Chapter 4

Synergistic Damage Response of the Double-Focus Eyespot in the Hindwing of the Peacock Pansy Butterfly

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Additional information is available at the end of the chapter

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Abstract

Eyespot color patterns in butterfly wings are determined by the putative morphogenic signals from organizers. Previous experiments using physical damage to the forewing eyespots of the peacock pansy butterfly, *Junonia almana* (Linnaeus, 1758), suggested that the morphogenic signals dynamically interact with each other, involving enhancement of activation signals and interactions between activation and inhibitory signals. Here, we focused on the large double-focus fusion eyespot on the hindwing of *J. almana* to test the involvement of the proposed signal interactions. Early damage at a single focus of the prospective double-focus eyespot produced a smaller but circular eyespot, suggesting the existence of synergistic interactions between the signals from two sources. Late damage at a single focus reduced the size of the inner core disk but simultaneously enlarged the outermost black ring. Damage at two nearby sites in the background induced an extensive black area, possibly as a result of the synergistic enhancement of the two induced signals. These results confirmed the previous forewing results and provided further evidence for the long-range and synergistic interactive nature of the morphogenic signals that may be explained by a reaction-diffusion mechanism as a part of the induction model for color-pattern formation in butterfly wings.

Keywords: butterfly wing, color-pattern formation, eyespot, induction model, *Junonia almana*, physical damage, reaction-diffusion model

1. Introduction

Animal bodies often have conspicuous color patterns such as stripes, dots, and eyespots. For example, various color patterns are notable in shells and fishes, and at least some of them have been explained well by some types of reaction-diffusion (RD) models [1–3]. In such models, activation and inhibitory signals interact based on local self-activation and lateral inhibition

[4–6]. A patterning process is initiated randomly, but some of the final outputs, such as zebrafish stripes, are stably constructed. Thus, there is no specific organizer or its associated prepattern that is required to construct the final pattern.

In contrast, eyespot patterns that emerge consistently at particular locations in some fish or other species may require organizers, or something similar, that initiate the determination process at particular locations. Although careful adjustment of boundary conditions for RD equations may be able to computationally specify eyespot locations consistently, such a model may not be robust enough to reproduce a given eyespot pattern in every individual under different environmental and genetic conditions during development. As a compromise, a developmental system that involves both predetermined classical organizers (i.e., sources of the putative morphogenic signals) and RD mechanisms might be more realistic. A potential example of such a system is the spotted mandarin fish, *Synchiropus picturatus* (Peters, 1877), which has many eyespots at distinct and consistent locations [7]. In this fish species, physical damage at the center of the eyespot cannot reduce the eyespot size, and surgical removal of a substantial portion of an eyespot initiates a regeneration process to reconstruct the entire eyespot [7]. These results suggest that the eyespot center does not function as an organizer and that lateral cellular interactions play an important role in constructing an eyespot in this fish species, although an initial specification mechanism of eyespot locations remains enigmatic. Interestingly, ectopic eyespots can be induced by physical damage to the background area between eyespots [7].

Another developmental system that may require both classical organizers and RD mechanisms is the butterfly color-pattern determination system. Butterfly color patterns are constructed based on three major symmetry systems, two peripheral systems, and other accessory systems [8–14]. Each symmetry system is composed of a collection of color-pattern elements. Among these elements, eyespots that belong to the border symmetry system are probably most conspicuous, at least to human eyes, and developmental mechanisms of eyespots have been studied relatively well. The initial specification of the central location of an eyespot has been successfully described by an RD model based on signals from wing veins in developing wing disk [8], although this model may be too fine-tuned to explain the developmental robustness of actual eyespots [15]. Interestingly, the subsequent determination process of an eyespot after the determination of its central location has been explained by a concentration gradient model, a non-RD model [8, 16, 17]. One of the reasons that the butterfly eyespot formation (except for the initial specification) has been considered a non-RD system may be that the center of the prospective eyespot has been known to behave as an organizer, as demonstrated by the following experiments. Cautery-based damage at the center of the prospective eyespot reduces or completely abolishes the prospective eyespot [18, 19], and transplantation of the central cells produces an ectopic eyespot at the transplanted site [18, 20, 21].

However, it has been noted that gradient models cannot explain the extreme diversity of eyespot morphology in nymphalid butterflies [22]. Moreover, gradient models cannot explain the morphological diversity of serial eyespots on the identical wing surface [23]. Furthermore, the status of parafocal elements as a part of the border symmetry system [10, 11, 22, 23] has not been explained by the previous models. Nijhout [15] recently proposed the grass fire model,

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in which parafocal elements can be produced together with eyespots by a simple RD system. It would not be surprising for the entire process of the butterfly eyespot determination to be solely based on RD mechanisms. However, another way of thinking about the system is that because an RD model in general does not require the existence of organizers, the butterfly color-pattern formation system may be something more than a simple RD system.

Accordingly, a model that includes both an organizer and the essence of an RD system has been proposed, and it is called the induction model [22–24]. For convenience, the induction model can be divided into two stages: the early and late stages. The early stage involves signal expansion and settlement from an organizer, and the late stage involves short-range activation and long-range inhibition, the essence of an RD model. In this model, the activation signals activate themselves, and the activation signals and inhibitory signals interact with each other. When two activation signals from different sources meet, synergistic enhancement may occur.

It is important to stress that the induction model is based on "inductive reasoning," meaning that it is based on collective analysis of many actual butterfly eyespot patterns and physiologically induced color patterns [22, 23, 25]. Thus, the induction model can be applied to "non-typical" distorted eyespots and damage-induced changes, which are not explained well by the gradient models [22, 23]. The induction model is essentially a formal model based on observations, experimental results, and integrative logics, and it is not a computational model that introduces many unknown assumptions. It is true that the induction model proposes unknown mechanisms such as mechanistic waves [13], but these unknowns should be tested and replaced, if necessary, with alternative ideas.

Among the data supporting the induction model is that when a prospective large eyespot is damaged, an adjacent small eyespot becomes larger [26]. This result suggests an inhibitory effect from the prospective large eyespot to the small one. In the induction model, the inhibitory signal is upregulated in the edge of the activation signal, based on the principle of the local (short-range) self-activation and lateral (long-range) inhibition [5]. This inhibition signal works on activation signals not only from its own eyespot but also from other eyespot. Because both activation and inhibition signals behave autonomously once released from organizing cells, the inhibitory signal does not have to affect the signal source to make an eyespot smaller.

Another finding supporting the induction model is that the outermost black ring can be uncoupled from its inner core disk when a prospective eyespot is damaged late [25]. This is also explained by autonomous nature of signals that the induction model proposes. An alternative explanation is that two different chemical morphogens are released. This is not compatible with the conventional gradient model [22], and autonomous behavior of parafocal elements, an equivalent element to "eyespot ring," prefers the induction model [23, 24].

