

# High accuracy of color-generating nanoarchitectures is kept in lowland and mountainous populations of *Polyommatus dorylas* (Lepidoptera: Lycaenidae: Polyommatinae)

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## ABSTRACT

It is known that the size of the scales covering the surface of the Lepidoptera wings is in correlation with body size: larger species possess larger scales. However, butterfly individuals representing the various generations of the same species but differing in body size were not investigated in this respect. Similarly, the question whether different scale size may influence structural color generation based on nanoarchitectures in the scale lumen was never addressed. Populations of lowland (environment of Budapest, Hungary) and upland (Carpathian Mountains, Romania) *Polyommatus dorylas* were compared in terms of voltinism, wing and scale size, and the structural origin of blue coloration. Data analysis showed that the univoltine upland population exhibits a larger wing and scale size. On the other hand, the nano-morphology of the blue color-generating scales was identical when compared between univoltine and bivoltine populations. Coloration was also identical when measured with a spectrophotometer under ultraviolet and visible light. This high accuracy present in the male structural coloration suggests that it is controlled genetically. Body size alteration for enhanced thermal fitness has no influence on the fine structure of the nanoarchitecture present in the scale lumen.

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## 1. Introduction

The Blue butterflies, the tribe Polyommatini of the Lycaenidae, is one of the most typical day flying lepidopteran tribes in the northern hemisphere (Talavera et al., 2013; Vila et al., 2011). They are important members of habitats characterized by open landscapes in the Carpathian Basin (Szabó, 1956). In the wind-swept meadows, polyommatine lycaenid females spend most of their time feeding and basking at ground level. They find their mate(s) because of the special prezygotic strategy the tribe employs: females detect species-specific optical signals present on the dorsal wing surfaces of males. Females prefer brightly colored males (Imafuku and Kitamura, 2018). The male sexual signaling colors are species-specific and precisely tuned spectrally (Bálint et al., 2012). The physical background of this signal is provided by photonic nanoarchitectures located in the lumen of the scales (Schmidt and

Paulus, 1970; Tilley and Eliot, 2002; Wilts et al., 2009; Biró and Vigneron, 2010). Due to the role this color generation mechanism plays in the mating strategy, the physical color of the lycaenids is subjected to strict selection (Piszter et al., 2016), and exhibits a stress stability which by far exceeds the stability of the pigment-based pattern found on the ventral side of the wings (Kertész et al., 2017). Moreover, as our previous experiments revealed, the sexual signaling color of *Polyommatus icarus* (Rottemburg, 1775) exhibits an amazing spectral stability, both in space and in time (Kertész et al., 2019).

An important strategy in the adaptation to the environment is the regulation of generation numbers. For example, the widely distributed typical grassland species, *P. icarus*, may have a single brood in northern latitudes (Henriksen and Kreutzer, 1982) but produces up to four broods in regions close to the Mediterranean Sea (Tshikolovtes, 2011). Therefore, the voltinism of this species is highly influenced by various local conditions. When the environment turns less favorable, some of the populations are forced to reduce their brood number in order to adopt a more economical way to use the available resources for breeding. Because of this, the

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size of the imagines in populations with different number of generations can also differ in body weight and wing surface size, as previously documented for *P. icarus* (Nygren et al., 2008).

On the other hand, according to recent investigations in Lepidoptera, there is a strong correlation between the wing surface area, location and the size of the scales (Simonsen and Kristensen, 2003; Dhungel and Otaki, 2014). Here, one has to observe that in the case of photonic nanoarchitectures, a very close relationship exists between the geometric size of the wing scale nanoarchitectures at the nanometer level and the spectral properties, which provide the species-specific signals for polyommata lycaenids (Bálint et al., 2012). Therefore, it is very useful to investigate species in which the switching from two generations to a single generation is associated with an easily measurable change in body and wing size (Bálint, 1987; Nygren et al., 2008), and the consequences of the change in wing size, both in terms of the associated scale size and the eventual spectral modifications.

