Chapter 5

Contact-Mediated Eyespot Color-Pattern Changes in the Peacock Pansy Butterfly: Contributions of Mechanical Force and Extracellular Matrix to Morphogenic Signal Propagation

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Additional information is available at the end of the chapter

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Abstract

Butterfly wing color patterns are developmentally determined by morphogenic signals from organizers in the early pupal stage. However, the precise mechanism of color-pattern determination remains elusive. Here, mechanical and surface disturbances were applied to the pupal hindwing of the peacock pansy butterfly *Junonia almana* (Linnaeus, 1758) to examine their effects on color-pattern determination. Using the forewing-lift method immediately after pupation, a small stainless ball was placed on the prospective major eyespot or background of the developing dorsal hindwing to cause a wing epithelial distortion, resulting in deformation of the major eyespot. When the exposed dorsal hindwing was covered with a piece of plastic film or placed on a surface of a glass slide, an adhesive tape, or a silicone-coated glassine paper, the major eyespot was effectively reduced in size without a direct contact with the covering materials. The latter two treatments additionally induced the size reduction of the minor eyespot and proximal displacement and broadening of parafocal elements through a direct contact, being reminiscent of the temperature-shock-type modifications. These results suggest the importance of mechanical force and physicochemical properties of planar epithelial contact surface (i.e., extracellular matrix) to propagate morphogenic signals for color-pattern determination in butterfly wings.

Keywords: butterfly wing, color-pattern formation, distortion hypothesis, eyespot, induction model, mechanical distortion, morphogen

1. Introduction

In any biological systems, cells are placed in an environment where not only chemical information but also mechanical information change over time. The biologically relevant chemical

and mechanical information is to be extracted by cells in real time. Chemical information is obtained via receptor molecules that are often specific to soluble chemicals such as hormones, cytokines, growth factors, neurotransmitters, and morphogens. Mechanical information is obtained via integrins and other membrane-spanning molecules that connect the extracellular matrix molecules with the intracellular actomyosin filaments [1]. In this sense, physicochemical properties of the extracellular matrix contribute to information signaling. At the organismal level, chemical information and mechanical information are obtained through the olfactory and gustatory systems and the mechanosensory system, respectively. Because both "modalities" are necessary for any cellular systems, immature cells may take advantage of both modalities to "sense" their environment to determine their own fate for differentiation during development.

Morphogenesis is sequential processes that involve three-dimensional changes of epithelial sheets [2, 3]. In other words, mechanical changes are necessarily involved during morphogenesis. However, a conventional understanding of the developmental fate determination process almost exclusively focuses on chemical signals and their reception, which is manifested, for example, as the gradient model for positional information [4, 5]. By contrast, mechanical signals and their reception have not been acknowledged well in developmental biology. Recent advancement of mechanobiology [1] will help to understand mechanical aspects of cells and tissues during development. However, a pattern formation system that relies on mechanical aspects of tissues has not been investigated sufficiently yet.

Butterfly wings exhibit extreme diversity of color patterns based on developmental and evolutionary modifications of the nymphalid groundplan [6–11]. The butterfly wing system is largely a two-dimensional entity as depicted in the nymphalid groundplan, but strictly speaking, it is three-dimensional; organizers for color patterns are located at the bottom (or top) of an indentation (or a bump) of the wing epithelium in the pupal stage, and this epithelial structure is reflected as pupal cuticle spots [12–14]. Furthermore, this three-dimensionality is reflected in adult wings [13]. Considering these facts, the distortion hypothesis has been proposed, in which mechanical waves generated by oscillatory physical disturbances of the wing epithelial tissue behave as morphogenic (morphogen-like) signals [3].

In this study, the possibility that mechanical and physicochemical properties of extracellular milieu of the epithelial tissue play an important role in morphogenic signal propagation was explored. It has been suggested that some extracellularly secreted molecules such as the Wnt family and TGF-β family proteins behave as chemical morphogens for color-pattern determination in butterflies [15, 16], although how and where these chemical morphogens are distributed are not known. Furthermore, other molecules that could regulate color patterns such as a transcription factor Distal-less have been studied in butterfly wings [17–20]. These molecular signals and regulators are certainly important and compatible with mechanical signal transduction; in a recent model, gene expression regulations are elicited in response to mechanical signals [3].

Here, this study concentrates on the dorsal hindwing of the peacock pansy butterfly, *Junonia almana* (Linnaeus, 1758). This butterfly has a large double-focus eyespot on the dorsal side of the hindwing. This eyespot is a fusion of the two original eyespots, but it is called the major eyespot as a singular entity. The dorsal hindwing also has a much smaller eyespot called the minor eyespot, which is sometimes nonexistent, and the parafocal elements, which,

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together with eyespots, belong to the border symmetry system. Importantly, the background area has a light orange coloration and does not harbor anything like semi-element or pseudo-element, which probably exists in the blue pansy butterfly, *J. orithya* (Linnaeus, 1758) [21–23]. Furthermore, several versions of color-pattern modifications in response to various treatments have already been known in the peacock pansy butterfly; it has been used for the injections of sodium tungstate, and ecdysteroid and for temperature shock [24] and for physical damage [25, 26]. The scale-size and scale-color distributions have also been recorded in detail in this species [27].

In the present study, the forewing-lift operation was employed, which has been developed and used for several experiments [8, 18, 21, 27–30]. This operation made it possible to insert a small stainless ball between the forewing and the hindwing to disturb the planar epithelial surface. Furthermore, the operation made it possible to cover the hindwing surface with various covering materials. It is likely that the hindwing surface is covered only with a thin layer, if any, of cuticle. This means that the cellular environment of the extracellular matrix can be manipulated directly. Here, various color-pattern modifications were successfully obtained, including the high-level size reduction of the major eyespot, on the dorsal hindwing by the forewing-lift method using small stainless balls and various covering materials. Importantly, modifications of the minor eyespot and parafocal elements were also obtained, which were reminiscent of the temperature-shock-type (TS-type) modifications known in this species [24].

