex adaptation of phenotypes (Wagner et al. 2007; Klingenberg 2008). A previous conceptual study proposed that modules evolved through the opposite processes of integration (coupling) and parcellation (uncoupling) (Wagner and Altenberg 1996). This conceptual framework seems to be crucial to comprehend the evolution of butterfly and moth wing patterns through modifications of the NGP. Contrasting to the early establishment of the conceptual basis, how *de novo* modules originated still remains poorly understood (Moczek et al. 2015). The question here is how modules of complex camouflage patterns originated within the context of morphological integration and parcellation.

To address this question, the modular architecture of the leaf vein-like pattern of *O. excavata* (Figs. 3.1a and 3.5c) was investigated using the morphological correlation network method (Suzuki 2013). This study revealed that the leaf pattern of *O. excavata* is highly modularized, with each module corresponding to each component of the leaf vein, implying the functional modules (Fig. 3.7b). To examine the extent of the association between these functional modules and the developmental modules of the NGP, the morphological correlation network of the *O. excavata* wing pattern was replotted (Fig. 3.7c). Interestingly, functional modules were generated by the coupling and uncoupling of NGP developmental modules. For example, the functional module of the left lateral vein (i.e., module 2) originated from coupling two distinct modules of the central and border symmetry systems, and the developmental module of the border symmetry system was uncoupled into three functional modules (i.e., modules 2, 3, 4). Thus, this analysis clearly demonstrated that, at least in the evolution of complex camouflage patterns such as leaf masquerade, *de novo* modules originated through the reintegration of NGP developmental modules (Fig. 3.7a).

Unlike the previous studies in which the NGP was considered to comprise autonomous units (Fig. 3.6; Nijhout 1991, 1994, 2001; Beldade and Brakefield 2002), the modules in the *O. excavata* leaf pattern originated through reintegration to new modules (Fig. 3.7). This discrepancy could be due to differences between simple and complex patterns (Figs. 3.6a and 3.7a). Previous studies often

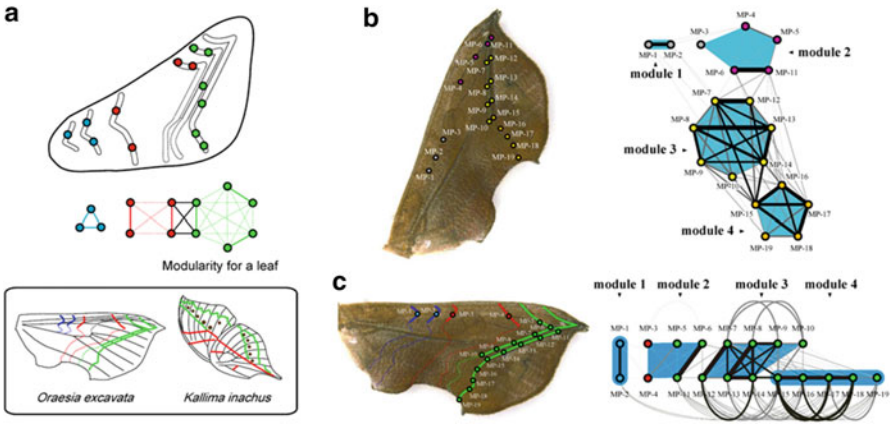

Fig. 3.7 Modularity of the leaf vein-like pattern of *Oraesia excavata*. (**a**) Schematic illustration of divergence strategy in moth and butterfly wing patterns. The modularity of complex patterns evolved through rewiring the original developmental modules of the NGP. (**b**) Forewings of *O. excavata* and its morphological correlation network. In this correlation network, nodes represent measurement points and lines represent correlations between measurement points (larger correlation coefficients are indicated by *thicker arrow edges* and *darker lines*). The modules detected are illustrated as *light-blue* areas. (**c**) Replot of the correlation network of *O. excavata* wing pattern based on the NGP (This figure was reproduced with modification from Suzuki (2013))

emphasized that the genetically and developmentally autonomous units of the NGP allowed further uncoupling pattern elements (e.g., dislocation), and such individualization is thought to allow establishing separate evolutionary trajectories, thereby contributing to the evolvability of lepidopteran wing patterns. In addition to this previous perspective, the present review emphasizes the importance of coupling of pattern elements in wing morphological diversification and proposes a new organizing principle, a "rewiring" strategy (i.e., coupling and uncoupling) of the NGP, in which a combination of decoupling and coupling processes "rewires" the correlations among common parts (Fig. 3.7a; Suzuki 2013).

3.7 Next Research Programs

Quantitative analyses, together with the scheme of the NGP, have begun to set a new path for understanding camouflage patterns of butterfly and moth wings. The NGP provides a foundation for the evolutionary pathways, evolvability, and genetic/developmental architecture underlying the complex and diversified camouflage patterns, through which the ground plan is modified. In this final section, further research programs are discussed.

3.7.1 Macroevolutionary Pathways Toward Camouflage Patterns

Diversification based on NGP modifications is not a random process but occurs in a certain sequential order. As shown above, mathematical methods using Bayesian statistics enabled analyzing the evolutionary origin and sequential steps toward the various camouflage patterns (Suzuki et al. 2014; Suzuki 2017). This approach allows to test whether camouflage patterns originated gradually or suddenly and to analyze the evolutionary process through which modifications were accumulated generating camouflage patterns.

Furthermore, comparing multiple evolution processes allows examining evolutionary pathways considering whether processes within them are possible or not. For example, comparing the evolutionary processes involved in butterfly leaf masquerade and lichen cryptic patterns may reveal common/different evolutionary mechanisms between the different camouflage patterns. Similarly, comparing the evolutionary processes of leaf masquerade among distinct taxa may reveal how many pathways are involved in the evolution of lepidopteran leaf patterns and/or addressing the mechanisms allowing the multiple origins of leaf mimicry in Lepidoptera. To date, studies considering macroevolution discussed only the tempo, mode, and trends of evolution (Simpson 1944; Carroll 2001). In addition to these research directions, studying the evolutionary processes and pathways involved in complex and diversified traits is expected to add a new direction in the research field of macroevolution.

3.7.2 Macro-evolvability of the NGP

The deep involvement between body plan and evolvability has often been discussed (Vermeij 1973; Riedl 1978; Kirschner and Gerhart 1998; Graham et al. 2000). Regarding evolvability, Vermeij (1973) proposed the concept of versatility, which focuses on the number and range of independent parameters controlling morphological form. As described above, the evolution of the *O. excavata* leaf pattern involved the reintegration of the original developmental modules of the NGP (Fig. 3.7), suggesting that the increase in the number of parameters controlling shape allowed new adaptations, reflecting the versatility of the NGP (Suzuki 2013). In addition, the flexible logic of leaf mimicry patterns suggests a new component (e.g. tinkering) in the evolvability of the ground plan (Fig. 3.2b and 3.5). It has been pointed out that evolvability has various definitions, and Pigliucci proposed its classification in an evolutionary time scale (Pigliucci 2008). Following his definition, I would like to propose the term "macro-evolvability" to define the long time scale evolution that generates various forms through modifications of the ground plan.

Furthermore, one extreme case when examining the macro-evolvability of the ground plan is to determine under which circumstances the ground plan is partially or fully broken. In other words, this approach provides an insight into evolvable limitation of the NGP. Unlike that considered before Darwin's theory of evolution,

the ground plan is also subject to natural selection, and therefore some or all of it might be broken with the evolutionary emergence of a specific form derived from the ground plan. Are there possibilities that the NGP was broken? The wing pattern of a mimicry butterfly, *Heliconius* sp., might be considered (Jiggins et al. 2017) a possible example of such a situation. Under this consideration, several questions are raised: How was the NGP deconstructed in *Heliconius* butterflies? What kind of natural selection promotes NGP loss? Does the evolutionary acquisition of Müllerian mimicry affect the loss of the NGP? To address such questions, it will be necessary to combine morphological and molecular studies to verify NGP integrity (Martin et al. 2012; Martin and Reed 2014), because the NGP might be difficult to identify in these butterflies.