To be sure, this approach is not intended to undermine computational models. Computational models can propose mathematically defined assumption that may be tested systematically, whereas the collective color-pattern analyses were mostly descriptive. However, both approaches are necessary to understand the complexity of butterfly color patterns. A novel

and important way to distinguish between the induction model and the gradient model is to examine a fusion eyespot that has two signal sources. A fusion of two eyespots can be explained either by the conventional gradient model or by the induction model. However, the synergistic enhancement by activation signals from two different sources could occur if the induction model (or a similar model) operates (**Figure 1A**). The synergistic enhancement in

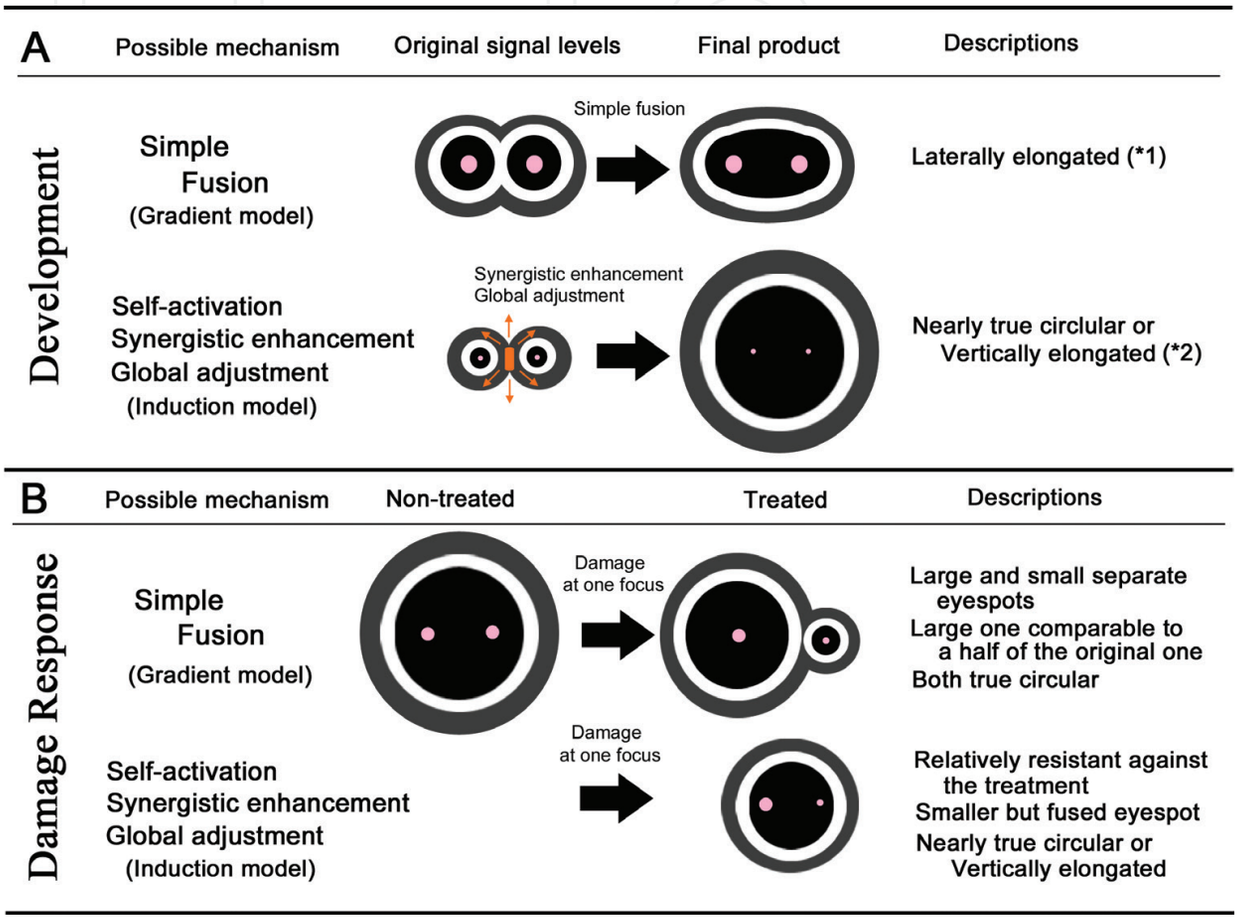

Figure 1. Conceptual distinction between simple fusion (the gradient model) and non-simple fusion (the induction model). Central circular areas indicate organizing centers that emit signals. (A) Distinction of the two modes during development. The simple fusion process produces a laterally elongated eyespot with relatively large numbers of organizing cells (which do not necessarily correspond to the white spots in actual butterflies). In contrast, the non-simple fusion process involves self-activation, synergistic enhancement, and global adjustment and produces a nearly true circular eyespot or vertically elongated eyespot from a relatively small number of organizing cells. The area in which the signals from two sources come into contact (shown in the vertical bar between the two organizing centers) acts as a new "source" of activation signal for the entire eyespot. However, even in the simple fusion, when two sources are relatively closely positioned in comparison with signal levels, the final eyespot may form a nearly true circle (Note *1). Likewise, when self-activation and synergistic enhancement are delayed and inhibited by the emerging inhibitory signals, the final eyespot remains laterally elongated even when the induction model is correct (Note *2). Moreover, signal distribution patterns at the early stage (shown as "Original signal levels") are highly similar between the two models. (B) Distinction of the two modes based on the damage response. If a double-focus eyespot is produced by simple fusion, as predicted by the gradient model, damage at one organizing center of a double-focus eyespot produces separate eyespots, one large and one small. The large one is comparable to half of the original one. Both eyespots are truly circular. In contrast, if a double-focus eyespot is produced by self-activation, synergistic enhancement, and global adjustment, as predicted by the induction model, the eyespot is relatively resistant against the treatment. A small but fused circular or vertically elongated eyespot will result.

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the induction model can be achieved if activation signals merge together before the upregulation of inhibitory signals around the activation signals. In other words, the final size of a fusion eyespot is determined not by a simple summation of the two independent sources but by a synergistic enhancement process. Importantly, the synergistic enhancement is most active at the boundary between the two sources, and the resultant fused eyespot would thus tend to become nearly a true circle or slightly vertically elongated (**Figure 1A**). In contrast, a simple fusion process will often result in a laterally elongated fused eyespot (**Figure 1A**).

