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Local Pharmacological Effects of Tungstate on the Color-Pattern Determination of Butterfly Wings: A Possible Relationship Between the Eyespot and Parafocal Element

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Butterfly wing color patterns can be changed by the application of a temperature shock or pharmacological agents such as tungstate, producing a distinctive type of elemental modification called the TS (temperature shock) type. Heterochronic uncoupling between the signaling and reception steps during the color-pattern determination process has been proposed as a mechanism for TS-type changes. As an extension of this hypothesis, both the parafocal element (PFE) and the eyespot in the same wing compartment are considered to be determined by morphogenic signal(s) emitted from the same eyespot focus. However, these models need to be examined with additional experimental data. Furthermore, there is controversy as to whether the action of tungstate on wing color patterns is direct or indirect. Using a species of nymphalid butterfly (*Junonia orithya*), we have devised a simple method for the local application of pharmacological agents directly on developing wings of pupae. Local tungstate application resulted in reduced eyespots and circular dislocated PFEs in the eyespot-less compartments only on the treated wing, demonstrating that tungstate directly induces color-pattern changes on wings. We further examined the eyespot-PFE relationship in normal and cold-shocked individuals, showing that an eyespot can be superimposed on a PFE and vice versa, probably depending on the timing of their fate determination. Taken together, we propose a two-morphogen model for the normal color-pattern determination, in which the morphogenic signals for the eyespot and PFE are different from each other despite their identical origin. This two-morphogen model is compatible with the heterochronic uncoupling model for TS-type changes.

Key words: butterfly wing, color-pattern determination, eyespot, parafocal element, sodium tungstate, heterochronic uncoupling, morphogen

INTRODUCTION

Diverse color patterns on butterfly wings have attracted much attention from amateur and professional scientists alike. Based on a comparative color-pattern analysis, Nijhout (1990, 1991, 2001) proposed a basic framework for butterfly color patterns called the nymphalid groundplan. The nymphalid groundplan is an idealized color pattern and is not necessarily evolutionarily primitive. The groundplan can be understood as a wing-wide positioning of "elements" against a plane "background", although the definition of background needs to be reconsidered (Kusaba and Otaki, 2009). Real butterfly color patterns are supposed to be "derived" from the nymphalid groundplan by changes of elements in size, shape, color, and location. Hence, elucidating the physiological and genetic factors that can modify these elemental features is of great importance in understanding the development, evolution, and phenotypic plasticity of wing color patterns in butterflies (Otaki, 2008a).

Application of pharmacological agents (such as sodium tungstate) or temperature shock to pupae can systematically modify the color patterns in adults, producing a distinctive type of elemental change called the TS (temperature shock) type (Otaki, 2008a). The evolutionary importance of the TS type is exemplified by color-pattern evolution in the genus *Vanessa* (Otaki and Yamamoto, 2004a; Otaki et al., 2006a, b; Otaki, 2008b, c) and in some lycaenid butterflies (Otaki and Yamamoto, 2003). These studies proposed that the mechanism for developing the TS type is essentially similar to the mechanism of color-pattern evolution in at least some butterfly species.

Color-pattern changes induced by the systemic injection of tungstate are almost always found symmetrically on both the right and left wings, even when the injection is made on only the right or left wing (Otaki, 1998, 2008a; Serfas and Carroll, 2005). It is thought that tungstate induces hormonal secretion into the hemolymph, which circulates throughout the body, including the wings, and affects color patterns (Serfas and Carroll, 2005). In other words, the action of tungstate on wing color pattern is thought to be indirect.

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Although we pointed out circumstantial evidence for the direct action of tungstate on wings (Otaki, 1998, 2008a), direct evidence has been lacking.

Among color-pattern elements, the most conspicuous is probably an array of border ocelli, or eyespots, located along the anteroposterior axis of the wing. The eyespots constitute the border symmetry system together with parafocal elements (PFEs), which are located in the immediate vicinity of the eyespots (Nijhout, 2001; Otaki, 2009). PFEs are elements that are highly diverse in shape and highly plastic in response to external perturbations such as chemical and temperature applications. Thus PFEs have been the most difficult elements to understand with respect to their status in the nymphalid groundplan (Nijhout, 1991, 2001; Otaki, 2008a, 2009).