3.7.3 *Body plan Character Map: Genetic and Developmental Architectures of the NGP*

What kind of genetic and developmental architectures underlies the ground plan? In previous studies, this issue was discussed from various perspectives, including the perspectives of transcriptomics (Duboule 1994; Kalinka et al. 2010; Irie and Kuratani 2011, 2014; Quint et al. 2012; Levin et al. 2016) and gene regulatory networks (Davidson and Erwin 2006; Wagner 2007). From the morphological integration and modularity perspective, two major schemes were proposed: the genotype-phenotype map (G-P map; Fig. 3.8a; Wagner and Altenberg 1996) and developmental mapping (D map; Fig. 3.8b; Klingenberg 2008). Both schemes describe how modules of traits were generated through internal interactions, but while the G-P map is based on genetics, the D map is based on ecological

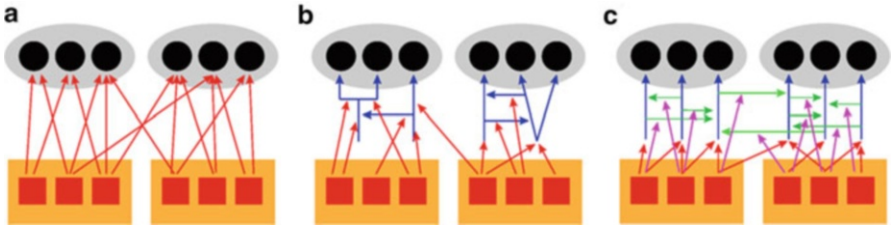

Fig. 3.8 Genetic and developmental architectures of a modularized phenotypic trait. (a) Genotype-phenotype map (G-P map). (b) Developmental map (D map). (c) Body plan character map (BC map). All schemes describe the relationship between genes (*red squares*) and the subcomponents (*black circles*) of a phenotypic trait, when the trait is modularized (*gray circles*). The G-P map describes the construction of modularity through changes in pleiotropic effects (*red arrows*), whereas the D map describes the modulated pathways of the developmental system (*blue arrows*) affected by changes in pleiotropy. The BC map describes the construction of modularity through the coupling (*green arrows*) and uncoupling (*light green arrows*) of the original developmental pathways of the ground plan (blue arrows), where subcomponents (*black circles*) are homologous parts, and each phenotypic trait is the ground plan of interest (Figure panel a was modified from Wagner and Altenberg (1996), and panel b was modified from Klingenberg (2008))

evolutionary developmental biology. These two schemes cover a broad spectrum of biological traits but are less likely to be practical for deciphering a specific genetic and developmental architecture of traits. From the perspective of comparative morphology, a specific scheme to comprehend the complexity and diversification of traits needs to be established.

How can the genetic and developmental architectures that create various forms by modification of the ground plan be depicted? Considering the experimental facts explained above, two major components seem to be involved: one arises from the original developmental modularity of the ground plan and the other from rewiring the developmental modules of the ground plan. In general, the ground plan is a sum of homologous parts, and it is thought that each homologous part constitutes one developmental module because each part is individually identifiable (Wagner 1989, 2014). An example of the component derived from rewiring the developmental modules of the NGP is the functional modules found in *O. excavata* leaf pattern (Suzuki 2013). In the present review, I propose a scheme for integrating the genetic and developmental architecture underlying the variations of a theme of the ground plan, termed Body plan Character Map (BC Map; Fig. 3.8c). This scheme describes the core generation process of the ground plan and the reorganization process that transforms it into various designs, which can only be revealed using the morphological approach described in this study combined with molecular data.

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Chapter 4
Morphological Evolution Repeatedly Caused by Mutations in Signaling Ligand Genes

Arnaud Martin and Virginie Courtier-Orgogozo

Abstract What types of genetic changes underlie evolution? Secreted signaling molecules (*syn*. ligands) can induce cells to switch states and thus largely contribute to the emergence of complex forms in multicellular organisms. It has been proposed that morphological evolution should preferentially involve changes in developmental toolkit genes such as signaling pathway components or transcription factors. However, this hypothesis has never been formally confronted to the bulk of accumulated experimental evidence. Here we examine the importance of ligand-coding genes for morphological evolution in animals. We use Gephebase (http://www.gephebase.org), a database of genotype-phenotype relationships for evolutionary changes, and survey the genetic studies that mapped signaling genes as causative loci of morphological variation. To date, 19 signaling genes represent 20% of the cases where an animal morphological change has been mapped to a gene (80/391). This includes the signaling gene *Agouti*, which harbors multiple cis-regulatory alleles linked to color variation in vertebrates, contrasting with the effects of coding variation in its target, the melanocortin receptor MC1R. In sticklebacks, genetic mapping approaches have identified 4 signaling genes out of 14 loci associated with lake adaptations. Finally, in butterflies, a total of 18 allelic variants of the *WntA* Wnt-family ligand cause color pattern adaptations related to wing mimicry, both within and between species. We discuss possible hypotheses explaining these cases of natural replication (genetic parallelism) and conclude that signaling ligand loci are an important source of sequence variation underlying morphological change in nature.

Keywords Signaling ligands • Genotype-phenotype relationships • Mutational target • cis-Regulatory alleles • Gephebase

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A key aim of developmental biology is to describe the molecular mechanisms underlying pattern formation, i.e., how gene expression patterns are established and how cell differentiation is orchestrated over time. Since the discovery of embryonic induction, which revealed that secreted molecules are capable of instructing and organizing cells in surrounding tissues (Waddington 1940; Spemann and Mangold 2001), cell-cell signaling has become a sine qua non mechanism of pattern formation in many (if not most) developmental systems (Meyerowitz 2002; Rogers and Schier 2011; Urdy 2012; Kicheva and Briscoe 2015). Experimental manipulations of extracellular signals can impact tissue patterning at a distance (Salazar-Ciudad 2006; Nahmad Bensusan 2011; Perrimon et al. 2012; Urdy et al. 2016). It follows that to understand how spatial information is deployed in differentiating tissues, it is critical to characterize the signals that mediate intercellular communication. A handful of genes coding extracellular proteins that act as signaling molecules between neighboring cells have been identified in animals (Nichols et al. 2006; Rokas 2008a; Perrimon et al. 2012): Wnt, TGF-beta, Hedgehog, Notch, EGF, RTK ligands, and TNFs, among other families. These signaling ligands are widely conserved and show highly regulated expression patterns (Salvador-Martínez and Salazar-Ciudad 2015).

In the 2000s it was proposed that the construction of multicellular organisms relies on a small set of conserved genes, referred to as the developmental genetic toolkit (DGT), which comprises a few hundred genes from a few dozen gene families involved in two major processes: cell differentiation and cell-cell communication (Carroll et al. 2005; Floyd and Bowman 2007; Rokas 2008b; Erwin 2009). On the other side, genes that are not part of the DGT were attached to vital routine functions such as metabolism, protein synthesis, or cell division. According to the DGT view, spatial information emerges from an interplay between genetic factors involved in signal transduction and transcriptional control. An inevitable consequence is that morphological evolution should be based, to a large extent, on reusing these toolkit components, and it follows that mutations in the DGT genes themselves should cause evolution of form (Carroll et al. 2005; Carroll 2008). Such proposition was formulated at the beginning of the twenty-first century, while few genes underlying morphological evolution had been identified – less than 50 cases in 2001 (Martin and Orgogozo 2013). As of today, the hypothesis that animal morphological evolution is mainly caused by mutations in DGT genes can now be tested further based on micro-evo-devo studies (Nunes et al. 2013) and the analyses of genotype-phenotype variation in nature (Orgogozo et al. 2015; Stern 2011). Here we investigate one aspect of the DGT view, the importance of genes encoding secreted signaling proteins in driving morphological evolution. We examine whether ligand-coding genes are preferential targets for the generation of morphological evolution. In addition, we confront existing data to predictions that the corresponding allelic variation should be (1) potentially adaptive (Barrett and Hoekstra 2011; Pardo-Diaz et al. 2015), (2) replicated over various phylogenetic levels (Gompel and Prud'homme 2009; Kopp 2009; Martin and Orgogozo 2013), and (3) cis-regulatory rather than coding (Prud'homme et al. 2007; Carroll 2008; Stern and Orgogozo 2008; Liao et al. 2010).

4.1 Gephebase: The Database of Genotype-Phenotype Variations

Experimental studies based on the manipulation of gene function in the laboratory – for instance, based on reverse genetics or on a mutant screen followed by forward genetics mapping – describe the overall architecture of the genotype-phenotype map in a given organism. However, the genetic causes of evolutionary change in nature do not necessarily equate to the mutations studied in the laboratory: evolutionary-relevant mutations may represent a particular subset of all possible mutations. To identify the genetic causes of natural differences between individuals, populations, and species, one can perform forward genetics studies that compare two naturally occurring phenotypic states – in general, using linkage mapping of quantitative trait loci or Mendelian genes or association mapping (Stern 2000). The so-called "loci of evolution" or "quantitative trait gene (QTG)" studies identify pairs of alleles linked to a specific phenotypic difference (Orgogozo et al. 2015), for instance between an ancestral and a derived state. These loci are typically genomic targets of selection when the variation is of adaptive or domesticating potential. Due to experimental limitations, the dataset is biased toward large-effect loci and thus misses a large fraction of what constitutes the total genetic template of evolution (Rockman 2012). Nevertheless, we think that it is crucial to gather the findings of this research program under the banner of a resource that would integrate, for comparative and meta-analytical purposes, our growing knowledge of genotype-phenotype relationships. To facilitate the curation and analysis of the relevant literature [see (Stern and Orgogozo 2008; Streisfeld and Rausher 2011; Martin and Orgogozo 2013) for previous examples], we have created Gephebase (http://www.gephebase.org), a database of genotype-phenotype relationships underlying natural and domesticated variation across Eukaryotes. Here, we use Gephebase to reflect on the importance of signaling ligand genes for morphological evolution in animals.