However, it is difficult to distinguish these two mechanistic possibilities simply based on the final morphology of the fusion eyespot alone, given that there may be conditions under which the typical morphology is not attained. For example, when two sources are positioned closely or when two signals are very strong, a simple fusion of the two would produce a near true circle. When the self-activation and synergistic enhancement processes failed to occur for some unexpected kinetic reasons before the upregulation of inhibitory signals, a laterally elongated fusion eyespot may result. Moreover, an essentially indistinguishable morphology will be exhibited by either mechanism at early fusing stages of a pair of eyespots (**Figure 1A**).

Nonetheless, physical damage at a single focus of a double-focus eyespot may resolve these two possibilities. Damage at a single focus would produce two circular eyespots, a large one and a small one, when a single gradient model is operating (**Figure 1B**). In contrast, damage at a single focus would produce a smaller but circular fusion eyespot with two foci if the induction model (or something similar) is operating, because of the synergistic enhancement and the global adjustment of the activation signals (**Figure 1B**). In other words, a double-focus eyespot would behave as if both foci were damaged. Therefore, characterization of the damage response of a double-focus eyespot that is constructed by fusion of two eyespots would test whether the induction model, or something similar, that involves the synergistic signal enhancement is more reasonable than the gradient model.

The best system to test this hypothesis is probably the large dorsal hindwing eyespot of the peacock pansy butterfly, *Junonia almana* (Linnaeus, 1758) (**Figure 2A**). In this paper, this eyespot is called the major eyespot (or the double-focus eyespot), simply because it is large in

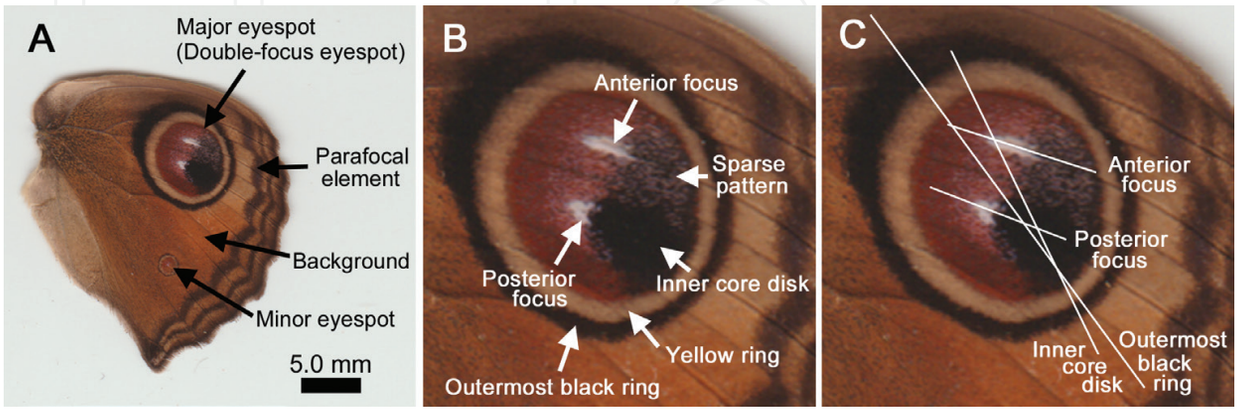

Figure 2. Nomenclature of the hindwing elements and sub-elements of the peacock pansy butterfly, *J. almana*. (A) An entire dorsal hindwing. (B) Higher magnification of the major eyespot and its surroundings. (C) Directions of elongation of sub-elements in the major eyespot. The directions are not in synchrony.

comparison with another eyespot (the minor eyespot) on the same wing surface (**Figure 2A**). Morphologically, the large circular shape with two distantly separate foci of this major eyespot already suggests the feasibility of the induction model (**Figure 1A**). Furthermore, each sub-element (components of an element, some of which are indicated in **Figure 2B**) of the eyespot appears to behave independently; their elongation directions are inconsistent (**Figure 2C**). Such independent behaviors of sub-elements within a given element are called uncoupling [13, 26].

If the large size of this hindwing eyespot is a product of the synergistic enhancement of the signals from two organizers, mechanical damage at a single organizer could reduce the size of the entire eyespot. That is, when one organizer is debilitated by damage, the other intact organizer would "help" to restore the entire eyespot, although small, from the merged center. The eyespot would be relatively resistant to damage because of the synergistic enhancement process. In the line of this argument, it is possible to test whether the prediction of the induction model is consistent with the damage response of the double-focus eyespots.

Here, the damage response of the dorsal hindwing major eyespot of *J. almana* was characterized. Damage to the background was also performed, which has been known to produce an ectopic black spot in the forewing of this species [26] and in other species of nymphalid butterflies [27, 28]. Hindwing damage in butterflies has never been reported except by Nijhout [8]. This is partly because the hindwing is covered by the forewing and is thus invisible from the outside at the pupal stage in butterflies. We have overcome this technical difficulty by directly observing the hindwing development using the forewing-lift method [29, 30]. Nijhout [8] briefly mentioned that the mechanism of eyespot formation in the hindwing may be different from that in the forewing based on the following results from *Junonia coenia* Hübner, 1822: the hindwing eyespot cannot be changed in size by cautery immediately after pupation, whereas the background is still responsive to cautery. This possibility has now also been tested in *J. almana* in this paper.

2. Materials and methods

2.1. Butterflies

The peacock pansy butterfly, *J. almana*, was used throughout this study. This study focused on the hindwing of this butterfly (**Figure 2**). The eggs were collected from females caught from the wild in Ishigaki-jima Island, Okinawa, Japan. Larvae were fed natural host plants in the laboratory at ambient temperature.

2.2. Damage applications and image analysis

After prepupation, pupation time was checked repeatedly at intervals of a few hours, and pupae were categorized into three groups based on time post-pupation: 3–6 h (early), 6–12 h (middle), and 12–18 h (or 12–20 h) (late). Mechanical damage was made at specific positions on the right pupal wings (without a forewing lift) using a stainless needle of 0.50 mm in diameter (Shiga Konchu, Tokyo, Japan). A needle was inserted down to approximately 3 mm in depth

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and moved up and down five or more times before being removed entirely. The contralateral (left) wing was not damaged because it served as an internal control. The damage sites of the hindwing were determined in advance using a different set of pupae by the forewing-lift method performed in this species [29, 31]. The damaged pupae were kept at ambient temperature until eclosion. The adults that eclosed were frozen immediately after pupation. Wing images were obtained using a Canon MG5730 scanner (Tokyo, Japan). Color-pattern changes of the treated wings were evaluated in reference to the normal color patterns of the non-treated wings of the same individuals.

2.3. Definition of focus

In this paper, an eyespot "focus" was defined as a white spot at the central region of an eyespot in a compartment. The white spots do not necessarily correspond to locations of organizers in this species [31] and also in other species [32]. However, because a white spot indicates an approximate location of an organizer in this species, the white spot is conventionally called the focus in this paper.