The PFEs indeed show somewhat perplexing responses to experimental manipulations. In response to tungstate or temperature application early in the pupal stage, the PFEs are dislocated closer to eyespots, and in extreme cases appear to be "absorbed" into the eyespots (Nijhout, 1984, 1991; Otaki, 1998, 2007, 2008a, 2008b; Otaki and Yamamoto, 2004b; Otaki et al., 2005a; Serfas and Carroll, 2005). This indicates that at the time of treatment, the location of PFEs was not yet determined, and the origins of positional information for PFEs are likely to be the prospective eyespot foci (Otaki, 2008a, 2009). Moreover, PFEs appear to be bi-sided. Although the conventional PFE is located on the distal side of the eyespot focus, an element equivalent to the conventional PFE exists in the proximal side of the focus in a given wing compartment, and is called the proximal PFE (Otaki, 2009). However, physical damage at the eyespot focus early in the pupal stage has so far not been reported to influence the PFEs, even if the damage resulted in a compromised eyespot (Nijhout, 1985, 1991; Brakefield and French, 1995; French and Brakefield, 1995). Furthermore, PFEs definitively exist even in the compartments lacking eyespots in at least some *Junonia* butterfly species such as *J. orithya*, *J. almana*, and *J. coenia*.

Physiological experiments, the bi-sided positioning of PFEs, and comparative color-pattern analyses suggest that PFEs are dependent on the eyespot foci. On the other hand, the results of focal damage experiments and the existence of PFEs in eyespot-less compartments suggest that PFEs are independent from the eyespots. We call this inconsistency the PFE paradox. We believe that a clue to solving the PFE paradox can be found in mechanisms such as TS-type modifications which cause experimental color-pattern changes.

Mechanistically, TS-type modifications have been explained by the threshold model, in which the treatment increases the threshold level for the putative morphogen without affecting organizing activities (Otaki, 1998; Serfas and Carroll, 2005). However, this model cannot explain the larger dislocated PFEs in locations closer to foci, changes in the color and shape of the dislocated PFEs, or the ectopic scales found in the "imaginary" foci. For this reason, Otaki (2008a) proposed the heterochronic uncoupling model as a mechanism for TS-type modifications. In this model, the process of color-pattern determination in normal and modified wings is divided into four sequential steps: signaling, reception, interpretation, and expression. Temperature and chemical treatments are thought to disrupt the normal coupling of timing between the signaling and reception steps. This uncoupling can be achieved either by accelerating the reception step or by delaying the signaling step.

The existence of PFEs in compartments lacking eyespots in some *Junonia* butterflies can be explained by two alternative models, as a theoretical extension of the heterochronic uncoupling model (Otaki, 2008a). One model states that the "imaginary" eyespot focus was once active to produce a morphogenic signal for a PFE, but that another morphogenic signal for the eyespot was not produced well in the eyespot-less compartment. In this model, the PFE signal and the eyespot signal are two different chemical entities, although both are produced from the same focus. We call this the two-morphogen model. The other model states that the morphogenic signals for an eyespot and a PFE are essentially identical, but after specifying the PFE, the signal specifying the eyespot either decreases in the compartment containing the eyespot, or completely disappears from compartments lacking an eyespot. In the latter model, dynamic changes in the single focal signal explain the differences between PFEs and eyespots and also between compartments with and without eyespots. We call this the single-morphogen model; however, we previously concluded that this latter model was not very convincing due to the scarcity of supporting evidence (Otaki, 2008a).

To determine the mode of action of tungstate (i.e., direct or indirect action on wings), it is necessary to apply tungstate locally on wings. Here we devised a simple method for the local chemical application on a developing pupal wing using a species of nymphalid butterfly, *Junonia orithya*, and showed that the local (but not systemic) application of tungstate on wings resulted in color patterns that have not been previously reported. Moreover, to infer the eyespot-PFE relationship in terms of morphogenic signaling, we examined the color patterns of this species (both natural and cold-shock-induced) in which eyespot and PFE are in physical contact with each other. Our results support the two-morphogen model for the eyespot-PFE relationship. We discuss our results together with a few mechanistic models that at least partly explain the TS-type modifications and color-pattern diversity of butterfly wings.

MATERIALS AND METHODS

Animals

Female adult individuals of the blue pansy butterfly *Junonia orithya* (Linnaeus, 1758) (Nymphalidae, à Lepidoptera) were caught on Okinawa-jima and Ishigaki-jima Islands in the Ryukyu Archipelago, Japan. Eggs were collected from these females. Alternatively, larvae were field-caught on these islands. Larvae were fed their natural host plants at ambient temperature. This species shows sexual dimorphism and high color-pattern variation among individuals. Normal (non-treated) hindwings are shown in Fig. 1A (male) and Fig. 1B (female).