4.2 Method: Construction of Gephebase and Identification of Signaling Genes

Gephebase is a quality-controlled, manually curated database of published associations between genes and phenotypes in Eukaryotes – containing a total of 1400 entries as of December 31, 2016. For now, genes responsible for human disease and for aberrant mutant phenotypes in laboratory model organisms are excluded and can be found in other databases (OMIM, OMIA, FlyBase, etc.). QTL mapping studies whose resolution did not reach the level of the nucleotide or of the transcriptional unit are also excluded. In Gephebase, each genotype-phenotype association is attributed to only one type of experimental evidence among three possibilities: "association mapping," "linkage mapping," or "candidate gene." This

choice is made by Gephebase curators based on the best evidence available for a given genotype-phenotype relationship. Gene-to-phenotype associations identified by linkage mapping with resolutions below 500 kb have priority in the dataset (see Supplementary Materials in Martin and Orgogozo 2013). Association mapping studies are included based on individual judgment, with a strong bias toward SNP-to-phenotype associations that have been confirmed in reverse genetic studies. In other words, Gephebase intends to be more stringent than a compilation of statistically significant SNPs, and attempts to select studies where a given genotype-phenotype association is relatively well supported or understood.

Gephebase presents itself as a collection of entries, where each entry corresponds to an allelic difference at a given gene, either between two closely related species or between two individuals, its associated phenotypic change, and the relevant publications. As of today, the database contains a total of 391 entries related to animal morphological changes: 174 for domesticated or artificially selected traits, 172 for intraspecific trait variations, and 45 for interspecific changes (Table S1, available at http://virginiecourtier.wordpress.com/publications/. We identify 80 cases of natural morphological evolution and domestication in animals (out of 391) that involve 21 different ligand genes (Table 4.1; Table S2, available at http://virginiecourtier.wordpress.com/publications/).

To estimate the proportion of genes encoding signaling ligands in genomes (Table 4.2), we used the BioMart portal from Ensembl (Smedley et al. 2015). All the genes, which have both the following Gene Ontology (GO) annotations, "receptor binding" (Molecular Function, GO:0005102) and "extracellular region" (Cellular Component, GO:0005576), were considered as ligand genes. To count the number of genes with two GO annotations, we used BioMart to extract text files containing Ensembl Gene ID for each GO and each species. We then counted the number of genes having both GO in each species with the following Linux command: *comm -1 -2 <(sort human-GO0005102.txt) <(sort human-GO0005576.txt) | head -n -1 | wc -l* (note that the title line had to be excluded from the count).

Box 4.1: Definitions

Admixture Mapping: a method capitalizing on the current gene flow between two or more previously isolated populations to associate genetic loci to phenotypic traits. Admixture mapping is a form of association mapping.

Association Mapping: a forward genetics method for gephe identification based on a genome-wide statistical association between genetic variants and phenotypic traits, generally in a large cohort of unrelated individuals.

Candidate Gene Approach: a reverse genetics method that tests if a locus defined a priori, based on our current biological knowledge, underlies variation in a phenotype of interest. *Example*: opsin photoreceptor genes are typical candidate genes for differences in color vision.

(continued)

Box 4.1 (continued)

Forward genetics: set of methods used to identify the genetic cause(s) of a given phenotypic trait ("from the phenotype to genes").

Genetic hotspot: a group of orthologous loci that have been associated multiple times to phenotypic variation due to independent mutational events in each lineage (Martin and Orgogozo 2013).

Gephe (neologism for genotype-phenotype relationship; pronounced *jay-fee*): an abstract entity composed of three elements: a variation at a genetic locus (two alleles), its associated phenotypic change (two distinct phenotypic states, e.g., an ancestral and a derived state), and their relationship (Orgogozo et al. 2015). A gephe is usually defined for a given genetic background and environment.

Haplotype: a set of closely linked alleles found on the same chromosome, which is inherited as a single piece.

Heterotopy: change that occurred during evolution in the location of a particular molecular event within the developing organism.

Linkage Mapping: a forward genetics method for gephe identification based on chromosome shuffling and crossing-overs, using the progeny of a hybrid cross. This includes the mapping of quantitative trait loci (QTL) and Mendelian loci.

Mendelian Gene: a segregating genetic unit which is detected through phenotypic differences associated with different alleles at the same locus (Orgogozo et al. 2016).

Morphospace: an abstract representation of all possible morphologies and shapes of an organism.

Orthologous Loci: pieces of DNA that share ancestry because of a speciation event and that are thus found in different species.

Parallel Evolution: here defined as independent repeated sequence variation at a same locus, underlying variation in a similar phenotypic trait (Stern 2013). For other definitions, see (Scotland 2011).

Phenologue: a similar phenotype caused by a conserved genetic mechanism in distant lineages (McGary et al. 2010; Lehner 2013). Used here as the phenotypic counterpart of a gephe involving several cases of parallel evolution.

Quantitative Trait Locus: a portion of DNA (the locus) that is associated with variation in a quantitative phenotypic trait.

Reverse Genetics: set of methods used to alter a given gene in order to characterize its function ("from genes to phenotypes").

Table 4.1 Twenty-one ligand genes with known gephe variations identified in Gephebase – 19 of which related to cases of morphological evolution (accessed December 31, 2016)

Ligand gene	Trait variation	Nb. of natural gephes (intra-/interspecific) in	Nb. of artificially selected gephes (domesticated) in	Comments	Reference PubMed ID
Agouti (ASIP)	Pigmentation	Mammals/birds (**11**)	Mammals/birds (**14**)	See Fig. 4.1 and Gephebase	See Gephebase
BMP2	Fertility (females) + comb morphology (males)	–	Chicken (**1**)	Pleiotropic and joint effect with *HAO1*	22956912
					24655072
BMP3	Craniofacial skeleton	–	Dog (**1**)	Uncertain (possible role of *PRKG2*)	22876193
BMP6	Tooth number	Stickleback fish (**1**)	–	cis-Regulatory allele	25205810
					26062935
					25732776
BMP15	Fertility (not a morphological trait)	–	Sheep (**4**)	Coding alleles	10888873
					23637641
CBD103	Pigmentation	Wolf/coyote (**1**)	Dog (same allele)	Coding allele	19197024
					17947548
EDA	Armor plates + schooling behavior	Stickleback fish (**1**)	–	cis-Regulatory allele	15790847
					22481358
					25629660
EDN3	Pigmentation	–	Chicken (**1**)	Large duplication	22216010
					25344733
					25741364
FGF5	Hair length	–	Cat (**1**), dog (**1**), donkey (**2**)	Coding alleles	See Gephebase
GDF5	Body size	Human (**1**)	–	Allele unknown (SNP association)	18193045
GDF6	Skeletal traits	Stickleback fish (**1**); human (**1**)	–	cis-Regulatory alleles	26774823

GDF9	Fertility (not a morphological trait)	–	Sheep (**2**)	Coding alleles	19713444
					20528846
IGF1	Body size	–	Dog (**1**)	cis-Regulatory allele	17412960
IGF2	Muscle and fat content	–	Pig (**1**)	cis-Regulatory allele	14574411
KITLG	Pigmentation	Stickleback fish (**1**); human (**2**)	Cattle (**1**)	cis-Regulatory alleles	See text
Myostatin (GDF8)	Muscular growth	–	Mammals (**14**)	Coding (12) + cis-reg. (2) alleles	See Gephebase
Rspo2	Hair length	–	Dog (**1**)	cis-Regulatory allele	19713490
scabrous	Bristle number	Fruit fly (**2**)	–	Distinct alleles affect thorax and abdomen	7992053
upd-like	Wing size	Jewel wasp (**1**)	–	cis-Regulatory allele QTL fractionation	22363002
wingless (Wnt1)	Pigment patterns (wing, larva)	Fruit fly (**1**)	Silkworm (**1**)	cis-Regulatory complex alleles	23673642
					26034272
WntA	Pigment patterns (wing)	Butterflies (10)	–	cis-Regulatory alleles	See text