These results highlight the importance of mechanical force and extracellular matrix on which the wing tissue depends to execute normal wing development. Planar tissue surface with tension and specific physicochemical factors of the extracellular matrix may be required to propagate morphogenic signals properly. These results can be explained by the assumption that chemical morphogens such as Wnt propagate on the dorsal side of the extracellular space of the hindwing. Alternatively, but not mutually exclusively, these results can be interpreted from the viewpoint of the distortion hypothesis and the induction model [3, 31–33]. The induction model that is integrated with the distortion hypothesis involves both mechanical signals (early stage) that follow a Newtonian equation to propagate [33] and chemical signals (late stage) that follow a short-range activation and a long-range inhibition, an essence of reaction-diffusion model [34–36]. The model proposes that the mechanical morphogenic signals are distortions of the planar epithelial sheet, which are translated into chemical signals (i.e., calcium waves and oscillations) that induce the expression of developmental regulatory genes such as Wnt [3].

2. Materials and methods

2.1. Butterfly samples and manipulations

The peacock pansy butterfly, *J. almana*, was used throughout this study. They were obtained from Ishigaki-jima Island, Okinawa, Japan. Eggs were collected from females, and larvae were reared at an ambient temperature using their natural host plants. No permissions were necessary to use these butterflies in biological research in Japan.

For all experimental procedures, the right forewing was lifted within 30 min after pupation, according to the previous studies that used this operation [8, 18, 21, 27–30]. After the operation of placement of either a ball or a covering material, the operated pupae were confined independently in a plastic container with a lid and placed at an ambient temperature until eclosion. After eclosion, the adult butterflies were frozen, and the wing color patterns were examined visually. The wing images were scanned using a Canon MG5730 scanner (Tokyo, Japan).

2.2. Ball placement

For the ball placement experiments, the forewing was first lifted and a stainless ball of 0.5 mm in diameter (Tsubaki Precision Balls, Tsubaki Nakashima, Katsuragi, Nara, Japan) was placed on the surface of the dorsal hindwing (**Figure 1A**). The forewing was then placed back to the original position. Thus, the ball was sandwiched between the forewing and the hindwing.

2.3. Contact treatments

For the contact experiments, a piece of transparent plastic film of polyvinylidene chloride (PVDC) for culinary use (Kurewrap, Kureha, Tokyo, Japan) was used to cover the wing surface with the operated wing upward (**Figure 1A**, **B**). The film was flexible enough to cover the entire surface of the exposed hindwing except the major eyespot. The anterior portion of the major eyespot was not exposed in this operation, and the posterior portion was exposed but might not been covered completely, because there was a small but disturbing physical gap between the epithelial surface and the surface of the pupal case that was not lifted. A different set of individuals were similarly covered with a piece of the plastic film, and the operated wing was placed downward (**Figure 1C**). Likewise, the dorsal hindwing surface was mounted on a Superfrost micro-glass slide (Matsunami Glass, Kishiwada, Osaka, Japan) (**Figure 1D**). This glass slide has a smooth surface that attaches to tissues, and it is thus frequently used for immunohistochemical analysis. In this case, the pupal body was lightly pushed onto a glass surface (**Figure 1E**). This way, the hindwing made a successful contact. Adult butterflies emerged from the operated pupae with severe forewing damage and color-pattern modifications of the operated dorsal hindwing (**Figure 1F**).

Additionally, a medical adhesive "white tape W129" with acrylic adhesives (Nichiban, Tokyo, Japan) was employed to cover the exposed hindwing surface. A piece of glassine paper coated with silicone resin (here called silicone-glassine paper) for culinary use (CGC Japan, Tokyo, Japan) was also used, on which the dorsal hindwing was placed (**Figure 1G**, **H**). In these treatments, the operated wing was placed downward. The adhesive tape and the silicone-glassine paper are not as flexible as the plastic film, and when a portion of the tissue was attached, the attached portion was confirmed from a horizontal view and from a non-attached side of the paper (**Figure 1G**, **H**).

2.4. Statistical analysis

Statistical analysis was not performed for the results of the major eyespot in comparison to the no-treatment group, because the characteristically disturbed eyespots by the ball were

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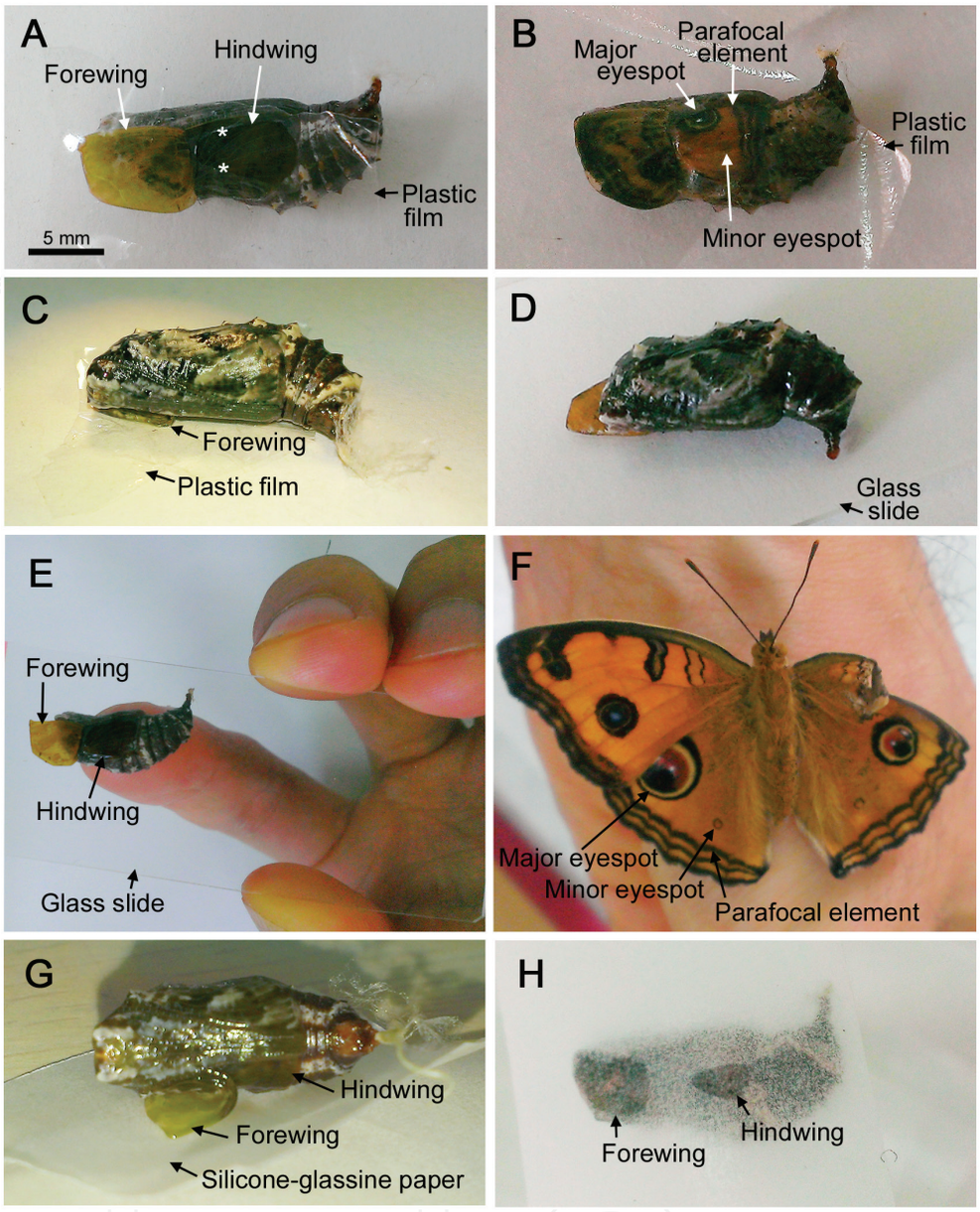