Experimental manipulations

To apply chemical solutions topically to immature scale cells without physical damage, we developed a novel method. Immediately after pupation (before pupal sclerotization), the pupal forewing was partly peeled off and carefully curled up, and a 0.5 μL droplet of a chemical solution was placed on the surface of the hindwing. The curled forewing was then returned to the original position. This

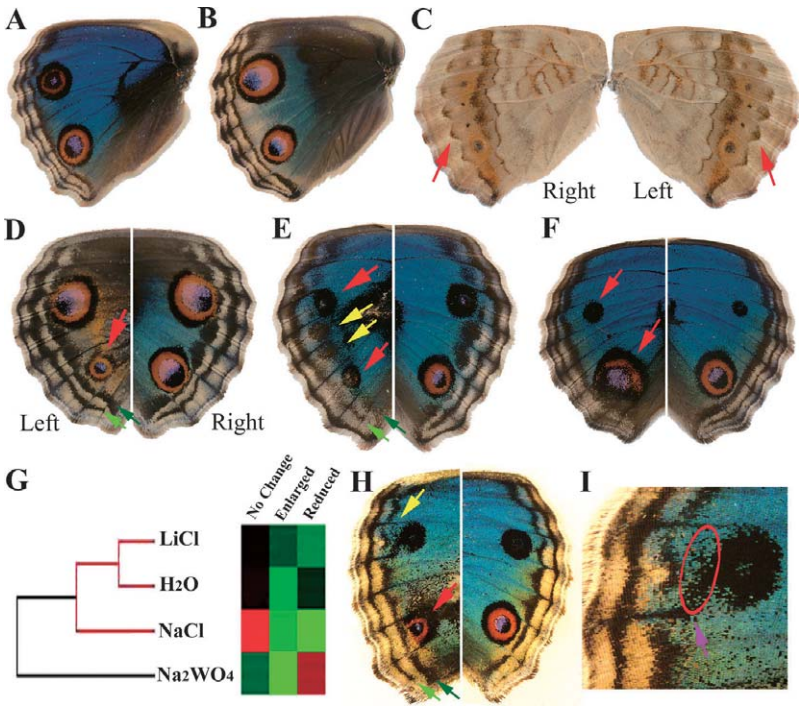

Fig. 1. Color-pattern changes induced in *J. orithya* by local applications of sodium tungstate or lithium chloride with the sandwich method. Left wing was treated. **(A)** Dorsal side of a non-treated (normal) male hindwing. **(B)** Dorsal side of a non-treated (normal) female hindwing. **(C)** Ventral side of hindwings of a tungstate-treated individual. The PFEs on the treated side (left) show characteristic elongation toward the eyespot foci. Red arrows indicate a pair of representative PFEs for comparison. **(D)** Reduced eyespot (red arrow) after tungstate treatment. Although a systemic effect on both wings was observed, the eyespot reduction was seen only on the left wing. The dark green arrow indicates the PFEs and the light green arrow points to the SMBs (submarginal bands). These dark and light green arrows are also shown in (E) and (H). Note that on the non-treated right wing, PFE dislocation (a systemic effect) was "blocked" by the large posterior eyespot, but this did not occur on the left wing. **(E)** Reduced eyespots (red arrows) and circular dislocated PFEs around the imaginary foci (yellow arrows) in tungstate-treated wings. Elongated PFEs due to the systemic effect are seen on the right wing. **(F)** Enlarged eyespots (red arrows) in lithium chloride-treated wings. This effect was not specific to lithium chloride, and is thus considered to be a physical or osmotic effect. **(G)** Dendrogram and heat map indicating relationships among the chemicals applied by the sandwich method. In the heat map, the brightest red indicates the maximal value, while the brightest green indicates the minimal value. Dark colors indicate intermediate values. **(H)** Ectopic PFE-like black element (yellow arrow) located between a normal PFE and eyespot. The normal PFE is also somewhat deformed, compared to the corresponding PFE on the right wing. The red arrow indicates the reduced eyespot. **(I)** Higher magnification view of part of (H). The end point of the continuous black scales along the wing vein normally signifies the position of a PFE, indicated by a purple arrow. The ectopic PFE-like black element is circled.

resulted in the solution being sandwiched between the pupal forewing and hindwing, and hence we call it the sandwich method. The operation was carried out only on the right or left wing, and the other wing was left untouched as an internal control. Eyespots on the non-treated wing of a treated individual have the similar size to those of non-treated (normal) individuals, as shown in Fig. 1A (non-treated individual) and Fig. 1D–F (treated individuals). Operational skill was required in order to avoid physical damage of wings and other parts of the pupae.