3. Results

3.1. Anterior damage to the major eyespot

The anterior focus of the major eyespot was damaged at 3–6 h post-pupation (n = 15). In 12 out of 15 cases (80%), the major eyespot was reduced in overall size (**Figure 3A–C**). Importantly, the entire eyespot (not only the anterior side but also the posterior side) decreased in size in most cases, although the damage was placed only at the anterior focus, suggesting dynamic interactions between the anterior and posterior signals during development to determine the final size and shape of the major eyespot. One individual exhibited minor size reduction at the anterior side but not clearly at the posterior side (**Figure 3C**). In 3 out of 15 cases (20%), the reduction was not clear. The failure of the size reduction was probably because the damage was mistakenly (but unavoidably) placed at the semi-focal point. In these semi-focal damage samples, coloration inside the core disk was disrupted, an ectopic yellow area emerged, and the anterior focus (white spot) was elongated toward the damage site (**Figure 3D**).

Similarly, the anterior focus of the major eyespot was damaged, but much later, at 12–20 h post-pupation (n = 4). In all 4 cases (100%), the outer black ring was enlarged in all directions, but the inner core disk was reduced in size, although to a small degree (**Figure 3E**), showing an uncoupling response between these two sub-elements.

3.2. Posterior damage to the major eyespot

The posterior focus of the major eyespot was damaged at 3–6 h post-pupation (n = 9). In 2 out of 9 cases (22%), the overall double-focus eyespot was moderately reduced in size (**Figure 3F**). The overall shape remained circular. No effect was observed in 7 out of 9 cases (78%), indicating the relatively low sensitivity of the posterior focus to damage in comparison with the anterior focus.

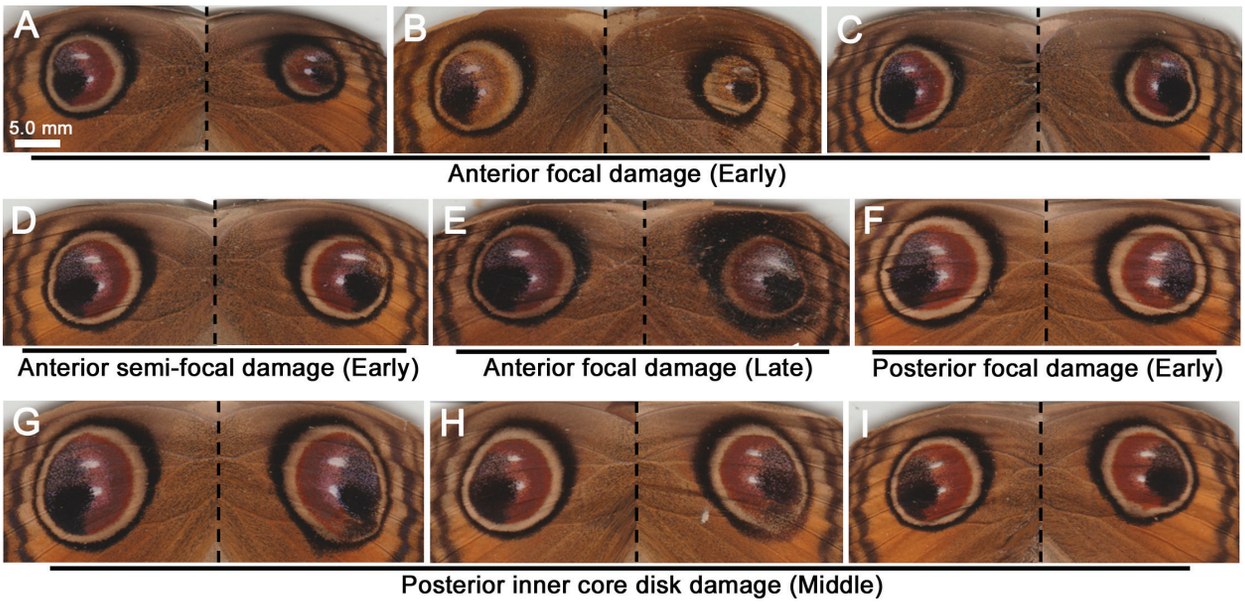

Figure 3. Damage-induced color-pattern changes in the major eyespot of the hindwing of *J. almana*. (A and B) Early damage at the anterior focus. In these typical cases, the entire eyespot was reduced in size, but it was still circular. (C) Early damage at the anterior focus. In this exceptional case, only the treated anterior side was clearly reduced, although the reduction level was minor. (D) Early damage at the anterior semi-focal site. A yellow area emerged inside the inner core disk. The anterior focus (white dot) was elongated toward the damage site. (E) Late damage at the anterior focus. The outermost black ring expanded in all directions, but the inner core disk was reduced in size. (F) Early damage at the posterior focus. The entire eyespot size was reduced slightly in size. (G-I) Middle (mid-term) damage at the posterior inner core disk. Response was largely local. Difference from the anterior focal damage shown in E is notable.

Then, the core disk was damaged in the posterior side, avoiding the posterior focus, at 6–12 h post-pupation ($n = 18$). In 10 out of 18 individuals (56%), the treatment induced the formation of a yellow area inside the inner core disk and made the coloration boundaries fuzzy (**Figure 3G** and **H**). Even in these cases, the overall size of the double-focus eyespot did not change. That is, changes were restricted to the immediate vicinity of the damage site. No or very minor effect was observed in 8 out of 18 cases (44%) (**Figure 3I**).

3.3. Damage to the outermost black ring of the major eyespot or in its close vicinity

The outermost black ring of the major eyespot was damaged at 6–12 h post-pupation. Because eyespot size and shape were slightly different from individual to individual, damage was made without distinction at the outermost black ring, at the yellow ring, or at the background immediately close to the outermost black ring ($n = 73$). Among these 73 treated individuals, a small black dot with a yellow area inside ($n = 21$; 29%) or without a yellow area ($n = 17$; 23%) emerged in a close proximity to the major eyespot. In another set of individuals, such a dot emerged on the yellow ring that accompanied the extrusion of the outermost black ring ($n = 10$; 14%) (**Figure 4A**). In more extensive cases ($n = 13$; 18%), entire eyespot shape was disrupted, with the extrusion of the inner core disk toward the damage site (**Figure 4B** and **C**), with a broken outermost ring (**Figure 4D**), or with the enlargement of the yellow area (**Figure 4E**). In many of these cases, both the outermost black ring and the core disk were distorted toward the damage site.