Figure 1. Mechanical disturbances applied to pupal hindwing in this study. (A) Operated pupa whose hindwing was covered with a piece of plastic film immediately after pupation. Asterisks indicate anterior (prospective eyespot) and posterior (prospective background) locations on which a 0.5-mm stainless ball was placed; in that case, the lifted forewing was placed back to the original position. (B) Adult hindwing color pattern seen through a piece of plastic film immediately before eclosion. Note that the posterior eyespot focus of the major eyespot is visible, but the anterior eyespot focus of the major eyespot is not visible because it is covered with the pupal case. The minor eyespot is found at the center of the exposed portion of the hindwing. Parafocal elements are also visible. (C) Operated pupa whose hindwing surface was covered with a piece of plastic film and placed down immediately after pupation. (D) Operated pupa whose hindwing surface was placed down onto a surface of a glass slide immediately after pupation. (E) Operated pupa as shown in D that was lightly pushed onto a glass surface. (F) An adult that eclosed from an operated pupa. Note that the hindwing major eyespot of the operated (right) side is smaller than that of the non-operated (left) side. (G) Operated pupa whose hindwing surface was placed down onto a piece of silicone-glassine paper. Only a central portion of the hindwing containing the minor eyespot and parafocal elements has a contact with the paper surface, and the prospective major eyespot has no physical contact. (H) The bottom image of a silicone-glassine paper that had an operated pupa. Only a central portion of the hindwing is attached to the paper. This attached portion contains the minor eyespot and parafocal elements, but not the major eyespot.

evident by their deformed shapes and because the covering operations were highly effective in almost all individuals treated (nearly 100%); high-level deformation and size reduction of the major eyespot were observed unilaterally. An exception was the film upward treatment, for which two-sided Fisher's exact test was performed in comparison to the film downward treatment and to the silicone-glassine paper treatment, using JSTAT 13.0 (2012) (Yokohama, Japan). There was no single case where such changes were obtained without an operation (no-treatment control here was $n = 76$ in this study alone, but such a case of changes has never been observed in many years of *J. almana* studies involving several hundred unoperated individuals). Similarly, statistical analysis for parafocal elements was not performed, because in the treatments using the adhesive tape and the silicone-glassine paper, changes of parafocal elements were seen in the majority of individuals (nearly 100%), whereas no such changes were observed in other treatments (0%). By contrast, Fisher's exact tests (two-sided results) were performed for the results of the minor eyespot in contrast to the data from no treatment, using JSTAT 13.0 (2012) (Yokohama, Japan).

3. Results

3.1. No-treatment control and forewing-lift control

The individuals without treatment (the no-treatment group) were first examined for their color-pattern symmetry or asymmetry of the major eyespot, the minor eyespot, and the parafocal elements between the right and the left hindwings in terms of their size and shape ($n = 76$). No extensive asymmetry was observed for the major eyespot (0%) and parafocal elements (0%). For the minor eyespot, however, 6 individuals out of 76 (8%) exhibited minor asymmetry. Similarly, as a basis of all experimental procedures that were performed in this study, a forewing-lift control experiment was performed, in which the forewing of a pupa was lifted and then placed back to the original position within a few minutes, and color patterns of these operated individuals were visually examined ($n = 20$). No change of color patterns was observed in the major eyespot (0%) and parafocal elements (0%) (**Figure 2A–C**). By contrast, 2 individuals out of 20 (10%) exhibited small-size asymmetry of the minor eyespot (**Figure 2A–C**). However, this asymmetry was not statistically significant in comparison to the non-treated individuals ($P = 1.0$).

3.2. Ball placement

A 0.5-mm ball was placed on the prospective major eyespot of the dorsal hindwing (**Figure 1A**). Because the exposure was limited to the posterior side of the major eyespot (**Figure 1B**), the ball was most likely placed on the posterior side of the major eyespot ($n = 21$). Ten treated individuals out of 21 (48%) exhibited irregular changes of the major eyespot (**Figure 2D–F**).

Likewise, a 0.5-mm ball was placed in the central background position of the dorsal hindwing (**Figure 1A**). The ball had no physical contact with the major eyespot ($n = 21$). Three treated individuals out of 21 (14%) exhibited irregular minor changes of the major eyespot in its anterior side, a remote place from the ball (**Figure 2G–I**).