Chemicals

We topically applied sodium tungstate (1.0 M; Sigma-Aldrich, St. Louis, USA), lithium chloride (1.0 M; Wako Chemicals, Osaka, Japan), sodium chloride (1.0 M; Wako Chemicals, Osaka, Japan), and distilled water by the sandwich method. Sodium tungstate induces distinctive color-pattern changes that feature the dislocation of PFEs toward the corresponding eyespot foci (Otaki, 1998, 2007, 2008a, b; Otaki and Yamamoto, 2004b; Otaki et al., 2005a). It has been shown previously that sodium chloride, lithium chloride, and water do not change the color pattern at all when injected into pupae (Otaki, 1998; 2007).

Evaluation of the color-pattern changes

After the local application of chemicals, we visually classified individuals into three categories: no change, reduction of eyespots, and enlargement of eyespots. Scores were assigned to each category, 0 for no change, –1 for reduction, and +1 for enlargement. In some cases, a systemic effect was observed after treatment by the sandwich method, but this effect was seen equally in both wings, even if the injection was made in one wing (Otaki et al., 2005a; Otaki, 2008a; Serfas and Carroll, 2005). Importantly, the treatment acts on PFEs, and virtually no systemic effect was seen on the eyespots in non-treated wings. Modified eyespots and PFEs can be easily distinguished from non-modified ones by a fuzzy pattern boundary and diffused scale distribution. Emerged adults with wing damage or deformation were excluded from the analysis.

To analyze the change scores, we used the Kruskal-Wallis test (a non-parametric multiple test with the Scheffe method) implemented in JSTAT version 10.0 (Yokohama, Japan). The result of each treatment was expressed as the number of individuals in the three categories. Percentages of individuals were used for a cluster analysis, implemented in Avadis Profetic version 4.2 (Strand Life Sciences, Bangalore, India), that produced a dendrogram and a heat map. Cluster analyses detect natural groupings among data sets. We performed the cluster analysis as an exclusive method, in which the same sample was not allowed to appear in two different clusters. The Euclidean distance metric (root mean squared distance) with the complete linkage method was employed to evaluate relationships among data sets. A hierarchical method was used to construct a tree diagram where a cluster could contain other clusters.

Cold-shock treatment and color-pattern analysis

Pupae were transferred to an incubator at –2±1°C about 24 hours after pupation and kept in the incubator for 3.5 days. This protocol differed slightly from the standard one previously used for this species (Otaki, 2008a; Otaki et al., 2005a). These conditions appeared to be useful in producing modified PFEs with minimal changes in eyespots. Eclosed adults were readily frozen and subjected to color-pattern analysis. Images were processed using Adobe Photoshop Elements software.

For quantitative morphological comparisons of PFEs, eyespots, and ectopic black elements, acquired digital images were enlarged and printed out. Elemental lengths parallel and perpendicular to the proximal-distal wing veins were manually measured using Digital Caliper (Shinwa Rules, Niigata, Japan). The size ratio of an element between the parallel length and perpendicular length was then calculated. The two-sided paired *t*-test was performed using JSTAT version 10.0 (Yokohama, Japan).

RESULTS

Reduced eyespots produced by the local application of tungstate

Local tungstate application to pupal wings by the sandwich method produced local pattern changes only on the treated wings, although some systemic changes were unavoidably found in both treated and non-treated wings. Among 32 individuals treated with tungstate, six individuals (18.8%) showed minor but clear modifications of PFEs on the ventral surface (in addition to the dorsal surface) of the hindwings only on the treated side (Fig. 1C), strongly suggesting that tungstate penetrated into the wing tissue and changed the color pattern directly and locally. On the dorsal surface of the hindwings, 65.6% of the treated individuals (n=21) showed a reduction in eyespot size (Table 1; Fig. 1D, E). In addition, 6.3% of the individuals (n=2) had enlarged eyespots, which may be attributed to non-specific physical effects, and 28.1% (n=9) showed no change at all.

