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Spatial patterns of correlated scale size and scale color in relation to color pattern elements in butterfly wings

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ABSTRACT

Complex butterfly wing color patterns are coordinated throughout a wing by unknown mechanisms that provide undifferentiated immature scale cells with positional information for scale color. Because there is a reasonable level of correspondence between the color pattern element and scale size at least in *Junonia orithya* and *Junonia oenone*, a single morphogenic signal may contain positional information for both color and size. However, this color–size relationship has not been demonstrated in other species of the family Nymphalidae. Here, we investigated the distribution patterns of scale size in relation to color pattern elements on the hindwings of the peacock pansy butterfly *Junonia almana*, together with other nymphalid butterflies, *Vanessa indica* and *Danaus chrysippus*. In these species, we observed a general decrease in scale size from the basal to the distal areas, although the size gradient was small in *D. chrysippus*. Scales of dark color in color pattern elements, including eyespot black rings, parafocal elements, and submarginal bands, were larger than those of their surroundings. Within an eyespot, the largest scales were found at the focal white area, although there were exceptional cases. Similarly, ectopic eyespots that were induced by physical damage on the *J. almana* background area had larger scales than in the surrounding area. These results are consistent with the previous finding that scale color and size coordinate to form color pattern elements. We propose a ploidy hypothesis to explain the color–size relationship in which the putative morphogenic signal induces the polyploidization (genome amplification) of immature scale cells and that the degrees of ploidy (gene dosage) determine scale color and scale size simultaneously in butterfly wings.

1. Introduction

Complex and diverse butterfly wing color patterns are believed to arise through modifications of a basic color pattern model called the nymphalid groundplan (Schwanwitsch, 1924; Süffert, 1927; Nijhout, 1991, 2001; Otaki, 2009, 2012a). The nymphalid groundplan comprises 3 major symmetry systems (the central, basal, and border symmetry systems) and 2 additional peripheral systems (the wing root and marginal band systems) (Otaki, 2012a). Each unit of a single symmetry system has a core element at the center and a pair of paracore elements on both the proximal and distal sides of a core element (Otaki, 2012a). The developmental mechanisms of these 5 systems are likely similar (Otaki, 2012a; Taira et al., 2015).

Among color pattern elements, eyespots are perhaps most conspicuous; they are the core elements of the border symmetry system. Transplantation and physical damage experiments have shown that a prospective eyespot focus functions as a source of morphogen for the eyespot (French and Brakefield, 1992, 1995; Nijhout, 1980a, 1985, 1991; Brakefield et al., 1996; Otaki et al., 2005; Otaki, 2011c). The prospective eyespot foci correspond to the areas of expression of some genes such as *Distal-less*, *Notch*, *engrailed*, and *spalt* during late larval wing development (Carroll et al., 1994; Keys et al., 1999; Monteiro et al., 2006, 2013), although the function of these genes in the context of eyespot development is not well understood.

According to the concentration gradient model for positional information, a putative morphogen diffuses from an organizing center and forms eyespot patterns based on the concentration gradient of morphogen molecules and based on thresholds for morphogen concentration that are predetermined in immature scale-building cells (or simply, scale cells) (Nijhout, 1978, 1981, 1991). However, this model is intended to explain an ideal concentric eyespot ring, and actual butterfly eyespots are too complex to be

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explained by this simple model (Otaki, 2011a). As an alternative model, the induction model has been proposed based on observations of actual diverse butterfly eyespot patterns (Otaki, 2011b). Thus, the induction model is more capable of explaining actual diverse butterfly wing color patterns and damage-induced and temperature-induced color pattern changes than is the gradient model (Otaki, 2011c, 2012a,b; Taira et al., 2015; Iwata et al., 2013, 2015). The induction model is mechanistically based on repetitive and nested pattern generators that enhance a nearby signal and inhibit a distant signal simultaneously, similar to a reaction–diffusion model (Otaki, 2011b, 2012b).

Although it is difficult to identify a morphogen for color patterns by simple observational studies, a hint of the mechanisms of color pattern determination may be obtained by examining scale size distribution patterns. A few studies have shown the proximodistal size gradient of scales in lepidopteran insects (Kristensen and Simonsen, 2003; Simonsen and Kristensen, 2003) and a correspondence between scale color and scale microstructure (Jansen et al., 2001). The proximodistal size gradient of scales is likely generally observed in Lepidoptera (Kristensen and Simonsen, 2003; Simonsen and Kristensen, 2003). However, spatial scale color–size relationships on a single wing were first discovered by Kusaba and Otaki (2009), in which a reasonable correspondence between scale color and scale size was detected in *J. orithya* and *J. oenone*. Scale size distribution analyses have revealed a size gap between the black and non-black rings of a single eyespot and that eyespot foci have scales that are larger than their surroundings (Kusaba and Otaki, 2009). We also found a proximodistal size gradient in *Vanessa cardui* and *Danaus chrysippus*, although the gradient of the latter species was small (Dhungel and Otaki, 2014). Based on these data, we proposed that a morphogenic signal that is released from a prospective eyespot focus determines scale size as well as color (Kusaba and Otaki, 2009) and that this signal is a train of wave-like pulses, as described in the induction model (Otaki, 2011a–c). Because scale size is probably determined by the size of scale cells, and because cell size can be altered by cell ploidy, we speculated that morphogenic signals for scale size and color may be identical to ploidy signals (Kusaba and Otaki, 2009; Dhungel and Otaki, 2014). From this perspective, the characterization of scale cell development is required to understand the nature of the putative morphogenic signal. Although scale cell development has been observed in some studies (Nardi and Magee-Adams, 1986; Cho and Nijhout, 2013; Iwata et al., 2014; Ohno and Otaki, 2015a), basic information on developmental morphological changes remains scarce, and exact relationships and interactions between scale size and scale color have been reported only in *J. orithya* and partially in *J. oenone* (Kusaba and Otaki, 2009).