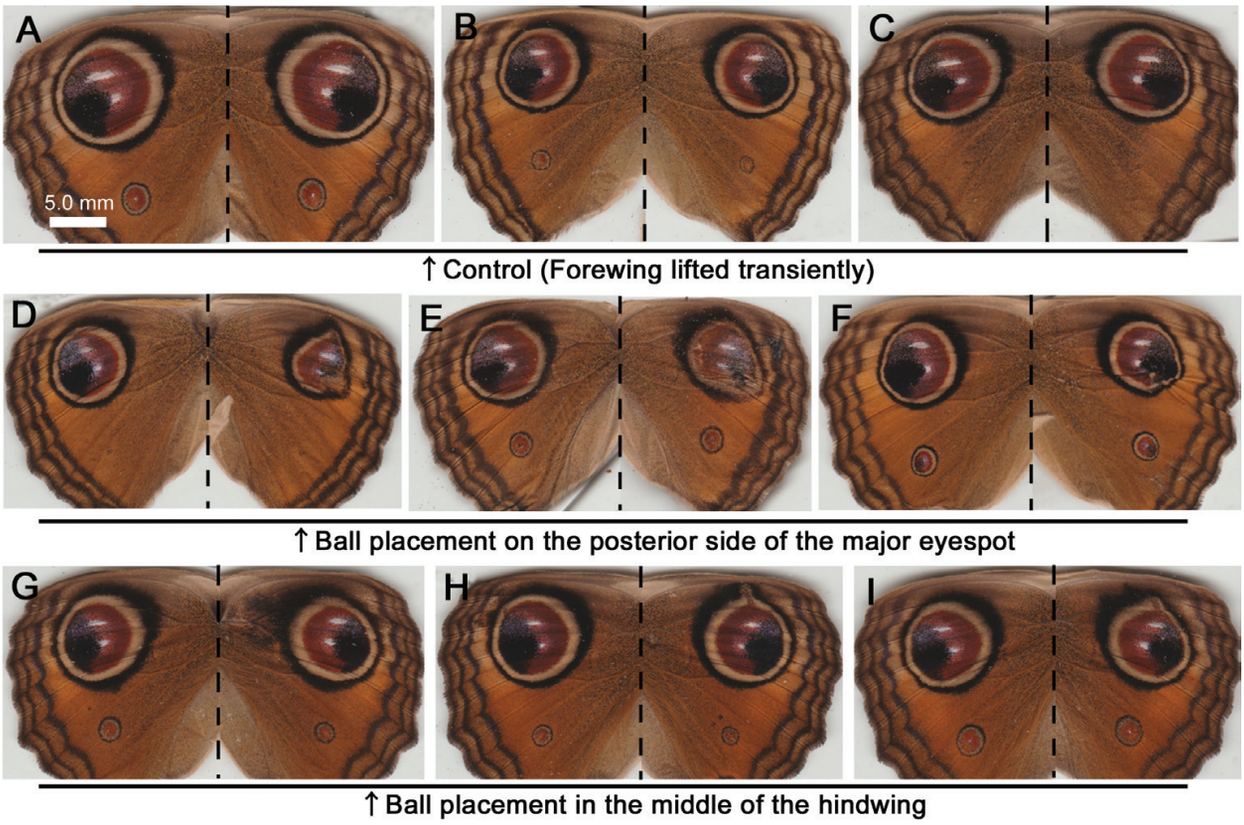

Figure 2. Forewing-lift control and ball placement. The right hindwing was operated and the left hindwing of the same individual was shown in every panel as the internal control for color-pattern comparison. (A–C) Control individuals. The forewing was lifted transiently and placed back to the original position. No changes of the color patterns are observed except that in B the right minor eyespot is smaller than the left one. However, this is not statistically significant in comparison to the no-treatment group (P = 1.0). (D–F) Ball placement on the posterior side of the major eyespot. Extensive deformation of the major eyespots is observed. (G–I) Ball placement in the middle of the hindwing. Color-pattern modifications are observed in the proximal (G) or anterior (H, I) part of the major eyespot.

3.3. Plastic film over the hindwing

After the forewing-lift procedure, the exposed hindwing was covered with a piece of transparent plastic film (n = 27) (**Figure 1A**, **B**). The operated right side was placed upward so that pressure on the hindwing surface was minimal. High-level size reduction with deformation of the major eyespot was observed in 20 treated individuals out of 27 (74%) (**Figure 3A–C**). Low-level size reduction or deformation was observed in 4 out of 27 (15%) (**Figure 3D**). Together, 24 out of 27 (89%) showed a change of the major eyespot. Even in the cases of the high-level reduction, the white spots inside the major eyespot were not affected much, and parafocal elements did not change, either. In the case of the minor eyespot, 3 individuals out of 20 (15%) that were visually judged clearly showed size reduction (1 individual; **Figure 2A**) and size enlargement (2 individuals; **Figure 2B**, **C**). However, these changes of the minor eyespot were not statistically significant in comparison to the no-treatment group (P = 0.39).

To examine if a light pressure on the hindwing due to its own weight may change color patterns, the exposed hindwing that was covered with a piece of plastic film was placed downward on a solid surface (n = 14) (**Figure 1D**, **E**). All 13 individuals out of 14 (93%) showed high-level

reduction of the major eyespot (**Figure 3E, F**). As an exception, one individual did not show any change. The downward configuration did not show significant difference from the upward configuration when the high-level and low-level changes were not treated as distinguished categories (P = 1.0). Even when only the high-level changes were compared, the downward configuration did not show significant difference from the upward configuration (P = 0.23).