To confirm that this eyespot size reduction was due to the local pharmacological action of tungstate, we locally applied sodium chloride, lithium chloride, and distilled water for comparison (Table 1). The sodium chloride application resulted in 80.0% of the treated individuals (n=32) with no color-pattern change at all, although we still observed a small proportion of the treated individuals with enlarged (12.5%; n=5) and reduced (7.5%; n=3) eyespots. This is in sharp contrast to the tungstate application. Somewhat surprisingly, the water application produced higher rates of enlarged (16.7%; n=5) or reduced (36.7%; n=11) eyespots. Similar results were obtained by the application of lithium chloride (Table 1; Fig. 1F). Relatively high rates of eyespot size change appeared to be inevitable and might have resulted from an osmotic effect of the applied chemicals or from operational disturbances.

The results of the four treatments were significantly different (Kruskal-Wallis test, p<0.0001). The results for sodium chloride and sodium tungstate were also significantly different (p<0.01). Similarly, the results for lithium chloride and sodium tungstate were different (p<0.01). The dendrogram and heat map indicated an independent position of sodium tungstate among the four treatments (Fig. 1G).

Circular dislocated PFEs and ectopic PFE-like elements

As a result of the local application of tungstate, we obtained three individuals that had circular black dots in the supposedly eyespot-less compartments, together with reduced eyespots (Fig. 1E). The circular black dots were highly likely dislocated PFEs. Similar but less intense dislocation was found in non-treated wings due to the systemic effect. However, we never before obtained such circular dislocated PFEs in the systemic treatment of this species (Otaki et al., 2005a). Notably, the circular dislocated PFEs and the compromised eyespots were similar to one another in size and in color.

Furthermore, we obtained three individuals in which an ectopic black element emerged between a PFE and an eyespot (Fig. 1H, I). The anterior eyespot retained its circular outline, and hence the ectopic black element was not likely to have been derived from deformation of the eyespot. The end point of the continuous black scales along the wing veins usually signifies the position of PFEs in normal color patterns. The ectopic black element was indeed located in the position signified by the end point of the black scales along the wing veins. To quantitatively evaluate this ectopic black element, we calculated the ratios between the parallel and perpendicular distances of elements to the proximal-distal wing veins for the anterior eyespots (0.95±0.18), PFEs (0.31±0.11), and ectopic black elements (0.15±0.08) located in the same compartment (shown as the mean±SD). The ectopic element was different from the eyespot at the 5% significance level (p=0.039), whereas it was not different from the PFE (p=0.25). Therefore, the ectopic black element was morphologically indistinguishable from the normal PFE. These results suggest that the ectopic black element can be considered as a PFE-like element.

Color-pattern analysis for the eyespot-PFE relationship

To gain insights into a possible relationship between eyespots and PFEs, we examined the natural and cold-shock-induced color patterns of *J. orithya* (Fig. 2). In non-treated female individuals (n=161), eyespots did not touch PFEs in most cases (70.2%; n=113), but it was not difficult to find cases where eyespots had physical contact with PFEs (29.8%; n=48) (Fig. 2A–F). Most of these color patterns (27.3%; n=44) looked as if the orange ring of the anterior eyespot was "ruptured" upon the physical interaction with the corresponding PFE (Fig. 2A, B). In rare cases (2.5%; n=4), the orange scales "spread" along the PFEs and were also found to a certain degree between the PFE and the submarginal band (SMB) (Fig. 2C, D). However, no "rupture" and "spread" of the outermost black ring were found. Intriguingly, we found a case in which the orange

Table 1. Results of the local application of inorganic salts by the sandwich method in *J. orithya*. Color-pattern changes in chemically treated wings were examined by visual inspection. The number of individuals in each category is given, followed by sex ratio in parentheses and percentage in square brackets.

Chemical Species	Successfully treated	No change	Eyespot enlargement	Eyespot reduction	Eyespot-like black spots	Ectopic PFEs
Sodium tungstate (Na2WO4, 1.0 M)	32(♂19,♀13)	9(♂7,♀2) [28.1%]	2(♂1,♀1) [6.3%]	21(♂11,♀12) [65.6%]	3(♂3,♀0) [9.4%]	3(♂3,♀0) [9.4%]
Sodium chloride (NaCl, 1.0 M)	40(♂20,♀20)	32(♂15,♀17) [80.0%]	5(♂2,♀3) [12.5%]	3(♂3,♀0) [7.5%]	0	0
Lithium chloride (LiCl, 1.0 M)	38(♂23,♀16)	17(♂10,♀7) [44.7%]	12(♂7,♀5) [31.6%]	9(♂6,♀3) [23.7%]	0	0
Water, distilled (H2O)	30(♂14,♀16)	14(♂6,♀8) [46.7%]	5(♀1,♀4) [16.7%]	11(♀7,♀4) [36.7%]	0	0

