

# Structural Variations in Wing Patterning of Seasonal Polyphenic *Melanitis leda* (Satyrinae)

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## Abstract

Seasonal polyphenism is a common phenomenon observed among members of the Lepidopteran subfamily Satyrinae. *Melanitis leda*, being a member of that subfamily, exhibits seasonal variation in terms of wing patterning. In butterflies, wing patterning is due to the nanostructural architecture of the scales, which reflects and refracts incident light, with or without the combination of pigments. The current scanning electron, fluorescence and optical microscope study divulge fine structural and signal changes that occur with different season in the scales of *M. leda* and give rise to the different wing pattern in butterfly. The structural and consequent signal changes are likely to be correlated with behavioural processes such as mate selection and escape from predation.

## Keywords

*Melanitis leda*, Wing Coloration, Polyphenism, Scales

## 1. Introduction

Among insects, Lepidoptera possess a wide dissimilitude in terms of wing patterning. The wing pattern is species specific and helps in inter as well as intra signal communication [1] [2]. Besides food uptake at the caterpillar stage, factors like temperature and humidity influence pigmentation and the nanostructural architecture of wing colour patterns in butterflies [3] [4] [5]. In pigmented butterflies, scales present in the upper lamina are of irregular structures and because of that cause random scattering of light, while the pigment inside the scales determines the colour via selective spectral filtering of the scattered light. Scale colouration produced by nanostructures is due to optical interference which enhances light reflection in a specific wavelength and suppresses complementary wavelengths, to generate a distinct colour [6] [7] [8]. Nanostructure

associated wing patterning has been reported in various species [2] [9]. In butterflies like *Papilio xuthus*, *Battus philenor* and other nymphaline species, scale structure and pigment expression are under enzymatic control [4] [10] and often environmental influences on hormone levels also bring out variations in butterfly coloration [11].

Environmental, *i.e.* seasonal variation in wing patterning in butterflies is referred as polyphenism [12] [13]. Tropical satyrid butterflies often exhibit seasonal variation in underside wing patterning. The dry form has reduced or almost no eyespots in comparison to the wet form [12] [14]. Although scales have a key role in pattern formation, the fine structural changes underlying polyphenism is not well understood. Furthermore, the effect of change in patterning in signal communication is also unanswered. The current study addresses the fine structural and signalling changes in wing scales of the tropical butterfly *Melanitis leda* in connection with the seasonally distinct morphs.

## 2. Materials and Methods

### 2.1. Sample Collection

More than 100 *Melanitis leda* were collected round the year (April 2015-April 2016) from the campus of NIT Rourkela, Odisha, India (22.25N, 84.90E). Wet and dry forms were stored in individual envelopes at 4°C throughout the experiment to avoid withering of scales. Out of the 100 butterflies, identical pattern and colour were handpicked for analysis, irrespective of the sex, as reported by Brakefield [15].

### 2.2. Scanning Electron Microscopy

Scale structure and arrangement was observed by scanning electron microscopy. Sections from each colour region of the eyespots were cut out using a scalpel and mounted on stub. The mounted samples were sputter coated with platinum and observed under JEOL JSM-6084LV scanning electron microscope. The samples were imaged in two different magnifications accounting for scale arrangement (200×) and architecture (5000×). Image J 1.48v software is used for the analysis of the distance between the ridges and area of 10 air pockets from different colored scales.

### 2.3. Optical and Florescence Microscopy

Eyespot regions were scanned under an optical microscope (Carl Zeiss, Germany/Axiotech, 100HD-3D). For fluorescence microscopy, sections of the respective wings were cut out using a scalpel and were pressed on a clean glass slide. The detached scales remained adhered on the surface of the slide which were then observed under red, green and blue filters of fluorescence microscope (Olympus IX71). UV signal of intact butterflies were imaged using UV light of Gel-doc system (Bio-Rad).