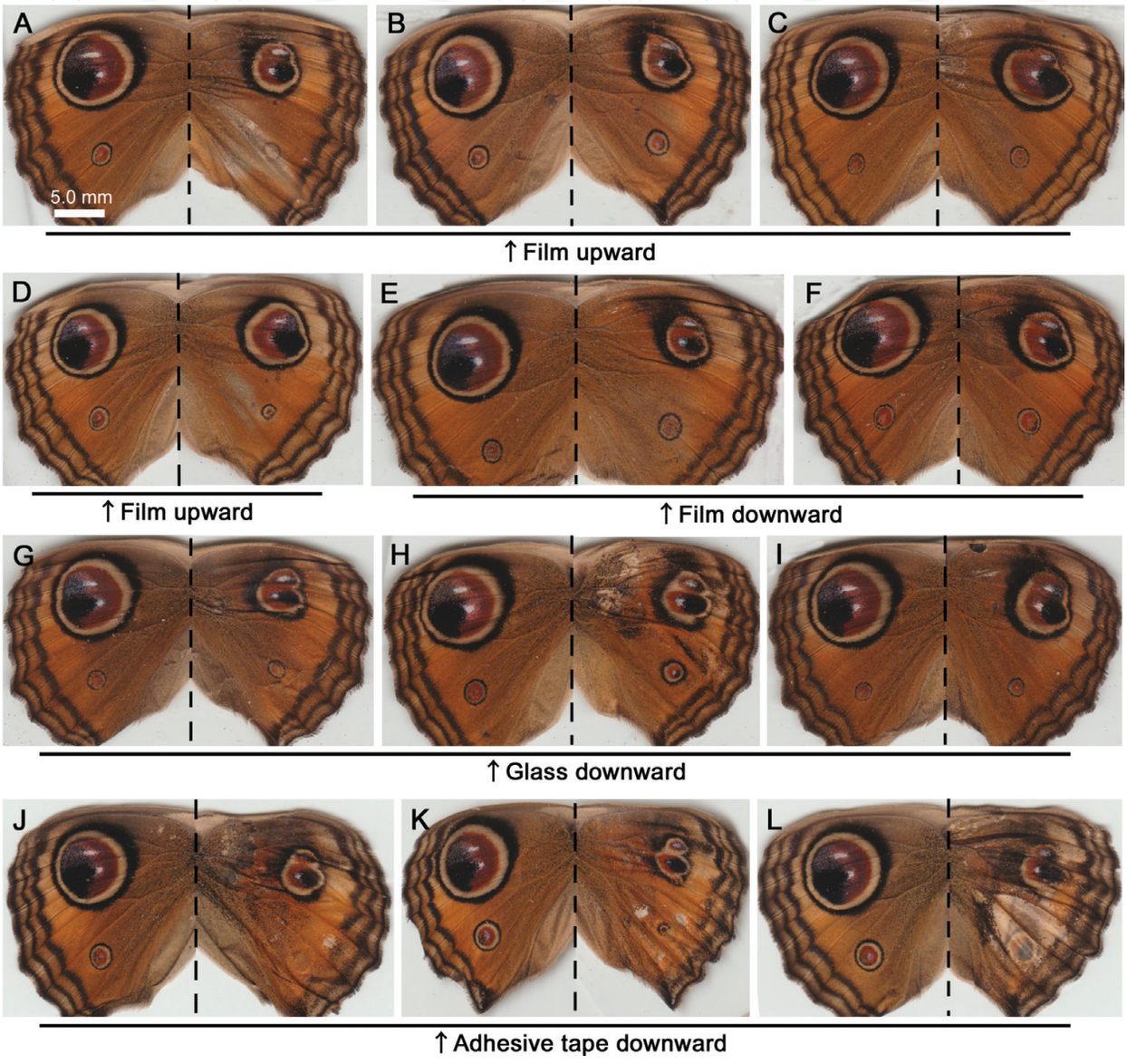

Figure 3. Film, glass, and adhesive tape experiments. The right hindwing was operated and the left hindwing of the same individual was shown in every panel as the internal control for color-pattern comparison. In all cases shown, the right major eyespot was deformed and reduced in size. (A–D) The hindwing surface was covered with a piece of plastic film and the treated wing was placed upward. The right minor eyespot was either reduced (A, D) or enlarged (B, C) in size. (E, F) The hindwing surface was covered with a piece of plastic film and the treated wing was placed downward. The right minor eyespot was enlarged in these individuals. (G–I) The hindwing surface was mounted on a piece of glass slide. The minor eyespot was reduced in size (G), showed the white spot inside (H), or showed no change (I). (J–L) The hindwing surface was mounted on a piece of adhesive tape. The minor eyespot was reduced in size in (K), but the scales of the minor eyespot in (J) and (L) were removed, which made observations of the minor eyespot impossible in these individuals. In (L), the scale removal is extensive; the ventral side is seen through.

Again, parafocal elements did not change, but 3 individuals (2 enlargements, **Figure 3E**, **F**; and 1 reduction, **Figure 3D**) out of 14 (21%) showed changes in the minor eyespot. However, these changes of the minor eyespot were not statistically significant in comparison to the no-treatment group ($P = 0.14$).

3.4. Hindwing placement on a glass slide

To examine the possibility that the covering materials may affect color patterns, the exposed hindwing was directly placed on the surface of a glass slide (**Figure 1D**). The hindwing was lightly pushed on the glass surface so that the hindwing could make a direct contact with a glass surface at least at that time point (**Figure 1E**). Thus, the operated side was placed downward ($n = 15$). Because the glass surface is rigid in contrast to the flexible film, the major eyespot may not have maintained a contact with a glass surface, as with the case of the white adhesive tape and the silicone-glassine paper (see subsequent experiments).

After the glass treatment, high-level changes with deformation of the major eyespot were observed in all 15 treated individuals (100%) (**Figure 3G–I**). Although not quantitative, the level of size reduction was also likely more severe than the previous film treatments. No change was observed in parafocal elements. The minor eyespot was affected in 5 out of 14 (36%). Among them, 3 showed reduction (**Figure 3G**) and the other 2 showed white spot emergence (**Figure 3H**). The minor eyespot changes were statistically significant in comparison to the no-treatment group ($P = 0.012$). Separately, the size reduction ($P = 0.0031$) and the white spot appearance ($P = 0.023$) were both significant. The surface rigidity or physicochemical nature of the glass slide might have contributed to these color-pattern changes of the minor eyespot.