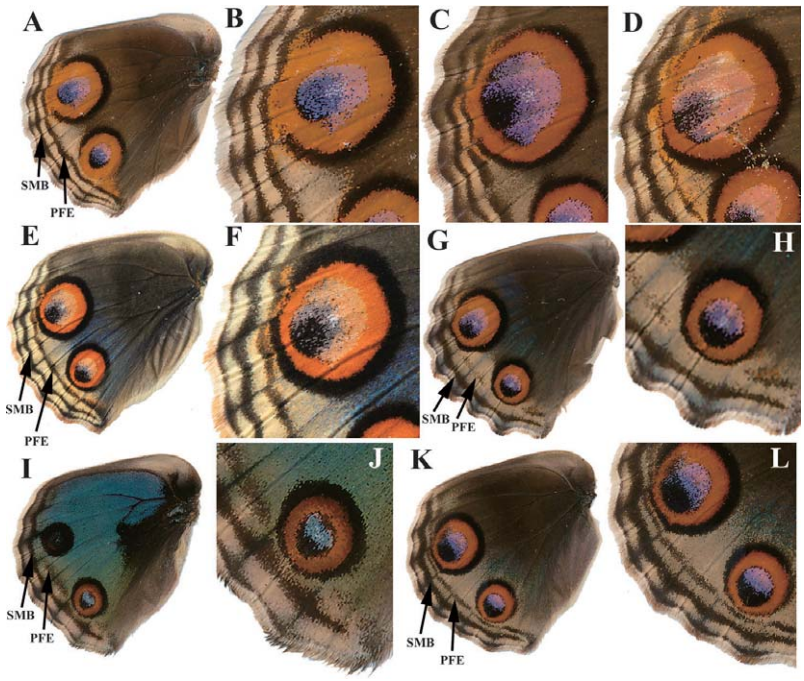

Fig. 2. Natural and cold-shocked dorsal hindwing color patterns in *J. orithya*. **(A)** An entire hindwing with relatively large eyespots. The anterior eyespot touches the PFEs. **(B)** Higher magnification view of part of A. Orange scales are evident along the PFEs. **(C)** Anterior eyespot of a different individual with orange scales along the PFEs. A small number of orange scales are evident between the PFEs and SMBs. **(D)** The anterior eyespot of yet another individual with a similar phenotype. **(E)** An entire hindwing with relatively large eyespots showing orange scales along the PFEs. Note that there are orange scales along the PFEs in proximity to either the anterior or posterior eyespot. **(F)** Higher magnification view of part of E. Note that orange scales are superimposed on the outermost black ring of the anterior eyespot. **(G)** Entire hindwing of a cold-shocked female individual. The SMBs are thick and the PFEs are blurred. The PFEs appear to be dislocated toward the eyespots. In the wing compartment having the posterior eyespot, a PFE is absent due to occupation of the compartment by the eyespot. **(H)** Higher magnification view of part of G. **(I)** Entire hindwing of a cold-shocked male individual. The PFE is completely absent in the wing compartment having the posterior eyespot due to occupation of the compartment by the eyespot. **(J)** Higher magnification view of part of I. **(K)** Another cold-shocked female individual. The posterior eyespot "pushes away" the PFE. **(L)** Higher magnification of part of K.

scales were distributed along PFEs but were simply superimposed on the outermost black ring (Fig. 2E, F). Notably, in this individual, the orange scales along the PFEs were distinguishable from the orange ring of the eyespot, and it is evident that these orange scales were superimposed on the eyespot in this wing. Therefore, it appears that the orange scales along PFEs are not produced from the "ruptured" eyespot and that they may be induced when an eyespot has physical contact with a PFE.

In the cold-shocked individuals (n=54), the eyespots were superimposed on PFEs (n=26; 48.1%) (Fig. 2G–L), in contrast to the natural eyespots discussed above. PFEs appeared to be "blocked" or eliminated by eyespots. Similar cases were occasionally found in individuals treated with tungstate by the sandwich method (see Fig. 1 and Discussion).