Table 4.2 Proportion of signaling ligand-encoding genes in the genome of several model species

	Homo sapiens (GRCh38.p7)	*Mus musculus* (GRCm38.p4)	*G. aculeatus* (BROADS1)	*D. melanogaster* (BDGP6)	*C. elegans* (WBcel235)
Nb. of protein-coding genes	22,285	22,222	20,787	18	20,362
Nb. of genes with GO (molecular function) = "receptor binding"	1592	1435	247	200	198
Nb. of genes with GO (molecular function) = "extracellular region"	4814	4225	334	1016	562
Nb. of genes with GO (molecular function) = "extracellular region" and "receptor binding"	930	771	115	105	86
Proportion of signaling ligand genes	4.17%	3.47%	0.55%	0.75%	0.42%

Ligand-encoding genes are defined as the protein-coding genes associated with both "receptor binding" and "extracellular region" Gene Ontology terms

4.3 A Few Select Genes for Body-Wide Switches in Melanin Production in Tetrapods

Among 294 Gephebase morphology entries for tetrapods (Gephebase search term "Tetrapoda," including mammals and reptiles sensu *largo*), 206 genotype-phenotype relationships relate to pigment variation, including 193 entries identifying components of the melanocyte differentiation pathway. Both sampling and ascertainment biases explain this unusual enrichment. First, pigmentation shows a bulk of variation accessible to breeders and natural selection altogether (Protas and Patel 2008; Linderholm and Larson 2013). In combination with the fact that coloration variation often involves few genes, these features have made pigmentation a favorite target for exploring genotype-phenotype relationships (sampling bias). Second, there is predictability in the genetic basis of melanin pigment variation, as illustrated by the fact that the melanocortin 1 receptor (MC1R), a major regulator of melanocyte activation, is the most represented gene in Gephebase with 84 entries (6% of all 1400 entries). Interestingly, 80% of *MC1R* gephes (67/84) were identified by a candidate gene approach. This pattern illustrates well a latent ascertainment bias in the study of vertebrate pigment variation: when interested in the genetic basis of a color variation involving shifts in melanin types (mammalian coat, bird plumage, etc.), it has become a knee-jerk reflex for biologists to look for amino acid changes in MC1R, in particular in domains that had been functionally characterized. As a matter of fact, all of the 67 MC1R gephes based on a candidate gene approach involve mutations affecting the gene-coding region. Thus, both the phenotypic diversity of vertebrate pigmentation traits and their simple genetic basis explain the overrepresentation of MC1R to a large extent. This said, the fact that the remaining 20% of *MC1R* entries were identified by linkage or association mapping validates the idea that *MC1R* is a bona fide driver of color variation in vertebrates. As an explanation for this trend, it is likely that the MC1R protein hosts tuning sites that can modulate pigmentation without affecting other traits and that its mutations can show a dominant effect prone to a rapid adaptive spread (Mundy 2005; Kopp 2009; Kronforst et al. 2012; Reissmann and Ludwig 2013; Wolf Horrell et al. 2016). Other components of the melanocyte activation cascade also form gephes involved in natural and artificial selection of coloration traits (Fig. 4.1). This includes downstream targets of MC1R signal transduction such as the transcription factor gene *MITF* and the melanogenic genes *TYR*, *TYRP1*, and *Pmel17*, all involved in the biogenesis of eumelanosomes.

Upstream of MC1R, two signaling molecules that interact with receptor function are known as allelic sources of color variation in vertebrates. In particular, the antagonist ligand *Agouti*/*ASIP* is a genetic hotspot for pigment variation with a total of 28 entries in Gephebase. This includes numerous cases where this gene was identified by linkage or association mapping, both in natural and domesticated contexts (Fig. 4.1a–c), making *Agouti* one of the most commonly mapped genes in our dataset. Coding alleles of *Agouti* are recessive loss-of-function mutations

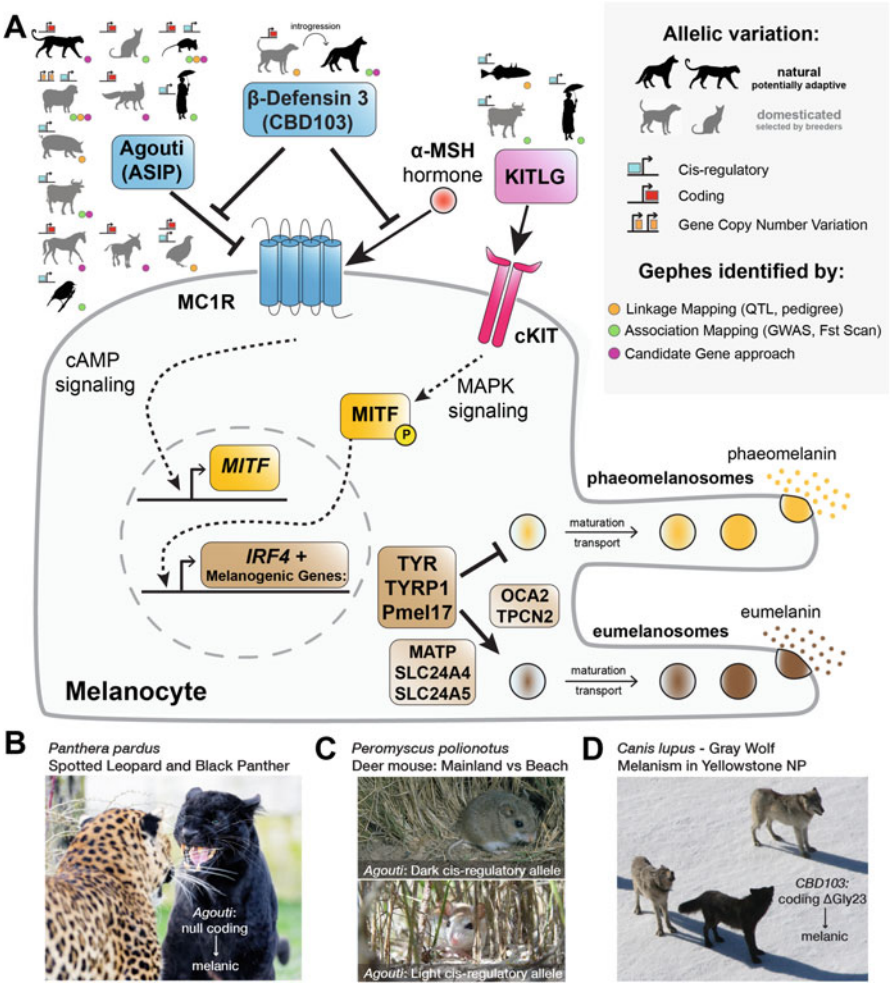

Fig. 4.1 Alleles of secreted ligands associated to pigment variation in vertebrates. (**a**) The MC1R and cKIT signaling pathways each activate a signal transduction regulatory cascade converging on the MITF transcription factor that modulates the expression of melanogenic genes and ultimately activates the maturation and transport of dark eumelanin in melanosomes. Agouti and β-defensin3 are secreted extracellular modulators of MC1R, and KITLG is the agonist ligand of cKIT. Allelic variation at these three genes is associated to pigment variation in vertebrates. (**b**) Black panthers are leopards that carry a null mutation in *Agouti*. (**c**) Adaptive pigment variation in deer mice (*Peromyscus* spp.) has repeatedly involved sequence modifications at the *Agouti* locus. For instance, distinct populations of *P. polionotus* adapted to dark (mainland Florida; *top panel*) and light (coastal Florida; *bottom panel*) color backgrounds via cis-regulatory variants that modulate *Agouti* skin expression. (**d**) Black wolves can be seen at increasing frequencies in packs of the Yellowstone National Park (USA). The melanic allele corresponds to a single amino acid deletion, which was originally selected in domestic dogs and later introgressed in wild in North American wolves and coyotes by hybridization. *Photo credits* – (**b**) Emmanuel Keller (License CC BY-ND 2.0), (**c**) Roger Barbour (License CC BY-ND 2.0), (**d**) Doug Smith (Public Domain)