To test if the scale color–size relationship is a general feature in butterflies and to understand the relationship more deeply, in the present study, we mainly focused on scale size distribution on the hindwings of the peacock pansy butterfly *J. almana* (Nymphalidae, Nymphalinae). This butterfly provides us with an excellent system to investigate wing color pattern determination for the following reasons: (1) it has distinct large eyespots with sharp color boundaries, (2) it has a relatively large orange background area without a color pattern element, (3) it is useful for physiological and damage experiments (Otaki, 2007, 2011c), (4) it has a relatively large wing size resulting in high-resolution analysis (Otaki, 2007, 2011c), and (5) its scale size can easily be measured under a digital microscope because its wing surface is not hairy. For comparison, we also used the dorsal hindwings of other 2 nymphalid species, *V. indica* and *D. chrysippus*, which do not have eyespots. We establish here the relationship between scale color and scale size in nymphalid butterflies, which is probably applicable to other butterflies in general. We also discuss the nature of morphogenic signal for scale color and scale size based on the present and previous results.

2. Materials and methods

2.1. Butterflies

Three species of nymphalid butterflies were used for scale size measurements in this study: *J. almana* (Nymphalinae), *V. indica* (Nymphalinae), and *D. chrysippus* (Danainae). Adults or larvae were collected on Okinawa-jima Island or Ishigaki-jima Island of the Ryukyu Archipelago, Japan. Eggs were obtained from field-caught adult females. Larvae were fed their natural host plants at approximately 27 °C. Adults were frozen immediately after eclosion to avoid physical damage to their wings.

We mainly focused on the hindwing of *J. almana* (Fig. 1), which is phylogenetically related to *J. orithya* (Kodandaramaiah and Wahlberg, 2007). This species has a relatively large eyespot called the major eyespot in the R_s and M_1 compartments. The scale size of *V. indica* and *D. chrysippus* was also measured for comparison; these species do not have any eyespot. Color pattern elements and their components were defined as shown in Figs. 1 and 3, based on the terminology of Nijhout (1991), Scott (1986) and Otaki (2007).

2.2. Scale observations and measurements

We observed scales under a Keyence VHX-1000 digital microscope with or without a polarization illumination adaptor (Osaka, Japan). Scale measurements on hindwings were made following the protocol established by our previous study (Kusaba and Otaki, 2009). Briefly, we measured the maximum width of cover scales as scale size (see Fig. 1A). In some exceptional regions where identification of cover scales from ground scales was difficult, scales that were measurable in our system were considered cover scales. For the wing-wide measurements, 5 scales were selected to measure the scale size every 1 mm from wing base to wing margin along straight lines. The raw datasets of 5 scales were averaged as the scale size of that particular location. These measurements were made from 3 individuals per species. To examine a general size distribution pattern for each species, the data from 3 individuals were combined and normalized to 1.0 at 0 mm (at the wing base). In addition, 5 scales were selected for measurements of every scale row within the eyespot and marginal areas. For this analysis, 2 individuals were used per species.

2.3. Physical damage experiment

Only one side of the wings of *J. almana* was damaged with a stainless needle (0.50 or 0.65 mm in diameter) within 18 h postpupation to induce an ectopic eyespot. The contralateral wing was left untouched and used as an internal control. The operated pupae were kept at approximately 27 °C. The operated adults were readily frozen immediately after pupation, and 11 individuals that had ectopic color patterns were subjected to morphological analyses for scale shape, color, and size using the digital microscope described above. A quantitative comparison of scale size between the treated and non-treated dorsal hindwings (right and left wings) in the same individual was made every 1 mm from the wing base to the wing margin along straight lines, as mentioned above, using 3 individuals. To make it easier to compare scale size distribution patterns between the right and left wings, these raw data were normalized so that the scale size at the starting point for measurements at 0 mm (at the wing base) was 1.0. We also examined 4 individuals that showed no or small color pattern changes. For this purpose, physical damage was applied to the basal location where ectopic induction of color patterns is known to be difficult (Brakefield and French, 1995).