DISCUSSION

Direct action of tungstate and limited applicability of the sandwich method

In this study we observed a statistically significant pharmacological effect of sodium tungstate on butterfly wing color pattern following local tungstate application by the sandwich method. Eyespot size reduction was observed only on the treated side in the majority of treated individuals. In addition, the tungstate application produced other local effects, i.e., circular dislocated PFEs and ectopic PFE-like elements. These results demonstrate that tungstate acts directly on wings.

Considering that the wing surface is likely to be water resistant even immediately after pupation, the fact that the color-pattern changes can be induced by the sandwich method may be somewhat surprising. The induction is likely to be due to the direct penetration of tungstate into the wing tissue, because we observed characteristic pattern changes induced by tungstate even on the ventral surface of the treated hindwings in addition to the dorsal surface. Although substances of high molecular weight such as DNA and protein may not be able to penetrate into the wing tissues, relatively small hydrophobic substances such as ecdysteroids could be applied directly on wings by the sandwich method.

For comparison, we locally applied sodium chloride, lithium chloride, and water. Unfortunately, we also observed a certain degree of eyespot reduction and enlargement with these treatments. The origin of these modifications may be attributed to osmotic pressure and is not of a pharmacological nature per se. This relatively high noise limited the general applicability of the sandwich method. Moreover, the sandwich method requires operational accuracy, because we obtained disrupted color patterns with less accurate operations (not shown). Because these operational artifacts were easily introduced, careful and skillful manipulations are necessary to be able to correctly interpret the resulting modified color patterns.

Lithium chloride is known as an inhibitor of the important developmental transcription factor Distal-less in *Xenopus* embryos (Kao et al., 1986; Beanan et al., 2000). In butterflies, Distal-less has been proposed to play a role in determining the focal site in wing compartments with an eyespot, because it is expressed at prospective eyespot foci but is not expressed at imaginary foci in compartments lacking an eyespot (Carroll et al., 1994; Brakefield et al., 1996; Reed and Serfas, 2004). In this study, the pharmacological effect of lithium chloride was not clear. We do not know whether its null effect is due to limitations in our methodology. It should be noted that systemic injection of lithium chloride also did not result in color-pattern changes (Otaki, 2007).

Local effect of tungstate and heterochronic uncoupling model

We have reported unique color patterns induced by the local application of tungstate by the sandwich method. We found that the systemic and local effects are quantitatively different. The systemic treatment mostly affects PFEs to a greater degree than eyespots, although both PFEs and eyespots are modified simultaneously to some extent (Otaki, 2008a). Local treatment affects eyespots more than PFEs, at least on the dorsal hindwings, as shown in this paper, although we obtained the circular dislocated PFEs and ectopic PFE-like black elements by the sandwich method. It is reasonable to think that this difference originates from the tungstate concentration difference in the wing tissue. Since the difference is quantitative and not qualitative, both local and systemic effects may be interpreted using the same mechanistic model. However, it is also important to recognize that the sensitivity difference between eyespots and PFEs at different concentrations of tungstate may stem from a possible sensitivity difference in morphogens or their secreting cells.

We think that the circular dislocation of PFEs around the imaginary eyespot foci in the compartments lacking an eyespot is produced by the combined effect of local and systemic actions of tungstate. Systemic effects, which were clearly observed on the non-treated wings, were unavoidable to some extent even if tungstate was applied by the sandwich method. In treated wings, the normal eyespots were reduced in size and became similar to the ectopic black dots in size and in color. Hence, the distinction between PFE and eyespot becomes rather difficult, which may suggest that both the eyespot and PFE originate from the eyespot foci. This idea is consistent with previous experimental and comparative analyses (Otaki, 2008a, 2009).

Our observation that an ectopic PFE-like element was produced between the normal PFE and eyespot is not trivial. To our knowledge, this is the first report of the artificial induction of such an element. The induction of this element is consistent with the notion that the location of a PFE is not determined by a signal from its own organizing center. In other words, there is no primary organizing center at the center of each PFE (Otaki et al., 2005b; Otaki, 2008a, 2009). Note that it is impossible to create ectopic eyespots or to dislocate the normal eyespot foci pharmacologically, probably because a primary organizing center exists at each focus, whose location is determined before pupation.