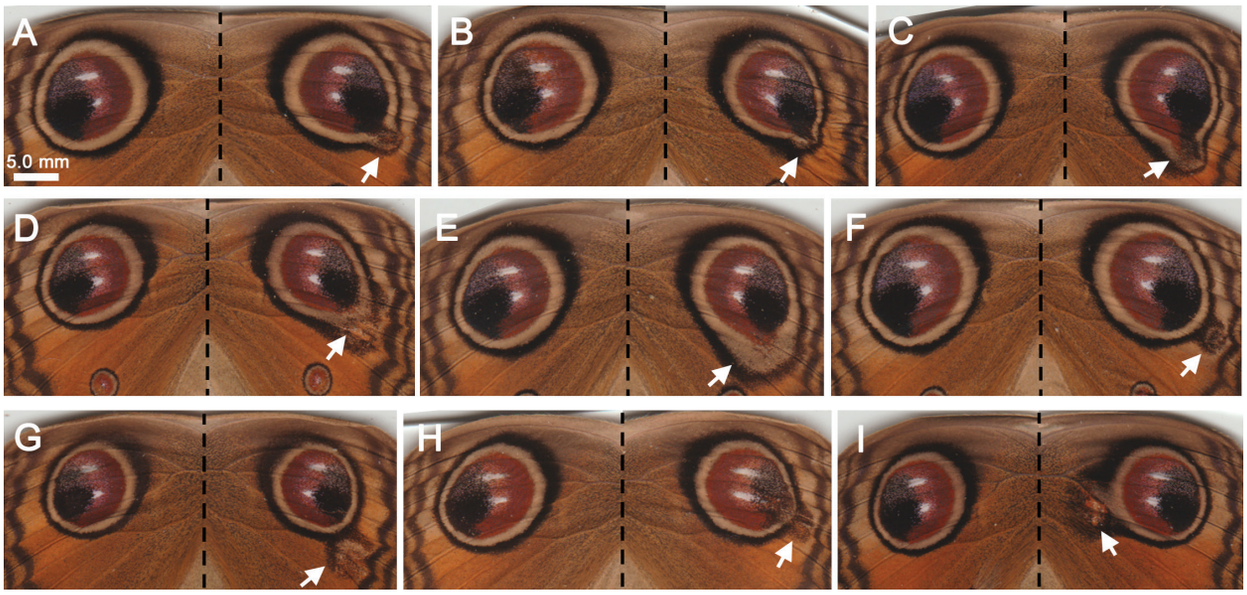

Figure 4. Damage at or around the outermost black ring of the major eyespot in the hindwing of *J. almana*. Damaged points are indicated by arrows. (A) Small ectopic black ring on the yellow ring. (B and C) Extrusion of the inner core disk and other structures. (D) Breaking of the outermost black ring. (E) Expansion of the black ring and yellow area. (F) Small ectopic arc and ring that fuse with the outermost black ring of the major eyespot but not with parafocal element. (G) Black arc (and vague black area inside) that fuses with the outermost black ring but not with parafocal elements. (H) Induced black area (arrow) and a sparse pattern that is induced in the posterior side of the inner core disk. A sparse pattern is present only in the anterior side of the inner core disk in non-treated individuals. (I) Large black area with an orange area inside induced by damage at the proximal side of the major eyespot.

In a different set of individuals (n = 11; 15%), a double black ring or a similar feature emerged in the background area immediately facing the eyespot (**Figure 4F** and **G**). Interestingly, in these cases, the double ring structure was not expressed clearly in the side facing the parafocal elements. In one case (n = 1; 1%), the damage-induced structure in the background was very minor, but a sparse pattern was induced in the posterior side of the black core disk (**Figure 4H**).

In addition to damage at the distal side of the major eyespot, the proximal side of the major eyespot was damaged at the outermost black ring at 12–18 h post-pupation (n = 21) (**Figure 4I**). Among these 21 treated individuals, an ectopic yellow region that was surrounded by a black area was produced in most individuals (n = 18; 86%). This induced black area fused with the outermost black ring of the eyespot smoothly, and the entire eyespot was distorted only slightly, if at all, toward the damage site. No effect was observed in 3 out of 21 cases (14%).

3.4. Damage to the background between the major and minor eyespots

To understand the reactivity of the background, the background between the major and minor eyespots was damaged at 12–18 h post-pupation (n = 9). A black area was induced in all treated individuals (100%) (**Figure 5A**). In the most severe cases, a yellow area emerged at the center (**Figure 5B**). In the individual shown in **Figure 5B**, the induced black area fused with the outermost black rings of the major and minor eyespots, and the minor eyespot was distorted toward the damage site, where a yellow area emerged inside the induced black area.

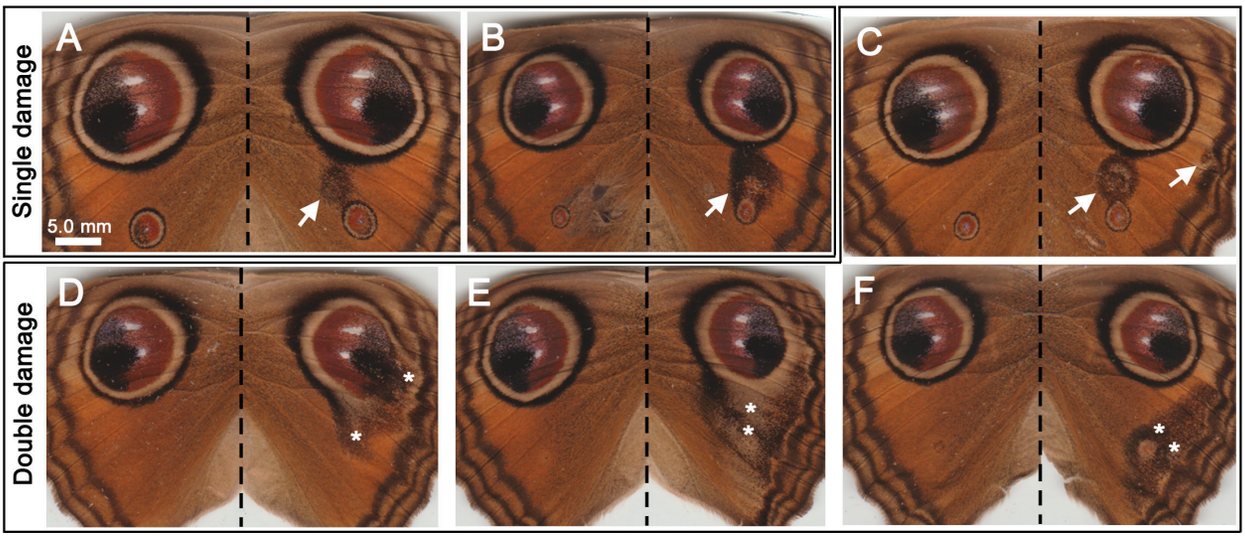

Figure 5. Damage to the background in the hindwing of *J. almana*. Damaged points are indicated by arrows or asterisks. (A and B) Single point of damage between the major and minor eyespots. A black area was induced. (C) Double damage at the area between the major and minor eyespots and near parafocal elements. A double ring structure and a small dot were induced. The two damage sites responded independently. (D) Double damage at the area between the major and minor eyespots and near parafocal elements. The major eyespot was ruptured. (E) Double damage between the major and minor eyespots, resulting in extensive modifications. (F) Double damage near the minor eyespot.