resulting in melanic phenotypes. This contrasts with the melanic gain-of-function coding alleles of MC1R which are dominant, a difference in allelic effects that is used to infer the genetic basis of melanism (Eizirik et al. 2003). The *Agouti* ligand inhibits the basal activation of the MC1R pathway. In an *Agouti*-null context, MC1R is hyper-activated by its active ligand, the pituitary melanocortin hormone α-MSH, which triggers a melanocyte regulatory cascade that culminates with eumelanin production. It has been proposed that wild-type Agouti can become an agonist of MC1R melanic variants (McRobie et al. 2014), suggesting that certain gain-of-function MC1R alleles reverse the responsiveness of the receptor to the Agouti ligand itself. In addition to Agouti, the β-defensin 3/CBD103 peptide is secreted by skin epithelia, strongly binds to MC1R, and was shown to be responsible for melanism in dogs (Candille et al. 2007). In certain melanic dog breeds, one amino acid deletion in β-defensin 3/CBD103 results in dominant melanism, possibly by blocking the inhibitory activity of Agouti or by losing its blocking of α-MSH stimulatory binding (Nix et al. 2013). Of note, the CBD103$^{\Delta G23}$ melanic allele is revealing a complex history that blurs the boundary between wild and domesticated. First, based on ancient DNA studies, it probably originated through domestication from a possible wolflike gene pool as early as 10,000 years ago (Ollivier et al. 2013), introgressing into modern dog breeds. Second, it propagated back in the wild, resulting in relatively recent segregation of melanic phenotypes in North American gray wolves, North American coyotes, and Italian gray wolves (Anderson et al. 2009). The melanic allele shows signatures of positive selection, but it remains unclear if this is due to a fitness effect of the melanic coat or, alternatively, to the antimicrobial properties of β-defensin 3. A few other cases of organism-wide color changes have been found to be positively selected (Vignieri et al. 2010; Barrett and Hoekstra 2011; Laurent et al. 2016).

In conclusion, mutations in MC1R and Agouti account for 54% (112/206) of the gephes dealing with tetrapod pigmentation variation in our current dataset. Such an overrepresentation cannot be explained by experimental bias alone and suggests that MC1R and Agouti are preferential targets for pigmentation evolution in tetrapods.

4.4 cis-Regulatory Evolution Drives Regional Specific Color Shifts

While ligand- and receptor-coding changes likely modulate the strength of signaling, and, thus, pigment synthesis in melanocytes, such changes are likely to affect all the body regions where these genes are expressed. In contrast, region-specific changes in coat, skin, or plumage coloration are more likely to involve cis-regulatory mutations. In a previous meta-analysis of the gephe literature, it was established empirically that localized morphological changes almost always involve cis-regulatory rather than coding variation (Stern and Orgogozo 2008).

Agouti is a hotspot of cis-regulatory evolution for pigment pattern modification and provides one of the most spectacular examples of QTL fractionation. Deer mice display extensive pigment variation matching the color of their environment (Manceau et al. 2010). Fine mapping of this variation revealed that not only the *Agouti* locus is the major driver of pigment variation (Manceau et al. 2011) but also this genetic region decomposes itself into multiple noncoding sub-loci, each tightly associated with parts of the total phenotype (Linnen et al. 2013). Various regulatory elements are involved in directing the expression of three alternative isoforms into different body regions (Mallarino et al. 2016). Each adaptive allele is a complex haplotype that is inherited as a package that underwent multiple local changes. This is of major importance to understand how small leaps in the morphospace occur, as it illustrates the principle that genetic hotspots, in addition to providing a somewhat predictable basis for phenotypic evolution between species, can also accumulate mutations that collectively result in large-effect variation within a single lineage (Stam and Laurie 1996; McGregor et al. 2007; Rebeiz et al. 2011; Martin and Orgogozo 2013; Linnen et al. 2013; Noon et al. 2016).

Thus, the studies of vertebrate pigment variation suggest that a receptor (*MC1R*) and its inverse agonist (*Agouti*/*ASIP*) are key regulators of melanocyte differentiation, driving adaptive variation in natural contexts as well as novel color features available to farmers and breeders. Coding evolution in either component results in body-wide color shifts, while cis-regulatory evolution of *Agouti*, by tuning the spatial deployment of an MC1R switch-off, permits subtle changes in morphology. The *Agouti*/*MC1R* axis is not a typical developmental pathway and plays little role during ontogenesis (e.g., see Gene Ontology annotations in Gephebase). In contrast, the *endothelin-3* ligand/*endothelin-receptor B* (EDN3/EDNRB) signaling axis has pleiotropic roles in the differentiation and migration of neural crest cells, and mutations in both EDN3 and EDNRB have been found to cause pigmentation changes in domesticated chicken, cattle, and horse (Santschi et al. 1998; Dorshorst et al. 2011; Qanbari et al. 2014). So far, only domesticated alleles of EDN3/EDNRB that may be under unrealistic selective regimes have been mapped. Thus, while it represents perhaps a genuine DGT component, it remains ambiguous if endothelin pathway genes can be a mutational target of evolution in a natural context. To truly assess the role of signaling ligand genes in morphological evolution, it is useful to focus on radiating lineages that allow a trait-by-trait dissection by forward genetics (i.e., taking advantage of natural variation between closely related lineages – populations and sister species) and, sometimes, natural experiments of replicated evolution (Kopp 2009; Powell and Mariscal 2015). In the next sections, we focus primarily on stickleback fishes and *Heliconius* butterflies, for which numerous linkage mapping efforts have been uncovering the genetic basis of several morphological adaptations.

4.5 Recent Stickleback Fish Adaptations Repeatedly Recruited Ligand Alleles

Three-spined sticklebacks (*Gasterosteus aculeatus*) are a species of marine fishes that repeatedly colonized freshwater environments following the retreat of the Pleistocene glaciers. Adapting to these novel niches involved numerous morphological, physiological, and behavioral modifications all available to genetic dissection by QTL mapping and population scans. Among the 14 gephes that have been mapped in sticklebacks (*Pitx1*, *TSHBeta2*, *KCNH4*, *KITLG*, *EDA*, *GDF6*, *BMP6*, *PRKCD*, *SOD3*, *KCNH4*, *ATP6V0A1*, *ATP1A1*, *Mucin*, *IGK*), 4 involve a secreted ligand gene. Analysis of well-annotated genomes indicates that secreted ligand genes represent less than 5% of the total number of genes within an animal genome (Table 4.2). The proportion of ligand gephes in sticklebacks (28%) is thus higher than expected with the null hypothesis that mutations responsible for phenotypic evolution occur randomly at any gene within a genome (chi^2 test: chi$^2 > 20$; $p < 10^{-5}$).

A single large-effect locus was identified as driving melanin pigment reduction in freshwater populations (Fig. 4.2a). Contrary to expectations, this trait mapped neither to the MC1R pathway nor at its downstream targets, but at the *Kit-ligand* (*KITLG*) locus (Miller et al. 2007; Jones et al. 2012), which encode the secreted signaling component of a parallel pathway (Fig. 4.1). KITLG is the ligand of the KIT receptor, which triggers a MAPK tyrosine kinase transduction cascade that modulates the differentiation and activity of melanocytes (Wehrle-Haller 2003). While the *KIT* receptor has been identified in a total of 17 color-related gephes, it is only linked to domesticated alleles in the cattle, pig, horse, donkey, domesticated fox, and domestic cat (see Advanced Search "Gene name and synonyms" = "KIT" at www.gephebase.org for a complete list). In contrast, cis-regulatory alleles of *KITLG* have been shown to underlie natural pigment variation not only in stickleback fishes but also in humans (Miller et al. 2007; Guenther et al. 2014). An Ala193Asp mutation in *KITLG* has also been shown to cause piebald coat color phenotypes in cattle breeds (Seitz et al. 1999; Qanbari et al. 2014). Of note, cis-regulatory *KITLG* variation may provide tissue-specific effects that limit its potential deleterious pleiotropic effects on cancer risks, as observed in other variant forms of this locus in humans (Karyadi et al. 2013; Litchfield et al. 2016).