In light of the heterochronic uncoupling model, the reduced eyespots and circular dislocated PFEs may be interpreted as "captured" images of the immature signaling stage when the eyespot signal and the PFE signal were still expanding from the prospective and imaginary foci, as speculated in Otaki (2008a). In other words, either the reception step in the treated individuals was carried out earlier than in the normal individuals or the signaling step in the treated individuals was delayed, resulting in the development of elements based on premature signals (Otaki, 2008a). The local application in the present study yielded smaller, relatively simple eyespots with fewer PFE modifications, which is more consistent with a delay (or slow onset) of the signaling step rather than an acceleration of the reception step. It seems reasonable to think that the cold shock simply delays the signaling step, whereas the heat shock simply accelerates it (Otaki, 2007). The delay of the signaling step relative to the reception step may be associated with weakened organizing activity due to the direct inhibitory action of tungstate on organizing centers or morphogens.

Eyespot-PFE relationship

Examination of the natural color patterns of *J. orithya* showed that in some females the anterior eyespot physically touches the corresponding PFE and competes with the PFE for the two-dimensional space. The orange scales were found along the PFEs in proximity to the eyespots in these cases, and they were superimposed on the black and orange rings of eyespots. This superimposition suggests that the PFEs were determined first and the eyespots were determined subsequently. Moreover, the fact that the orange scales were found only when the eyespots were large enough to have physical contact with PFEs suggests that the orange scales are likely to be the product of the eyespot-PFE interaction. Interestingly, similar orange scales along PFEs were found in other species of the genus *Junonia*, such as *J. coenia* and *J. evarete* (see Otaki et al., 2005a). In addition to passive interactions such as fusion of two adjacent eyespots, dynamic and active elemental interactions such as the orange scales along PFEs may play an important role in diversifying butterfly color patterns. Such a consideration may be necessary to "derive" a real butterfly color pattern from the nymphalid groundplan.

In contrast, in individuals treated with cold shock under specific conditions (i.e., one day after pupation at –2°C for 3.5 days), the posterior eyespot tended to block the inner dislocation of the PFE. In more extreme cases, the prospective position of the dislocated PFEs was completely occupied by the posterior eyespot. This is because the PFE signal is affected to a greater extent than the eyespot signal by this treatment. It is likely that the eyespot signal first occupied the prospective position, and subsequently the PFE signal reached there, but the immature scale cells had already committed to differentiate into an eyespot. This sequence of events is opposite from that of the natural color patterns discussed above.

A similar relationship was observed in the locally treated wings. As shown in Fig. 1D, PFEs were dislocated toward the real and imaginary eyespot foci on both the right and left wings due to the systemic effect of tungstate. On the right wing, however, the large posterior eyespot blocked the inner dislocation of the PFE. On the left wing, this blockage did not occur because of the smaller eyespot size induced by tungstate. On the other hand, as shown in Fig. 1H, the distal part of the reduced eyespot was cut off, probably by the superimposition of the corresponding PFE.

Considering these independent behaviors of PFEs and eyespots, i.e., superimposition and mutual exclusion that can be reversed by cold-shock treatment, the developmental determination process is difficult to explain using a single-morphogen model for both eyespot and PFE. Two different and independent morphogens, one for the eyespot and the other for the PFE, may be better able to explain these results. The quantitatively different sensitivity of PFEs and eyespots to systemic vs. local tungstate treatment is also consistent with a two-morphogen model.

According to the two-morphogen model, the PFE signal precedes the eyespot signal in the normal process of color-pattern determination. Because the two-morphogen model is necessarily heterochronic between two signals, this model is compatible with the heterochronic uncoupling model for the TS-type changes. In addition, the existence of PFEs in the eyespot-less compartments in *Junonia* butterflies (such as *J. orithya*, *J. almana*, and *J. coenia)* can be easily explained by this two-morphogen model. The PFE signal is released from the imaginary focus, but only a limited amount of the eyespot signal is released from the same focus in a compartment lacking an eyespot.

Taking the two-morphogen model into account, a simple solution for the PFE paradox is that the PFE signal is not like a conventional concentration gradient. Instead, the PFE signal may have a wave-like character. That is, once released from the real and imaginary eyespot foci, the PFE signal can autonomously propagate and does not depend on the activity of the source. For example, direct cell-to-cell interaction could serve as a slow autonomous signal. The eyespot signal is source-dependent, and hence the signals for eyespots and PFEs are fundamentally different in this model. Another solution is that the PFEs are produced not from the eyespot foci but from the marginal organizing centers, at least in *J. orithya*, and somehow interact with the foci to determine their relative position in the wing compartment. At this point, we do not prefer the latter model because it has not been reconciled with previous color-pattern analyses (Otaki, 2009).

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