3.5. Hindwing placement on a piece of adhesive tape

Here, it was hypothesized that surface adhesion may contribute to color-pattern changes. A piece of adhesive tape was used to cover the surface of the exposed hindwing. However, in this treatment, it was confirmed that there was no direct contact with the major eyespot. That is, the major eyespot was not physically covered with the tape. By contrast, the minor eyespot was completely covered. This configuration was the same as the silicone-glassine paper treatment (**Figure 1G**, **H**). Thus, the effects on the major eyespot are basically from no-covering material. But the effects on the minor eyespot are from a covering material on it.

In all 14 individuals that eclosed (including 3 individuals that formed complete adult wings in pupae but failed to exit from the pupal case), high-level reduction of the major eyespot was observed (100%) (**Figure 3J–L**). Although not quantitative, the level of reduction appeared to be more severe than the previous treatments. Interestingly, the minor eyespots were also reduced in all of these individuals ($n = 13$) (100%), although one individual cannot be judged because of the removal of scales of the minor eyespot upon eclosion. This result was statistically significant ($P < 0.0001$). Because of high adhesiveness of the tape, scales were often removed at the time of eclosion around the minor eyespot (**Figure 3J–L**). The scale removal demonstrated the direct (or nearly direct) adhesion of the tape to scales. Parafocal elements and submarginal bands were thickened and somewhat displaced proximally in all individuals (100%), which is reminiscent of the temperature-shock-type (TS-type) modifications [24, 38–40].

3.6. Hindwing placement on a sheet of silicone-glassine paper

To gain further insights into mechanical and physicochemical factors for color-pattern determination, the exposed hindwing surface was placed on a sheet of silicone-glassine paper ($n = 26$) (**Figure 1G**, **H**). The silicone-glassine paper was used here because it is not supposed to stick to the wing surface tightly, in contrast to the adhesive tape used earlier. Contrary to the expectation, results were similar to those of the adhesive tape. In all 26 individuals, high-level reduction of the major eyespot was observed (100%) (**Figure 4A–I**).

Interestingly, the minor eyespot changes in coloration and size were observed in 21 individuals out of 24 (2 individuals were not possible to judge because of breakage of the wings during eclosion and manipulation) in the silicone-glassine paper treatment (**Figure 4A–I**). This result was statistically significant ($P < 0.0001$). Similarly, parafocal elements changed in size and location, reminiscent of the TS-type modifications [24, 38–40], in 14 treated individuals out of 20 (6 individuals were not possible to judge because of breakage of the wings during eclosion and manipulation) (**Figure 4A–I**). In addition, small ectopic spots or ring structures were often observed in the background.

3.7. Response profiles of color-pattern elements

On the basis of the experimental results on the number of individuals that exhibited color-pattern changes, response profiles of the major eyespot, the minor eyespot, and parafocal

Figure 4. Silicone-glassine paper experiment. The right hindwing was operated and the left hindwing of the same individual was shown in every panel as the internal control for color-pattern comparison. In all cases, not only the major eyespot but also parafocal elements and the minor eyespot were affected. The minor eyespot modifications are different from that of the major eyespot. But overall, the modifications are reminiscent of those induced by tungstate injection or temperature-shock treatment [24, 38–40].

elements were obtained (**Figure 5A**). The major eyespot was always disrupted by any treatments; this is probably because no mode of treatment covered the prospective major eyespot area, with a possible exception of the film treatment. Indeed, when the high-level changes of the film upward treatment were compared to the silicone-glassine paper treatment, their difference was statistically significant ($P = 0.010$). This result likely means that the upward film treatment physically covered the exposed posterior portion of the major eyespot at least in some individuals and that this film cover functionally mimicked the natural extracellular matrix for the hindwing tissue to some extent.

In contrast to the major eyespot, the minor eyespot and parafocal elements were firmly covered by the covering materials, which mean that the effects on the minor eyespot and parafocal element may be caused by physicochemical properties of the materials. Parafocal elements were affected only by the adhesive tape and the silicone-glassine paper.

The response profiles of the minor eyespot were further obtained in terms of three types of color-pattern changes: size reduction, size enlargement, and appearance of the white spot at the center (**Figure 5B**). Among them, reduction was the most frequent change in the glass ($P = 0.0031$), the adhesive tape ($P < 0.0001$), and the silicone-glassine paper ($P < 0.0001$).

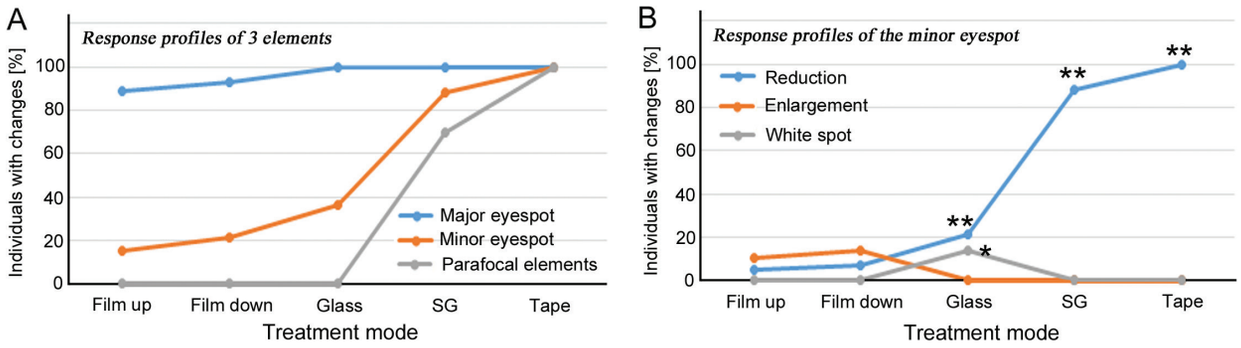

Figure 5. Response profiles of color-pattern elements. "SG" and "tape" indicate the silicone-glassine paper treatment and the adhesive tape treatment, respectively. (A) Profiles of three elements. For the film upward treatment, both high-level and low-level changes were included without distinction. The major eyespot is highly responsive to all the treatment modes. Parafocal elements are sensitive to the two modes, the adhesive tape and the silicone-glassine paper. These are highly significant results without doubt in comparison to the no-treatment group. (B) Profiles of the minor eyespot. Three different response patterns are recognized, and they are profiled in response to the treatment modes. Size reduction is prominent in the adhesive tape (**$P < 0.0001$) and the silicone-glassine paper (**$P < 0.0001$), but it can also be seen in the glass treatment (**$P = 0.0031$). These results are statistically significant. The white spot appearance in the glass treatment is also significant (*$P = 0.023$). By contrast, size reduction and enlargement seen in the film upward treatment ($P = 0.39$) and the film downward treatment ($P = 0.14$) are not statistically significant.