3.5. Double background damage

In the experiments described above, a single site per individual was damaged. Here, to understand possible interactions between damage-induced signals, two sites in the background were damaged. First, two distant sites in the background were damaged, one between the major and minor eyespots and one between the major eyespot and parafocal elements, at 12–18 h post-pupation (n = 6). In all 6 cases (100%), a black area was induced at both damage sites without any noticeable interaction. In one of these cases, the proximal site (an area between the major and minor eyespots) was more extensive than the distal site (an area near parafocal elements) (**Figure 5C**). Indeed, in this individual, the induced black spot at the distal site was very close to parafocal elements but did not fuse with them. Instead, the ectopic small black spot appeared to "repel" the nearby parafocal element. In 2 out of 6 cases (33%), including the individual shown in **Figure 5C**, a clear double ring appeared at the proximal site. In one extensive case, the major eyespot opened up with the extensive light black area (**Figure 5D**). In this individual, the induced black area again did not fuse with parafocal elements; there was a clear gap between them.

Then, two closely positioned sites in a wing between the major and minor eyespots were damaged at 12–18 h post-pupation (n = 5). All treated individuals (100%) showed marked disruption of the major eyespot. The outermost black ring was ruptured, and the induced black area covered extensive portions of the background (**Figure 5E**). However, the induced black area again could not invade parafocal elements. There was a narrow but distinct gap between the induced black area and parafocal elements.

When two closely positioned sites around the minor eyespots were damaged at 12–18 h post-pupation (n = 15), 14 out of 15 cases (93%) showed induction of a black area. One of

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them showed an extensive black area along the parafocal elements (**Figure 5F**). Although the ectopic black area fused smoothly with the outermost black ring of the major eyespot, there was no fusion between the ectopic black area and parafocal elements. Interestingly, the distinct minor eyespot was not present in the normal wing in this particular individual, but the induced black area did not enter the minor eyespot area, thereby demonstrating the existence of the imaginary ring at that site. No effect was observed in one case (7%).

4. Discussion

4.1. Hindwing eyespot response

In the present study, response profiles of the double-focus eyespot and its surrounding wing surface in the hindwing of *J. almana* were obtained. This eyespot on the dorsal hindwing is quite large, and simply because it has two foci in two compartments, one can discern that this eyespot is a fusion of two original eyespots. The response profile of the hindwing double-focus eyespot that was obtained in the present study is largely consistent with that of the forewing single-focus eyespot of *J. almana* reported previously [26]. It was confirmed that focal damage, but not non-focal damage, dramatically changed the overall eyespot size, supporting the idea that the focal cells function as organizing cells. However, it was found that the posterior focus was less sensitive than the anterior focus. This sensitivity difference probably reflects different developmental periods when organizing cells are active. The insensitivity of the hindwing eyespot to cautery-based damage in *J. coenia* [8] may simply be attributable to earlier species-specific differentiation of organizing cells, before the treatment time point. In that case, the insensitivity does not suggest any fundamental mechanistic difference between the forewing and hindwing.

4.2. Synergistic response to focal damage

The double-focus eyespot of *J. almana* provided an ideal system to test the dynamic interactions between the signals from two different sources. Importantly, the early anterior focal damage made the entire double-focus eyespot small as if both foci were damaged, and the treated eyespot kept its circular shape. These results suggest the existence of dynamic interaction, possibly synergistic enhancement, of the activation signals from two sites, confirming the feasibility of the induction model over the conventional gradient model. In one case, a minor size reduction only at the treated anterior side was observed. However, simply because the change was minor (likely due to incomplete damage), this case does not support the gradient model.

Additionally, the late anterior focal damage enlarged the outermost black ring but reduced the size of the inner core disk. The enhancement of the outermost black ring was not restricted to the anterior side; the enlargement was in all directions. This uncoupling behavior between the outermost black ring and the inner core disk within the same eyespot is indeed consistent with the late damage results of the forewing eyespot [26]. This uncoupling response can be explained if the normal signals are wave-like (which means that the

signals behave independently from their source once released) and if the induced signal was added to the normal signal for the outermost black ring. The normal signal for the inner core disk was being released at the time of damage, and because some of the organizing cells were destroyed by physical damage, the inner core disk became smaller. This uncoupling response is an indication of independence of the signal for each sub-element (i.e., the outer black ring and the inner core disk). The wave-like nature of signals is also highlighted in these results. These results are not explainable by the gradient model.

4.3. Response to other types of damage

Semi-focal damage at the anterior side produced a yellow area inside the inner core disk, which is also consistent with the forewing results [26]. This result is also difficult to explain using a gradient model. A threshold increase in response to damage may be a remedy, but a threshold decrease should also be introduced to explain the induced black area in the background. Furthermore, the induced double-ring structures in the background require multiple threshold sets to be explained by the gradient model. These damage-induced rings have been shown to have scale structures that are similar to those of normal eyespots [31]. These complicated threshold arrangements are too complex to accept as a theoretical framework for color-pattern determination in butterfly wings.

Interestingly, the white focal area was elongated toward the damage site in the semi-focal damage. Notably, developmental signals for the white "focal" spot and the eyespot body to which that white spot belongs do not have to be identical [31, 32]. Indeed, the white focal spot is likely uncoupled from the rest of the sub-elements [32].

Damage at or around the outer black ring produced various results. A small black ring was produced in the yellow ring in some cases, but in other cases, the inner core disk, the yellow ring, and the outer black ring were often "pinched off" from the normal shape of the eyespot, suggesting that the ectopically induced signals are able to merge with natural signals locally. In other words, spontaneous and artificially induced signals are indistinguishable to developing scale cells. Furthermore, the extrusion of both the outermost black ring and the inner core disk toward a damaged site suggests that serial lateral interactions keep their shapes, which is reminiscent of the eyespots of the spotted mandarin fish [7]. In addition to these local effects, overall shape changes of the treated eyespots were often observed, although not extensively in response to this manipulation.

4.4. Synergistic response to double background damage

Double-damage experiments that produced extensive black areas confirmed that the induced signals at two sites can be combined to produce strong effects. It is likely that when two sites of damage were close enough, the induced area was more than a simple summation of two areas induced independently by two single damage treatments. These results can be interpreted as evidence for synergistic enhancement of two artificially upregulated signals in the hindwing of this species. This synergistic enhancement process may also occur spontaneously in the double-focus eyespot during development.