Another locus, encoding the bone morphogenetic protein 6 (*BMP6*) ligand, was found to cause tooth gain in freshwater stickleback population (Cleves et al. 2014; Erickson et al. 2015) (Fig. 4.2b). The causal change is cis-regulatory and downregulates *BMP6* expression, late during oral development (see Cleves et al. 2014 correction). Surprisingly, genetic mapping of a second freshwater population revealed that another genomic locus has driven a similar phenotypic output (Ellis

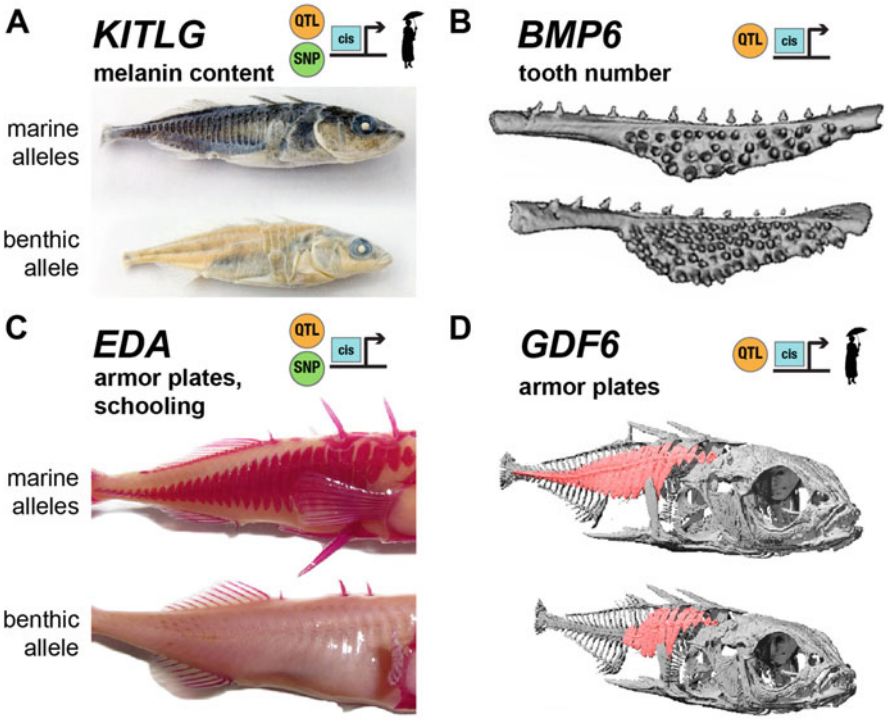

Fig. 4.2 Secreted ligand loci involved in marine-to-lake adaptations in sticklebacks. (**a**) A *KITLG* cis-regulatory variant causes reduced melanization in lake populations (*bottom*) compared to marine alleles (*top*). (**b**) MicroCT images of the tooth plates of a marine vs. a lake-adapted ecotype. The freshwater cis-regulatory *BMP6*-derived allele causes increased tooth area and density. (**c–d**) Armor plates are lateral bony structures, here stained by Alizarin Red (**c**) and false-colored in MicroCT rendering (**d**, *pink*), which were repeatedly reduced or lost in freshwater populations. cis-Regulatory alleles of *EDA* and GDF6 cause distinct effects on plate distribution, number, and size (*Photo credits* – (**a**) Frank Chan and David Kingsley, (**b**) Craig Miller and David Kingsley, (**c**) Nicholas Ellis and Craig Miller, (**d**) Catherine Guenther, Vahan Indjeian, and David Kingsley)

et al. 2015). BMP ligands belong to the TGF-β family, are shared by all bilaterian animals, and play important roles for the regulation of development (De Robertis 2008). Compilation of current data suggests that mutations in TGF-β family genes are often involved in the tinkering of reproductive and skeletal traits during evolution and domestication. Several BMP alleles have been associated to increased fertility in domestic sheeps (*BMP15* and its paralog *GDF9*) (Monestier et al. 2014) and to fecundity and bone allocation in chicken (*BMP2*) (Johnsson et al. 2012). Genetic studies of craniofacial diversity mapped a QTL interval containing the *BMP4* gene in cichlid fishes (Albertson et al. 2005) and found a strong association between a single amino acid change in BMP3 and brachycephalic (short-skulled) dog breeds (Schoenebeck et al. 2012).

Body armor loss, via the reduction of lateral bony plates, has been a recurring adaptation to freshwater in sticklebacks. Two major loci have been characterized. The tumor necrosis factor superfamily gene *Ectodysplasin A* (*EDA*) harbors cis-regulatory variation existing at low frequency in the marine population that has been repeatedly recruited in continental populations to drive plate number reduction (Colosimo et al. 2005; Jones et al. 2012; O'Brown et al. 2015). The same locus also triggers a change in schooling behavior, as fishes from lake habitats have lost the ability to precisely align their body axis when swimming in a group, an effect that is reversed by transgenic overexpression of EDA (Greenwood et al. 2016). In addition, a combination of QTL mapping and genome scan has identified a freshwater-specific allele at the *growth/differentiation factor 6* (*GDF6*) locus, which results in a gain of expression of that gene in the developing epithelium and, ultimately, in a reduction of lateral plate size (Indjeian et al. 2016). Like for *KITLG*, this case also opened a window into human evolution as it was found that a *GDF6* hindlimb-specific enhancer was lost in the human lineage, with skeletal modifications obtained in mice that suggest a potential role in the evolution of bipedalism (Indjeian et al. 2016).

Forward genetics efforts in sticklebacks thus show that ligand genes belonging to classical developmental pathways are an important source of morphological variation of adaptive relevance. Noticeably, all the stickleback gephes described here are cis-regulatory, in accordance with the prediction that tinkering of developmental genes is more likely to involve cis-regulatory changes than coding mutations (Carroll 2008; Stern and Orgogozo 2008). Next, we focus on how accumulated changes in signaling ligand loci have enlarged the landscape of possible morphologies in insect wings.

4.6 The Wnt Beneath My Wings

There are few case studies that characterize adaptive variation for a same set of traits both within and between species. Butterflies of the *Heliconius* genus provide a rich phylogenetic template for such micro-evo-devo studies (Papa et al. 2008; Supple et al. 2014; Kronforst and Papa 2015; Merrill et al. 2015). They display a range of highly variable wing color pattern phenotypes involved in Müllerian mimicry (the collaborative display of similar morphologies to predators from multiple unpalatable species) and sexual selection that are amenable to hybrid crosses followed by linkage mapping. In addition, their natural hybrid zones form a system of choice for high-resolution admixture mapping, looking for SNP-phenotype associations and the smoking guns of selection that are the handful of Mendelian loci that keep adjacent populations phenotypically distinct in the face of constant gene flow and recombination. The Wnt-family signaling ligand WntA has emerged as a key genetic driver of wing pattern evolution in butterflies. Originally discovered as a Mendelian locus responsible for discrete shifts in pattern shapes in the *Heliconius erato* mimetic radiation, this gene shows striking