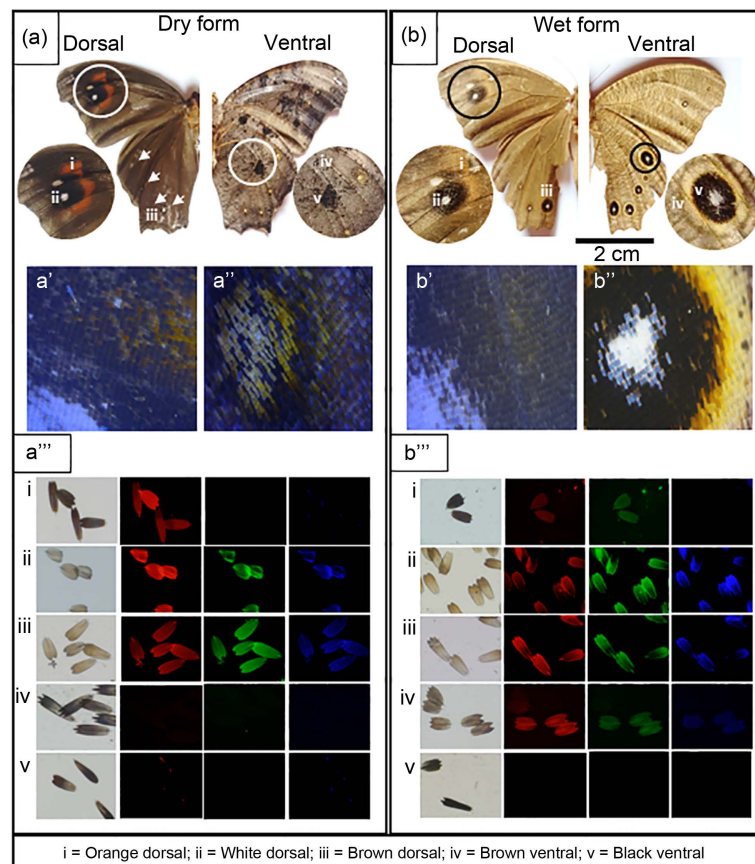
### 2.4. Statistical Analysis

The measurements obtained from the dry and wet form scales, bearing different colour are compared with unpaired student t-test with  $p < 0.05$  which is considered for the level of significance using Graph Pad Prism 6.0 software.

### 3. Results and Discussion

*M. leda* morphs of dry and wet seasons differed significantly from each other in terms of wing patterning. Dry season morphs are darker in comparison to the wet season morphs (**Figure 1(a)** and **Figure 1(b)**). The brown and black colouration of the wing is due to the presence of melanin [10]. Dark colouration of the dry form suggests a presence of greater amounts of melanin suppressing reflection and increasing absorption of more radiation (both UV and visible). In many cases, melanin further saturates the colour signals [4].

Besides colour, other significant differences include the presence/absence of various types of eyespot (so-called wing ocelli) in both forms. The dorsal side of the forewing in the dry morph has two ferrogenous lunules interiorly flanking black sub-apical spots with a white centre. This eyespot of the forewing is pale in the rainy season (**Figure 1(a)** (i, ii), **Figure 1(b)** (i, ii)). The dorsal side of the hind wing has two dark, white centred, fulvus-ringed ocelli in the wet form (**Figure 1(a)** (iii), **Figure 1(b)** (iii)) which are absent in the dry form. However, three or four miniature white spots appear in the



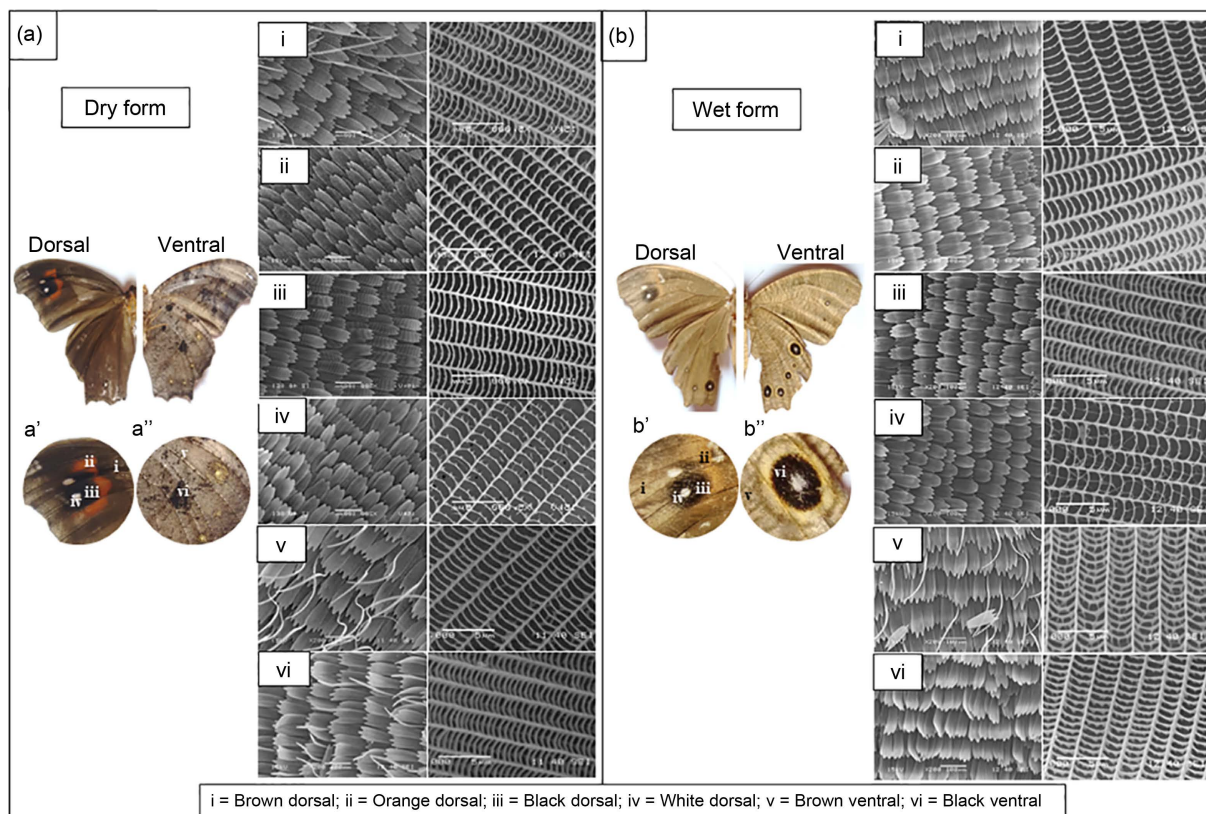
**Figure 1.** Polyphenism of *M. leda* depicting variations in wing patterning on dorsal and ventral side. (a) Dry morph, optical microscopy image of dorsal (a') and ventral (a'') eyespot, Fluorescence image of the marked scales (a'''). Arrowheads represent the presence of white spots on the dorsal side of hind wing; (b) wet morph, optical image of dorsal (b') and ventral (b'') eyespot, Fluorescence image of the marked scales (b''').