In some cases, the black signals induced by double damage merged with the outermost black ring of the natural eyespot, resulting in the rupture of the eyespot. This result again demonstrates the indistinguishability of the natural and induced signals. Somewhat surprisingly, the large black area highlighted one of the "inhibitory areas" that are usually invisible but associated with parafocal elements and the minor eyespot. That is, in an individual lacking the distinct minor eyespot, the induced black area could not invade the area surrounding the minor eyespot. This invisible area might have arisen if the inhibitory signal became stronger and larger in that area than the activation signal for black areas during development of the minor eyespot. Similarly, the induced black signal could not make contact with parafocal elements, suggesting that the inhibitory signals are present along parafocal elements. The similar inhibitory area that surrounds an eyespot has generally been termed the imaginary ring [13, 22]. The reason that the imaginary ring was not observed around the major eyespot is not well understood, but development of an imaginary ring around the major eyespot may require additional time before the end of the pattern determination period.

4.5. Possible mechanisms

Overall, these results strongly suggest that the signals that determine the final scale color of a given scale cell are highly dynamic. It is likely that a reaction-diffusion mechanism, as a part of the induction model, operates in the butterfly color-pattern determination system. The induction model consists of two stages. The early stage is a dissipation of signals from their source, and the late stage is essentially a reaction-diffusion mechanism that involves short-range activation and long-range inhibition. It is speculated that calcium signals play an important role in color-pattern determination in the late stage of the induction model; calcium signals traveling on the developing pupal wings have been observed [33]. On the basis of a linear relationship between scale size and cell size [34–36] and a relationship between scale color and scale size [31], it has been proposed that the putative morphogenic signals from organizers are ploidy signals that determine cellular size via polyploidization [31]. Calcium signals may play an important role in polyploidization.

The early stage of the induction model proposes that a signal moving slowly from its source is the original morphogen that subsequently triggers calcium waves as an activation signal. It is speculated that this slow-moving signal is waves of mechanical distortion [25]. Importantly, organizing centers are present as physical bumps or indentations [32, 37]. These organizing centers can be identified as the pupal cuticle spots in pupae [38, 39]. Based on this fact and other observations, the distortion hypothesis has been proposed, in which physical distortion of the wing tissue functions as the primary morphogenic signal [13]. Physical distortion of the wing epithelial sheet will be created when cells at the organizers selectively increase their sizes via an increase in cell number or polyploidization. Ecdysone receptor is expressed in focal cells in *J. coenia* [40], and such an increase of cells has been observed in eyespot centers of *Bicyclus anynana* (Butler, 1879) in response to ecdysone; this increase likely results in larger eyespots [41]. The finding that ecdysone injection into pupae of *J. almana* does not affect eyespot size but does change background coloration [42] is to be reconciled with the observation that ecdysone receptor is responsible for an increase in organizing cells in *B. anynana*.

In physical damage experiments, mechanical distortion of the wing epithelial sheet is probably introduced, nicely mimicking the natural developmental process that involves physical distortion waves. Although there are some classical histological studies on developing wing tissues of butterflies and moths [34, 43, 44], real-time live imaging studies have just begun on developing wing tissues and organizing cells [30, 37, 45]. The distortion hypothesis should be tested in the future in light of the importance of mechanical forces in development [46, 47]. Compatibility of these proposed mechanisms with other related mathematical models for eyespot focus determination [48–50] is also to be investigated in the future.

5. Conclusions

The present study provided experimental evidence that morphogenic signals for eyespot color patterns are able to synergistically interact with each other, focusing on the damage-induced color-pattern changes of the double-focus eyespot in the hindwing of the peacock pansy butterfly, *J. almana*. The present results may be explained by a reaction-diffusion mechanism as a part of the induction model, but not by the conventional gradient model. A different set of experiments that removed the surface contact from the posterior side of the hindwing major eyespot results in miniaturization of both the anterior and posterior sides of the eyespot [51], suggesting synergistic interactions between the two focal signals that are consistent with the present study.

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References

[1] Kondo S, Asai R. A reaction-diffusion wave on the skin of the marine angelfish *Pomacanthus*. Nature. 1995;**376**:765-768

[2] Meinhardt H. The Algorithmic Beauty of Sea Shells. 4th ed. Berlin: Springer-Verlag; 2009. p. 269

[3] Watanabe M, Kondo S. Is pigment patterning in fish skin determined by the Turing mechanism? Trends in Genetics. 2015;**31**:88-96

[4] Gierer A, Meinhardt H. A theory of biological pattern formation. Kybernetik. 1972; **12**:30-39

[5] Meinhardt H, Gierer A. Pattern formation by local self-activation and lateral inhibition. BioEssays. 2000;**22**:753-760

[6] Kondo S, Miura T. Reaction-diffusion model as a framework for understanding biological pattern formation. Science. 2010;**329**:1616-1620

[7] Ohno Y, Otaki JM. Eyespot colour pattern determination by serial induction in fish: Mechanistic convergence with butterfly eyespots. Scientific Reports. 2012;**2**:290

[8] Nijhout HF. The Development and Evolution of Butterfly Wing Patterns. Washington: Smithsonian Institution Press; 1991. p. 297

[9] Nijhout HF. Elements of butterfly wing patterns. Journal of Experimental Zoology. 2001;**291**:213-225

[10] Dhungel B, Otaki JM. Local pharmacological effects of tungstate on the color-pattern determination of butterfly wings: A possible relationship between the eyespot and parafocal element. Zoological Science. 2009;**26**:758-764

[11] Otaki JM. Colour-pattern analysis of parafocal elements in butterfly wings. Entomological Science. 2009;**12**:74-83

[12] Otaki JM. Color pattern analysis of nymphalid butterfly wings: Revision of the nymphalid groundplan. Zoological Science. 2012;**29**:568-576

[13] Otaki JM. Self-similarity, distortion waves, and the essence of morphogenesis: A generalized view of color pattern formation in butterfly wings. In: Sekimura T, Nijhout HF, editors. Integrative Approach to the Analysis of the Diversity and Evolution of Butterfly Wing Patterns. Singapore: Springer Nature Singapore; 2017. pp. 119-152

[14] Taira W, Kinjo S, Otaki JM. The marginal band system in nymphalid butterfly wings. Zoological Science. 2015;**32**:38-46

[15] Nijhout HF. The common developmental origin of eyespot and parafocal elements; and a new model-mechanism for color pattern formation. In: Sekimura T, Nijhout HF, editors. Integrative Approach to the Analysis of the Diversity and Evolution of Butterfly Wing Patterns. Singapore: Springer Nature Singapore; 2017. pp. 3-19

[16] Nijhout HF. The color patterns of butterflies and moths. Scientific American. 1981;**245**: 104-115