4. Discussion

4.1. Overview of this study

In this study, different types of mechanical distortions and adhesions on developing pupal hindwing tissues were introduced. The present study is composed of three parts that require independent interpretations: (1) the response of the major eyespot to the ball placement, (2) the response of the major eyespot to no-covering material via the forewing-lift method, and (3) the

response of the minor eyespot (together with the parafocal elements) to various covering materials. Collectively, however, the present results demonstrated that artificially introduced mechanical distortions and properties of contact surface affect the final color patterns in butterfly wings.

4.2. Ball placement and physical damage

The degrees of size reduction in the major eyespot in response to the ball treatment may be compared with the damage-induced changes in the previous study [26]. When the anterior eyespot focus was physically damaged by a stainless needle, the major eyespot was reduced in size not only in the anterior side but also in the posterior side, suggesting synergistic interactions of signals from two adjacent organizers. When the posterior eyespot focus was damaged, similar effect was observed, but it was much less effective [26]. In the present study, the ball placed on the posterior portion of the major eyespot appears to be at least as effective as physical damage at the posterior focus, suggesting the importance of distortion in developmental fate determination. Assuming that the ball placement did not kill epithelial cells, the present results suggest that necrotic cell death caused by physical damage is not necessary to induce color-pattern changes. The ball placement on the background was less influential, but interestingly, it induced irregular local extrusion of the major eyespot, suggesting that the mechanical distortion may impose a long-range effect on the major eyespot. On the other hand, a small degree of wing-wide pressure on the hindwing in the downward configuration with the plastic film coverage did not change color patterns at the anterior side, suggesting that a local distortion of the planar tissue may be more important than a wing-wide pressure (i.e., distortion) to cause changes in color patterns.

4.3. Extracellular environment of the dorsal hindwing surface

It is important to understand the extracellular environment of the hindwing tissue before discussing possible interpretations of the experimental results of various covering materials. The hindwing dorsal surface, when the forewing was lifted immediately after pupation, may not be covered with cuticles. If any, that cuticle coverage may be very thin. Alternatively, the forewing-lift operation and/or coverage with artificial materials may completely inhibit or reduce the cuticle formation process on the surface of the hindwing. To be consistent with this idea, a long-term hindwing exposure without any coverage after the operation makes them die from being dried [21]. This was also confirmed in the present study; all the operated pupae (n = 24) with an exposed hindwing without any coverage (but the ventral forewing was covered with a piece of plastic film) died without development of color patterns (100%). Furthermore, the adhesive tape treatment removed many scales from the dorsal hindwing upon eclosion, probably because the adhesive tape was sticky enough to bind scales directly and strongly. Thus, it is likely that the extracellular side of the hindwing tissue cells was directly exposed to covering materials.

4.4. Response of the major eyespot

The major eyespot of the dorsal hindwing was sensitive to the operations performed in this study. Use of various covering materials with different rigidity, adhesiveness, surface

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smoothness, and chemical composition resulted in miniaturization of the major eyespot. But in the adhesive tape and the silicone-glassine paper treatments (and probably also in the plastic film and glass treatments), the posterior side of the major eyespot was not in contact with anything. Because of curvature of the hindwing tissue and a physical gap between the surface of the hindwing tissue and the pupal case of the most ventral part, even the flexible plastic film cannot completely make a contact with the major eyespot. This configuration was clearly confirmed in the adhesive white tape and the silicone-glassine paper treatments. Furthermore, it is to be noted that the major eyespot in this butterfly could not be completely exposed by the forewing-lift method; the anterior portion was always under the pupal case. These facts likely explain that the results of various covering treatments were virtually identical for the major eyespot.

It is surprising that the miniaturization of the major eyespot by the present operations is more efficient than the physical damage treatment [26], despite the fact that the present operations are less invasive. Likely interpretations would be that the major eyespot organizers need extracellular supporting materials to propagate morphogenic signals and that the anterior side alone that was covered with the pupal case cannot expand without the help of the posterior side. These interpretations are consistent with the previous chapter that describes synergistic signal amplification and expansion processes in this eyespot [26]. Indeed, the anterior side of the major eyespot appeared to be more sensitive to the present treatments and also to physical damage [26] than the posterior side despite the fact that the anterior side is physically hidden. It is to be noted that the upward film treatment was the least effective to induce changes. And there is a possibility that this treatment covered the posterior part of the major eyespot at least in some individuals because of its flexibility. Therefore, for the morphogenic signals to propagate efficiently, a covering material is required. However, judging from the effects of various covering materials on the minor eyespot, the covering materials should have certain physicochemical properties to support normal propagation of morphogenic signals. In this sense, the plastic film that did not affect the minor eyespot significantly is ideal and may be similar to the normal extracellular matrix of the hindwing epithelium in *J. almana*.

As a general tendency, the proximal side of the major eyespot showed a fusion of the signals from the anterior and posterior organizers, whereas the distal side often showed a separation of the two. Signals may be more expandable to the proximal side. In the reduced major eyespot, the size of the white spots was not affected much in the film and glass treatments, suggesting an uncoupling behavior of the white spots from the rest of the eyespot. Similar uncoupling behavior of white spots has been shown in *Calisto* butterflies [37]. Similar to the white spots, the minor eyespot and parafocal elements were not affected very much by the film treatment. However, the glass treatment significantly induced the size reduction and the white spot induction of the minor eyespot. This induction may be specific to the glass surface physical chemistry, but the low level of pressure applied in this particular treatment may be a reason.