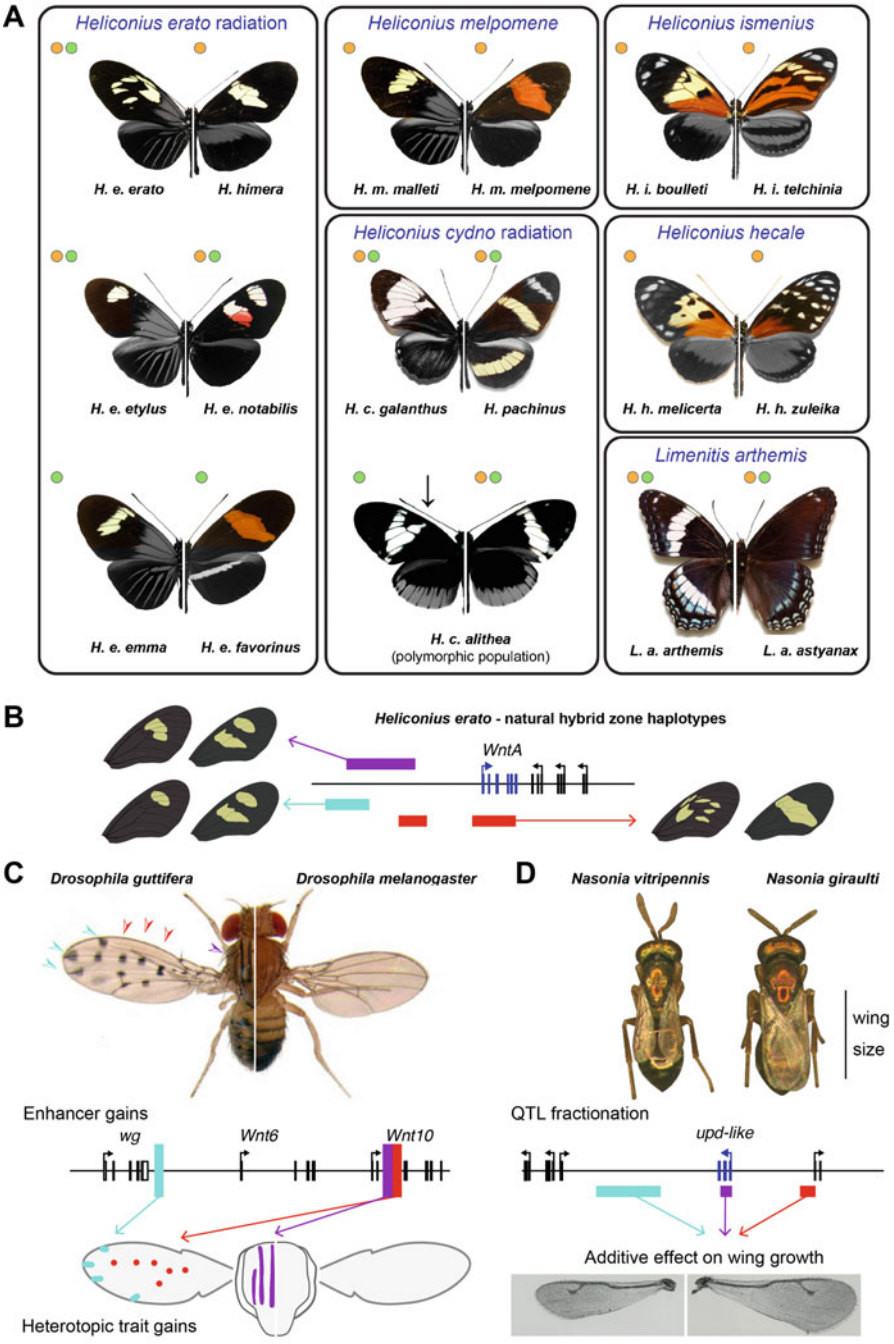

Fig. 4.3 Mapped cis-regulatory alleles of *WntA*, a genetic hotspot of wing pattern shape variation. (**a**) A total of 18 *WntA* cis-regulatory variants have been identified by linkage mapping (*orange dots*) and admixture mapping in natural hybrid zones (*green dots*). Each allele is associated with spatial shifts in *WntA* expression that drive pattern shape variations, in particular, in the median

expression differences in larval wing disks that correlate tightly with the position of presumptive color elements and defines the black contours of forewing color patterns (Martin et al. 2012). Both linkage and admixture mapping approaches have revealed that a versatile pool of *WntA* alleles underlie marked phenotypic differences in at least six geographic races of *H. erato* (Fig. 4.3a, b). Following this discovery, additional mapping efforts discovered that *WntA* variants control pattern variation in four other *Heliconius* species, as well as in *Limenitis arthemis*, a species which diverged from the *Heliconius* genus 65 million years ago (Fig. 4.3a) (Gallant et al. 2014; Huber et al. 2015). All the mapped *WntA* alleles not only underlie phenotypic divergence within species but also convergence between sympatric morphs that evolved in distinct species, thus providing clear examples of adaptive tinkering and repeated evolution of similar patterns. As expected, the causative changes are not found in the *WntA* coding exons, which show little variation in amino acid sequence, but in the adjoining regulatory loci that control *WntA* expression during wing development. The role of *WntA* cis-regulatory mutations may very well extend to much broader phylogenetic levels, as *WntA* expression, which shows spectacular shifts in expression in all the butterfly species assessed so far, always correlates with color pattern features (Martin and Reed 2014). With a total of 18 alleles in 7 species, all associated with wing color pattern variation, *WntA* can be seen as a genuine genetic hotspot of adaptation (Martin and Orgogozo 2013) and a case model for linking regulatory sequence variation, pattern formation, and morphological evolution at multiple time scales.

4.7 Ligand Gene Modularity Allows Interspecific Differences

The current data suggest that the *WntA* locus contains multiple control regions and haplotypes, each being able to reconfigurate part of *WntA* expression and the overall organization of wing patterns. Association mapping reveals at least three adjacent haplotype regions with distinct patterning effects in *H. erato* (Fig. 4.3b) and a single 1.8 kb indel perfectly associated to a polymorphic variant in a sympatric *H. cydno alithea* population (Gallant et al. 2014; Van Belleghem et al. 2017). This said, the

Fig. 4.3 (continued) region of butterfly forewings. Each half-butterfly corresponds to a natural morph. *WntA*-independent color patterns were manually masked and shaded in *gray* to better highlight the wing pattern areas influenced by *WntA*. (**b**) Fractionation of the *H. erato WntA locus* at several haplotypic blocks, each perfectly associated with pattern shape variation across three natural hybrid zones (Van Belleghem et al. 2017). (**c**) Three novel cis-regulatory regions underlie the evolution of novel pigmentation traits in *D. guttifera*. (**d**) Fine QTL mapping of wing size variation in male *Nasonia* wasps identifies three intervals responsible for the differential spatio-temporal recruitment of the *upd-like* growth factor (*Photo credit (use with permission)* – (**c**) Nicolas Gompel and Shigeyuki Koshikawa and (**d**) David Loehlin)

functional dissection of these genetic elements is reaching a technical limitation at this moment due to the inability to test for the function of each cis-regulatory region in butterflies, and we must gain insight into the evolution of ligand gene expression in analog models to explore the logic of cis-regulatory control. Interestingly, detailed analyses of the cis-regulatory region of another *Wnt* locus, this time encompassing *wingless* (syn. *Wnt1*; *wg*) and its tandem paralogs *Wnt6* and *Wnt10* (Fig. 4.3c), show that three novel, tissue-specific cis-regulatory elements drive *wingless* expression and underlie novel color patterns on the wings and thorax of *Drosophila guttifera* fruit flies (Werner et al. 2010; Koshikawa et al. 2015). While these studies lack the phylogenetic resolution and replication observed in butterflies, they provide one of the most detailed mechanistic accounts of truly novel traits, where the deployment of Wnt expression in three different body regions is driven by independent cis-regulatory changes. Of note, *wg* is also associated to color patterns and wing contours in both flies and butterflies (Macdonald et al. 2010; Martin and Reed 2010; Koshikawa et al. 2015), and a redeployment of this gene to new body regions is likely to drive the evolution of new patterns as well, as it seemed to have occurred during the evolution of larval cuticle patterns in Lepidoptera (Yamaguchi et al. 2013). We note that while Koshikawa et al. did not detect any pattern-related *Wnt6* and *Wnt10* expression in *D. guttifera* developing wings (Koshikawa et al. 2015; S. Koshikawa, personal communication), these two paralogs are co-deployed with *wg* in butterflies where they may underlie a more complex architecture, with partially redundant ligand activities (Martin and Reed 2014). Beyond their obvious parallels (wing pigmentation traits; *Wnt* loci), the butterfly and *D. guttifera* data collectively depict a modular landscape of pattern evolution where acquisitions and modifications of cis-regulatory elements allow a fine-tuning of color patterns (Koshikawa 2015).

Another case study provides further support for linking gene regulatory region modularity at a ligand locus and interspecific variation (Loehlin and Werren 2012). Using two *Nasonia* wasp sister species, Loehlin and Werren mapped a male wing size variation QTL to the JAK/STAT pathway ligand gene *unpaired-like* (*upd-like*) and, by a genetic tour de force, were able to genetically break down this locus into three regulatory intervals, each with complementary effects on wing size. In fact, each mapped interval affects various complementary spatiotemporal expression patterns of *upd-like*, ultimately affecting wing growth. Thus, whether the phenotypic output is a growth trait (the *upd-like* case) or a color pattern (the *WntA* and *wg* cases), we have empirical evidence that morphological evolvability depends in these cases on the capacity to modify an expression pattern. In a nutshell, the different case studies linking insect wing variation and ligand genes highlight the importance of modular cis-regulatory architecture in the tinkering of anatomy.

4.8 How, When, and Why Ligand Genes Are Likely Drivers of Pattern Variation, or Not

Our cumulative knowledge of evolutionary genetics foreshadows a relative predictability in the genetic mechanisms that drive phenotypic change (Stern and Orgogozo 2009; Martin and Orgogozo 2013; Orgogozo 2015): by laying out what seems to be common mechanisms or trends in the generation of novelty, we can formulate post hoc expectations that can be generalized over broad taxonomic ranges. The cases of Wnt-based color pattern variation discussed above, *WntA* in nymphalid butterflies and *wg* in *D. guttifera*, both provide a useful model framework for understanding the molecular logic of pattern evolution due to their relative simplicity, as they take place in the two-dimensional canvas of the insect wing epithelium. To the best of our knowledge, these patterning systems are uncoupled from tissue growth, which prevents the complex dynamics found in many other morphological contexts (Salazar-Ciudad 2006; Salazar-Ciudad 2009; Urdy et al. 2016). As simplified spatial output of cellular differentiation, color patterns can be used as a proxy for more complex morphologies, providing fundamental insights that can be applied across all animals. A simple ascertainment emerges from the fly and butterfly data: cis-regulatory evolution of pattern-inducing signaling genes has repeatedly driven the evolution of new patterns and derived pattern shapes. We can elaborate upon a simple gradient model of positional information (Wolpert 1969) generating threshold-dependent pattern boundaries (Fig. 4.4a), to derive five types of ligand gene signaling that can produce morphological outcomes (Fig. 4.4b–f). Since cis-regulatory variation modulates gene expression in time and space, it can affect tissue patterning in multiple ways, and its effect on a ligand gene can be sufficient to induce a new pattern (Fig. 4.4b) or simply change its shape (Fig. 4.4c). In addition, cis-regulatory acquisition of localized repressors can dislocate a pattern and thus affect both pattern number and shape (Fig. 4.4d). Pattern size can also be affected by quantitative or temporal changes in the expression of a secreted factor, without requiring a change in the number of source cells, or, alternatively, by *trans*-interactions upstream of the ligand that would affect its secretion and transport (Fig. 4.4e). Finally, modification in the tissue responsiveness to the signal or its concentration or time-dependent interpretation may modulate the pattern thresholds (e.g., color composition) without affecting the overall size and shape of the pattern (Fig. 4.4f).