[17] Monteiro A, French V, Smit G, Brakefield PM, Metz JA. Butterfly eyespot patterns: Evidence for specification by a morphogen diffusion gradient. Acta Biotheoretica. 2001;**49**:77-88

[18] Nijhout HF. Pattern formation on lepidopteran wings: Determination of an eyespot. Developmental Biology. 1980;**80**:267-274

[19] French V, Brakefield PM. The development of eyespot patterns on butterfly wings: Morphogen sources or sink? Development. 1992;**116**:103-109

[20] French V, Brakefield PM. Eyespot development on butterfly wings: The focal signal. Developmental Biology. 1995;**168**:112-123

[21] Brakefield PM, Gates J, Keys D, Kesbeke F, Wijngaarden PJ, Monteiro A, French V, Carroll SB. Development, plasticity and evolution of butterfly eyespot patterns. Nature. 1996;**384**:236-242

[22] Otaki JM. Color-pattern analysis of eyespots in butterfly wings: A critical examination of morphogen gradient models. Zoological Science. 2011;**28**:817-827

[23] Otaki JM. Generation of butterfly wing eyespot patterns: A model for morphological determination of eyespot and parafocal element. Zoological Science. 2011;**28**:817-827

[24] Otaki JM. Structural analysis of eyespots: Dynamics of morphogenic signals that govern elemental positions in butterfly wings. BMC Systems Biology. 2012;**6**:17

[25] Otaki JM. Physiologically induced color-pattern changes in butterfly wings: Mechanistic and evolutionary implications. Journal of Insect Physiology. 2008;**54**:1099-1112

[26] Otaki JM. Artificially induced changes of butterfly wing colour patterns: Dynamic signal interactions in eyespot development. Scientific Reports. 2011;**1**:111

[27] Nijhout HF. Cautery-induced color patterns in *Precis coenia* (Lepidoptera: Nymphalidae). Journal of Embryology and Experimental Morphology. 1985;**86**:191-203

[28] Brakefield PM, French V. Eyespot development on butterfly wings: The epidermal response to damage. Developmental Biology. 1995;**168**:98-111

[29] Kusaba K, Otaki JM. Positional dependence of scale size and shape in butterfly wings: Wing-wide phenotypic coordination of color-pattern elements and background. Journal of Insect Physiology. 2009;**55**:175-183

[30] Iwata M, Ohno Y, Otaki JM. Real-time *in vivo* imaging of butterfly wing development: Revealing the cellular dynamics of the pupal wing tissue. PLoS One. 2014;**9**:e89500

[31] Iwata M, Otaki JM. Spatial patterns of correlated scale size and scale color in relation to color pattern elements in butterfly wings. Journal of Insect Physiology. 2016;**85**:32-45

[32] Iwata M, Otaki JM. Focusing on butterfly eyespot focus: Uncoupling of white spots from eyespot bodies in nymphalid butterflies. SpringerPlus. 2016;**5**:1287

[33] Ohno Y, Otaki JM. Spontaneous long-range calcium waves in developing butterfly wings. BMC Developmental Biology. 2015;**15**:17

[34] Henke K. Ueber die verschiendenen Zellteilungsvorgänge in der Entwicklung des beschuppten Flügelepithelis der Mehlmotte *Ephestina kühniella* Z. Biologisches Zentralblatt. 1946;**65**:120-135

[35] Sondhi KH. The biological foundations of animal patterns. The Quarterly Review of Biology. 1963;**38**:289-327

[36] Cho EH, Nijhout HF. Development of polyploidy of scale-building cells in the wings of *Manduca sexta*. Arthropod Structure and Development. 2012;**42**:37-46

[37] Iwasaki M, Ohno Y, Otaki JM. Butterfly eyespot organiser: *In vivo* imaging of the prospective focal cells in pupal wing tissues. Scientific Reports. 2017;**7**:40705

[38] Otaki JM, Ogasawara T, Yamamoto H. Morphological comparison of pupal wing cuticle patterns in butterflies. Zoological Science. 2005;**22**:21-34

[39] Taira W, Otaki JM. Butterfly wings are three-dimensional: Pupal cuticle focal spots and their associated structures in *Junonia* butterflies. PLoS One. 2016;**11**:e0146348

[40] Koch PB, Merk R, Reinhardt R, Weber P. Localization of ecdysone receptor protein during colour pattern formation in wings of the butterfly *Precis coenia* (Lepidoptera: Nymphalidae) and co-expression with Distal-less protein. Development Genes and Evolution. 2003;**212**:571-584

[41] Monteiro A, Tong X, Bear A, Liew SF, Bhardwaj S, Wasik BR, Dinwiddie A, Bastianelli C, Cheong WF, Wenk MR, Cao H, Prudic KL. Differential expression of ecdysone receptor leads to variation in phenotypic plasticity across serial homologs. PLoS Genetics. 2015;**11**:e1005529

[42] Otaki JM. Reversed type of color-pattern modifications of butterfly wings: A physiological mechanism of wing-wide color-pattern determination. Journal of Insect Physiology. 2007;**53**:526-537

[43] Mayer AG. The development of the wing scales and their pigment in butterflies and moths. Bulletin of the Museum of Comparative Zoölogy at Harvard College. 1896;**XXIX**(5):219-236

[44] Nardi J, Magee-Adams SM. Formation of scale spacing patterns in a moth wing: I. Epithelial feet may mediate cell rearrangement. Developmental Biology. 1986;**116**:278-290

[45] Ohno Y, Otaki JM. Live cell imaging of butterfly pupal and larval wings *in vivo*. PLoS One. 2015;**10**:e0128332

[46] Iskratsch T, Wolfenson H, Sheetz MP. Appreciating force and shape—the rise of mechanotransduction in cell biology. Nature Reviews Molecular Cell Biology. 2014;**15**:825-833

[47] Gilmour D, Rembold M, Leptin M. From morphogen to morphogenesis and back. Nature. 2017;**541**:311-320

[48] Sekimura T, Venkataraman C, Madzvamuse A. A model for selection of eyespots on butterfly wings. PLoS One. 2015;**10**:e0141434

[49] Evans TM, Marcus JM. A simulation study of the genetic regulatory hierarchy for butterfly eyespot focus determination. Evolution and Development. 2006;**8**:273-283

[50] Marcus JM, Evans TM. A simulation study of mutations in the genetic regulatory hierarchy for butterfly eyespot focus determination. BioSystems. 2008;**93**:250-255

[51] Otaki JM. Contact-mediated eyespot color-pattern changes in the peacock pansy butterfly: Contributions of mechanical force and extracellular matrix to morphogenic signal propagation. In: Perveen FK, editor. Lepidoptera. Rijeka, Croatia: InTech; 2017

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