4.5. Adhesive tape and silicone-glassine paper treatments

In contrast to the major eyespot, the minor eyespot was in direct contact with the covering materials. Also in contrast to the film and glass treatments, which did not induce significant

changes in the minor eyespot, both the adhesive tape and the silicone-glassine paper treatments unexpectedly induced extensive modifications of the minor eyespot and parafocal elements, in addition to the reduction of the major eyespot. In these two treatments, the size reduction of the minor eyespot was statistically significant, suggesting the importance of a functional contact surface in expanding morphogenic signals for eyespots. Furthermore, size reduction of the white spot inside the major eyespot was prominent in the two treatments. If chemical morphogens are secreted to the apical extracellular side of epithelial cells, chemical morphogen transport would be disrupted by different covering materials. The present results using various covering materials do not contradict with this idea.

4.6. Similarity to the TS-type modifications

The overall phenotype, the displaced and diffused parafocal elements and the smaller major and minor eyespots induced by the adhesive tape and the silicone-glassine paper, is similar to the tungstate-injected phenotype, or more generally temperature-shock-type (TS-type) modifications that were demonstrated in this and other nymphalid butterfly species [22–24, 38–40]. The tungstate treatment and temperature-shock treatment have been known to induce characteristic wing-wide color-pattern modifications in this species [24]; eyespots became smaller and parafocal elements are diffused and dislocated proximally toward the eyespot focus. It appears that the adhesive tape and the silicone-glassine paper treatments were as effective as the injection of tungstate to produce the TS-type modifications or their similar ones in this species. This fact suggests that the mechanisms for the size reduction by covering materials and by tungstate injection may basically be similar. In that case, tungstate, cold shock, and the cold-shock hormone may act on the extracellular matrix of the wing tissue.

However, there is an important difference between the contact treatments and the tungstate and its related treatments. In the contact treatments, the minor eyespot appeared to be more sensitive than parafocal elements (note that a comparison to the major eyespot is irrelevant, because it was not in contact with anything). Parafocal elements were not modified in the glass treatment but the minor eyespot was. Morphogenic signals for parafocal elements had already been released by the time of the treatments, but signals for the minor eyespot had not [32]. It appears that the contact treatments affect the early phase of signals than the moving phase. Tungstate and its related treatments affect in an opposite way. In this sense, these two modes of treatments are different. The reason for this difference is unknown. A possible speculation is as follows. During development, the wing tissue shows a slow contraction cycles [28], which may contribute to an adjustment of the physical properties (including that of the extracellular matrix) of developing epithelial tissues. Because the epithelial tissue is covered by inflexible materials in the covering experiments (except for the film treatment), this contraction movement may be inhibited, affecting morphogenic signals to be released and propagate. Morphogenic signals that were released already may not be affected much, because it is less dependent on the contractive movement anymore.

Interestingly, heparin, chondroitin sulfates, and dextran sulfate that could act extracellularly are also able to induce TS-type modifications [41]. Because heparin sulfate proteoglycans play an important role in Wnt signaling [42–49], because Wnt family proteins are thought to be

chemical morphogens for butterfly wing color patterns [16], and because TS-type modifications may be attained by molecular changes of the extracellular matrix, various treatments that induce TS-type modifications may cause the reduction of the extracellular movement of Wnt family proteins. On the other hand, Wnt may be transmitted via membranous structures such as cytonemes [50–54] and argosomes [44] through intercellular spaces that are filled with hemolymph inside the tissue. Cytoneme-like structures were reported in the developing butterfly wing tissue [14, 29].

The distortion hypothesis and induction model (see subsequently) posit mechanical morphogens, but they do not deny (but incorporated) chemical morphogens such as Wnt family proteins that would play an important role in finalizing the adult color patterns. Mechanical signals may be released first from organizers, but they should readily be translated into chemical signals that act locally. Activation of TGF-β in the extracellular matrix is executed by mechanical forces medicated by integrins and other extracellular matrix molecules [55]. Interestingly, TGF-β has been considered a candidate morphogen in butterfly wings [15].

4.7. The induction model and the distortion hypothesis

The induction model has been proposed to explain processes of color-pattern determination in butterfly wings, based on several lines of evidence including color-pattern comparisons among many butterfly species [31, 32], experimentally induced color-pattern changes [25], scale-size distribution patterns [21, 27], morphological and histochemical analyses of pupal wings [12], mathematical modeling [33], and developmental real-time imaging [14, 28, 29]. In this model, morphogenic signals are released as slow decelerating wave pulses from organizers, and the locations of their settlement then act as the secondary organizers [3]. However, identity of the wave signals has been enigmatic. The present study has suggested that one possible candidate is mechanical distortions of the epithelial tissues and highlighted the importance of the extracellular matrix as a medium for mechanical or chemical signals.

The distortion hypothesis has been proposed, in which the putative wave signals were explained as mechanistic distortions of the wing epithelial tissues [3]. Cuticle spots are likely sources of distortions, and distortions slowly propagate radially with decelerating motion. Distorted immature scale cells are activated by calcium waves through a stretch-sensitive calcium channel. Distortions act as a ploidy signal, and the degrees of polyploidy of the epithelial cells determine the final coloration of a given scale [27]. This distortion hypothesis can explain the nature of morphogenic signals that have been proposed in the induction model of positional information in butterfly wings. To generate and propagate the wave signals, the planar epithelial sheet and its supporting materials (i.e., the extracellular matrix) with their appropriate physicochemical properties may be required.

Surface rigidity that is conferred by the extracellular matrix may play an important role in development in general by giving mechanical supports for cells [1]. In *Drosophila*, the apical surface of wing epithelial cells changes its morphology, and this morphology acts as a template to produce a rigid dorsal cuticle. After that, a flexible ventral cuticle is produced, which is then molded on the inner surface of the rigid dorsal cuticle [56, 57]. A similar mechanism has been proposed in the development of butterfly scales [58].

5. Conclusions

The present study provided experimental evidence that mechanical force and physicochemical properties of extracellular matrix contribute to morphogenic signal propagation, focusing on the hindwing color patterns of the peacock pansy butterfly. These results point to the importance of an appropriate tension and the extracellular milieu that the planar wing epithelium has. Mechanical distortions and physicochemical properties of the extracellular matrix may be functional mediators of long-range morphogenic signals in butterfly wings.

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