hind wing of the dry form (**Figure 1(a)**). The ventral side of the dry form is brown or greyish brown in colour, with occasional black patches and the ocelli are missing (**Figure 1(a)** and **Figure 1(b)**). In wet form, the underside is pale with dark brown striae followed by a series of marginal ocelli, four on the fore wing and six on the hind wing (**Figure 1(a)** and **Figure 1(b)**). The apical and sub-terminal wing spots are the largest.

The ocelli significantly fade with the change of the season. The role of the ocelli is to provide different signals for communication [16]. Now the question arises whether the signal value disappears together with the disappearance of the ocelli? To check the difference in signal, selected areas of eyespots of both dry and wet forms were investigated under the refraction microscope. Although the orange-patched eyespot of the dorsal forewing is more prominent in the dry form, there fraction-signalling pattern remains unaffected in either forms (**Figure 1 a', b'**). The eyespot on the ventral side of the wet morph reveals stronger signals under the refraction microscope (**Figure 1 a'', b''**). To investigate the signalling value further, all eyespots were observed for UV and fluorescent signalling. Like many other butterfly species, *M. leda* from wet form possesses more prominent UV signals than specimens from dry form (**Figure S1**) [17]. Scales from different regions were analysed under the fluorescence microscope (**Figure 1 a''', b'''**). In dry morphs, the orange patch displays a red fluorescence, where as specimens from the wet form exhibit both red and green fluorescence (**Figure 1 a'''(i), b'''(i)**). In either forms, the black patch does not appear to emit a colour signal while the white spot incorporates red, green, blue and UV signals (**Figure 1 a'''(ii, iii, v), b'''(ii, iii, v), Figure S1**). The dorsal eyespot patterning is required for mate selection while ventral patterning is aimed to prevent predation [18]. The disappearance of the green signal from the dorsal eyespot of the dry form suggests conservation of energy during the non-breeding season (**Figure 1 a'''(i), b'''(i)**).

Another significant difference is the disappearance of the wing ocelli in the ventral hind wing of the dry form. The ocelli of the wet-morphs possess all four colour signals (but lack the black ring) whereas in the dry form only a faint UV signal is found at the white spot region. The role of the ventral eyespot is to likely to be of value in protecting the butterfly from predation (e.g. birds). The eyespot of the wing gives a false eye-like impression, can startle a predator and allow the butterfly to escape [19]. In the season that fewer predators are around the eyespot is not needed and its disappearance (together with its signal) can be tolerated [19] [20]. Structural variations are observed in the eyespot as well as non-eyespot regions. Elongated scales are present in the non-eyespot region probably to strengthen the wings (**Figure 2**). The maintenance of the eyespot is likely to be an energetically costly phenomenon and thus to save energy its absence when not required could be advantageous. However, this hypothesis is yet to be tested since cold exposure during the pupal stage has been implicated in eyespot absences in the nymphalid *Aglais urticae*.