These distinct dimensions of pattern variation can be used to generate hypotheses on the molecular targets underlying a given phenotypic state. Below we illustrate this principle, building upon a set of observations made on the variable checkerspot (*Euphydryas chalcedona*). *E. chalcedona* checkerspots display a set of orange patterns outlined by black scales that are each expressing *WntA* or *wg*/*Wnt6*/*Wnt10* (Martin and Reed 2014). Each of these patterns can be contracted or expanded by an injection of dextran sulfate or heparin, respectively (Fig. 4.4g). These two sulfated polysaccharide compounds possess a high molecular weight, which restrict them to the extracellular space, and injections are only effective when

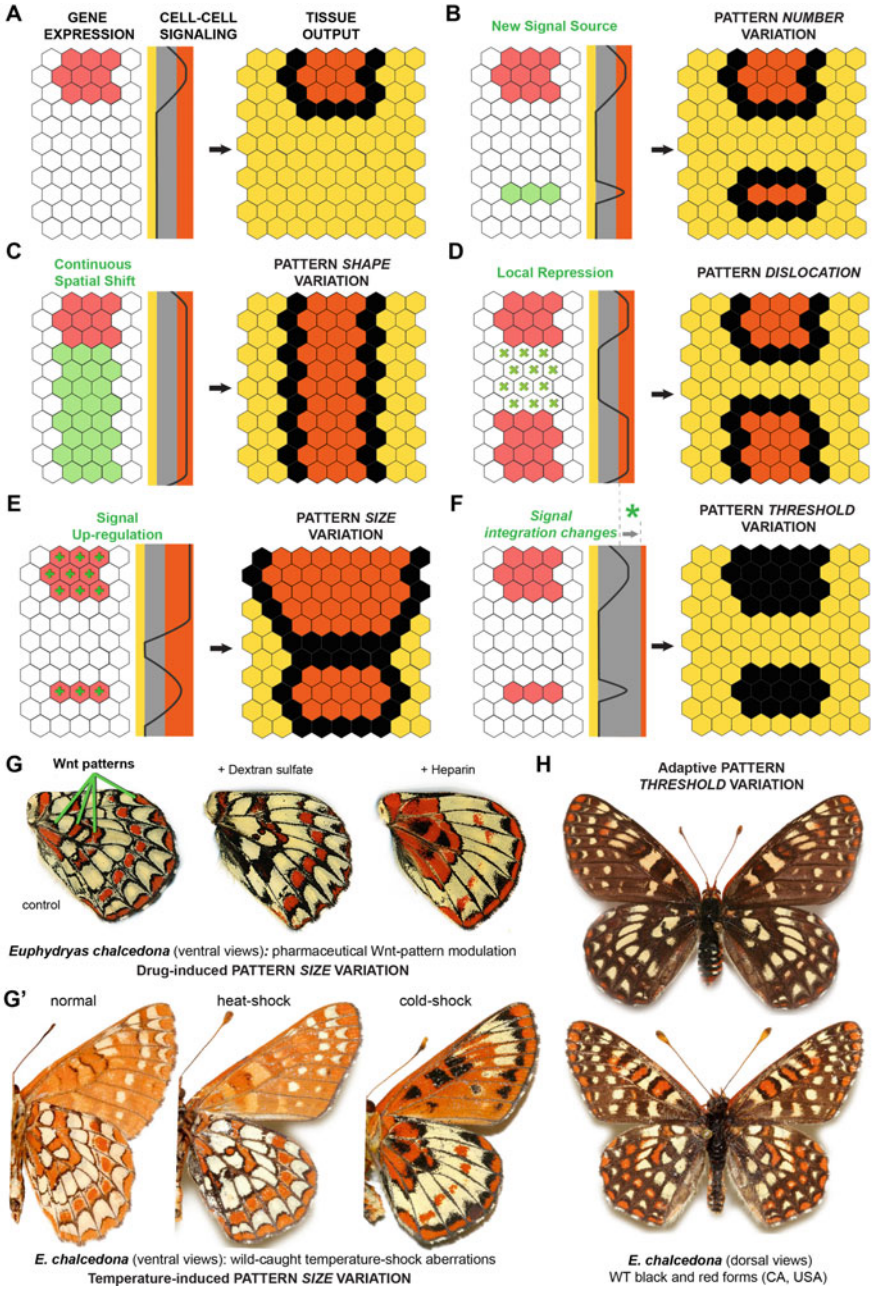

Fig. 4.4 Distinct aspects of pattern variation may rely on different modes of ligand gene modification. (**a**) A three-step model of pattern formation. Ligand-expressing cells (*red hexagons*) deploy a signal that is interpreted by neighboring cells in a concentration-dependent manner, resulting in a three-state output (*yellow*, low signal; *black*, intermediate; *orange*, high). (**b**) Discrete gain of a novel ligand gene expression domain can generate novel pattern elements. (**c**) Continuous spatial modulation of ligand expression can generate new pattern shapes. (**d**) Local

performed within 24 h after pupation, revealing a short time window for pattern formation (Serfas and Carroll 2005; Martin and Reed 2014). Finally, both heparin and endogenous, heparin-like heparan sulfate proteoglycans (HSPGs) are known to bind Wnt ligands in the extracellular space, where they are of critical importance for signal secretion, stability, and transport (Lin 2004). These observations provide a simple alternative mechanism for modifying pattern size: rather than affecting signal strength directly, variation at genes involved in HSPG synthesis could also modulate the spread of Wnt ligands. Similarly, temperature shocks experienced during early pupal life create analogous pattern aberrations (Fig. 4.4g'), suggesting that specific physiological conditions are critical for normal patterning and that, here again, a broad range of molecular mechanisms taking place during cell-cell signaling (e.g., signal secretion, transport, reception, and degradation) could affect pattern size. The variable checkerspot takes its name from the extensive color pattern variations (Bowers et al. 1985; Long et al. 2014b) that can be observed between populations (Fig. 4.4h). Can we predict whether a ligand locus is involved in driving the difference between these Wnt-positive black vs. red/black patterns? Based on the framework developed above, we believe this is in fact an unlikely scenario. Indeed, the variation involves little differences in pattern shape or number and instead consists in color composition differences. A difference in signal sensitivity rather than signal strength between the two forms is more likely to explain the phenomenon, resulting in a threshold trait variation (see Allen et al. 2008 for a discussion of pattern size vs. color composition). We thus predict that this polymorphism could map to a Wnt-pathway gene or to a gene that can modify the output of the Wnt signaling pathway and that this gene should be active during the extracellular signaling phase or shortly thereafter. Alternatively, the threshold traits could also depend on signal temporal dynamics (Sorre et al. 2014). To be formally tested, these competing hypotheses will require linkage or association mapping between natural morphs and illustrate how our current knowledge can guide a different set of predictions, based on the type of observed trait variation.

Fig. 4.4 (continued) loss of ligand expression can result in pattern dislocation. (**e**) Upregulation of a ligand gene can generate enlarged patterns. (**f**) Pattern composition may vary based on modifications of the signal interpretation process, downstream of the ligand gene itself (without affecting its expression or protein). (**g**) Sulfated polysaccharide injections in the variable checkerspot butterfly, performed within 24 h after pupation, affect the size of Wnt-positive patterns. Dextran sulfate results in Wnt pattern contractions, while heparin results in Wnt gain-of-function effects that expand the same patterns. Both compounds illustrate how genetic modulations of the extracellular environment can modulate pattern size. (**g'**) Temperature shocks during early pupal life result in pattern distortions (similar to G panel), indicating a sensitivity of the signaling step to physiological conditions. (**h**) The variable checkerspot is named after its color pattern polymorphism, involved in adaptive mimicry (Bowers et al. 1985; Long et al. 2014b). Differences in *red patterns* may be due to changes in genes modulating Wnt signal, rather than at a Wnt gene locus itself (see **f**)