Butterfly wings are carpeted with scales, which produce colour due to the summed reflection of stacked scales present on either side of the wing (dorsal and ventral). To



**Figure 2.** SEM image 200 $\times$  and 5000 $\times$  magnification of scales from dry (a) and wet form (b). Note the cross-ribs present in images of higher magnification.

the structural arrangement of different colored scales were observed by scanning electron microscopy (Figure 2). Variations were observed among the scales in terms of ridge separation and the area enclosed by consecutive cross ribs that was statistically found to be significant when compared for dry and wet form and coloration (Figure S2). The trabeculae originating from the cross-ribs are highly oriented in black scales, slightly less in brown, but are highly branched or webbed in white scales. Similarly, the gaps between two consecutive cross-ribs are greater in white than in black, brown or orange scales. Based on comparisons between scales from dry and wet morphs, we can relate the structural coloration are directly related to the pattern formed in the wings from different scales.

The colourations produced in butterfly are mainly physical phenomena. All these minute variations contribute to bring changes in the wing colouration in both forms of the butterfly. Besides wing colour significant variations were observed in patterning of eyespots. *distal-less* is the gene responsible for the eyespot formation in butterflies [21]. A comparative study of *distal-less* expression profiles from dry and wet morphs of *M. leda* could help unravelling the question behind the polyphenism of *M. leda*.

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## Competing Interests

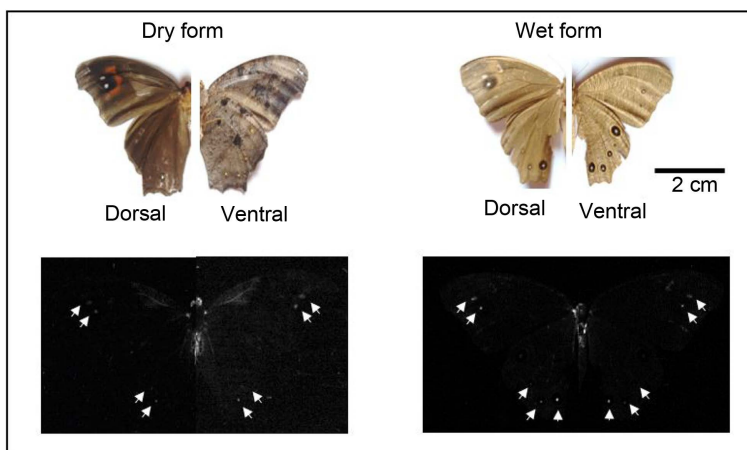
No competing interests.

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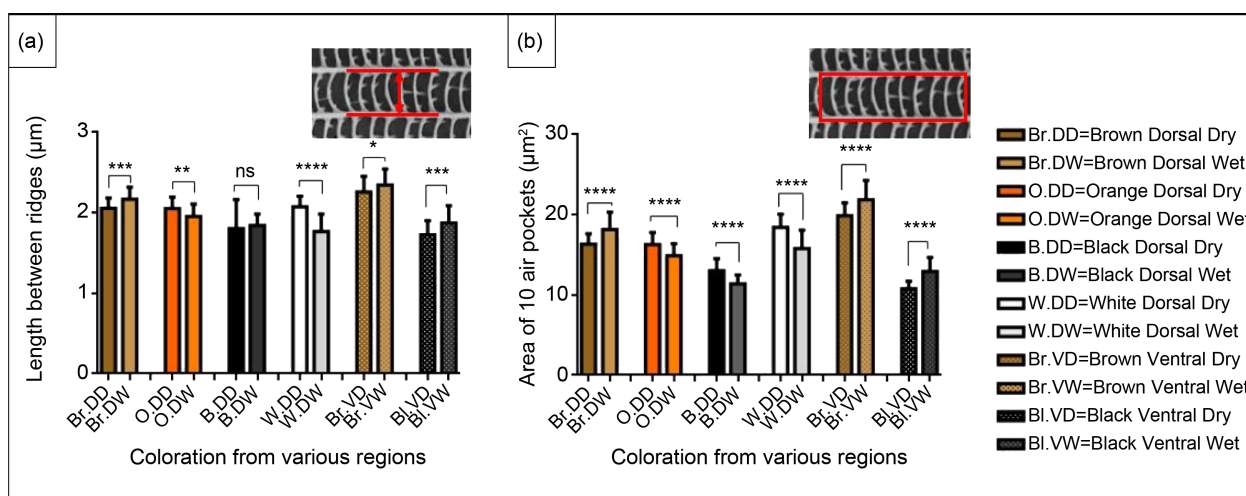
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### Supplementary Information



**Figure S1.** UV signals observed in dry and humid form of *M. leda*.



**Figure S2.** Comparative analysis of scales from various regions. (a) Distance between the ridges (b). Area covered by 10 air pockets. Significance for the results as compared with corresponding scales of the dry and wet form marked with asterisks (ns = non-significant, \*for P value = 0.038, \*\*for P value < 0.0010, \*\*\*for P value = 0.0001, \*\*\*\*for P-value of <0.0001).





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