In this paper, we studied one polyommata species that is typically found in calciferous open landscapes in the Western Palearctic region: *Polyommatus dorylas* (Denis and Schiffermüller, 1775). In general, this species is bivoltine across most of its range (Tolman and Levington, 1997; Tshikolovtes, 2011), but there are records of univoltine populations, some of which have been discriminated taxonomically (Bálint, 1985, 1987). It appears that the univoltine populations of this species produce imagines with conspicuously larger wing surface areas as compared to bivoltine populations. We aimed to compare the wing surface and the color-generating scale sizes of populations with different voltinisms. We also investigated their spectral properties and, in particular, attempted to determine whether there is any correlation between the size of the scales and the number of generations and whether the spectral properties of the populations with different voltinisms are common or different.

## 2. Materials and methods

### 2.1. Species investigated

*P. dorylas* is distributed westwards from the Dnieper Plain throughout Central Europe to the Pyrenees, and southwards to the Balkans and Anatolia, and the Caucasus region (Tshikolovtes, 2011). For Central Europe in general, this species is documented as being bivoltine (Szabó, 1956; Slamka, 2004). In mountainous areas, towards the edge of its range, populations of this species are univoltine (Geiger, 1987; Lafranchis et al., 2015).

### 2.2. Populations and specimens examined

We sampled and examined a univoltine population of *P. dorylas* (*Magna*) living in the eastern Carpathians (Transylvania, Romania) and a bivoltine population of *P. dorylas* (first generation: *Dorylas I* and second generation: *Dorylas II*) inhabiting the Budapest region (Pannonia, Hungary) (Fig. 1A). The specimens are deposited in the Lepidoptera collection of the Hungarian Natural History Museum (HNHM) (Budapest). Samples are also held in the Nanostructures Department, Institute of Technical Physics and Material Science, Centre for Energy Research, Hungarian Academy of Sciences (Budapest). For more details of the specimens, please refer to supplementary material, Table S1 (Fig. 2).

### 2.3. Measurements of wing dimensions

In total, we examined 32 male specimens of *Magna*, *Dorylas I*, and *Dorylas II* ( $\Sigma = 96$ ). In order to measure wing size, we acquired digital images in the laboratories of the HNHM, which documented

all specimens on a millimeter-scaled graph paper background. Images were acquired with a Camedia C 7070 (Olympus, Tokyo, Japan) digital camera.

Using CorelDRAW X6 (Corel, Ottawa, Ontario, Canada) software, three measurements of the left forewing were taken: (1) the length of the forewing costa measured from the wing base (vein erection) to the apex (vein R2 terminus); (2) the length of the outer margin measured from the apex (vein R2 terminus) to the tornus (anal vein terminus) and (3) the length of the anal margin measured from the wing base (vein erection) to the tornus (anal vein terminus) (Fig. 1B). Using these three measurements we calculated the area between the white lines on Fig. 1A.

Then, using optical microscope images of the right forewing (taken with a 2.5 × objective), we marked an area of 1.96 mm<sup>2</sup> between the vein M3 and the Cubitus. We determined the number of blue cover scales in this area on wings of five randomly selected male exemplars of *Magna*, *Dorylas I*, and *Dorylas II*.

### 2.4. Optical microscopy

Optical imaging of the wing scales was carried out using an Axio Imager A1 microscope (Carl Zeiss AG, Jena, Germany) using reflected light. The wing scales usually stand at an angle of approximately 15° relative to the wing membrane, so for better visibility, we used focus stacking (Adobe Photoshop, Adobe systems Inc., CA, USA) to compensate for the narrow depth of field of the high-resolution microscope objectives.

Single scales were removed using a sharp needle, and placed on a microscope glass slide. The scales were inspected in reflected light, and we measured their maximum width and length using CorelDRAW X6.

### 2.5. Scanning and transmission electron microscopy (SEM and TEM)

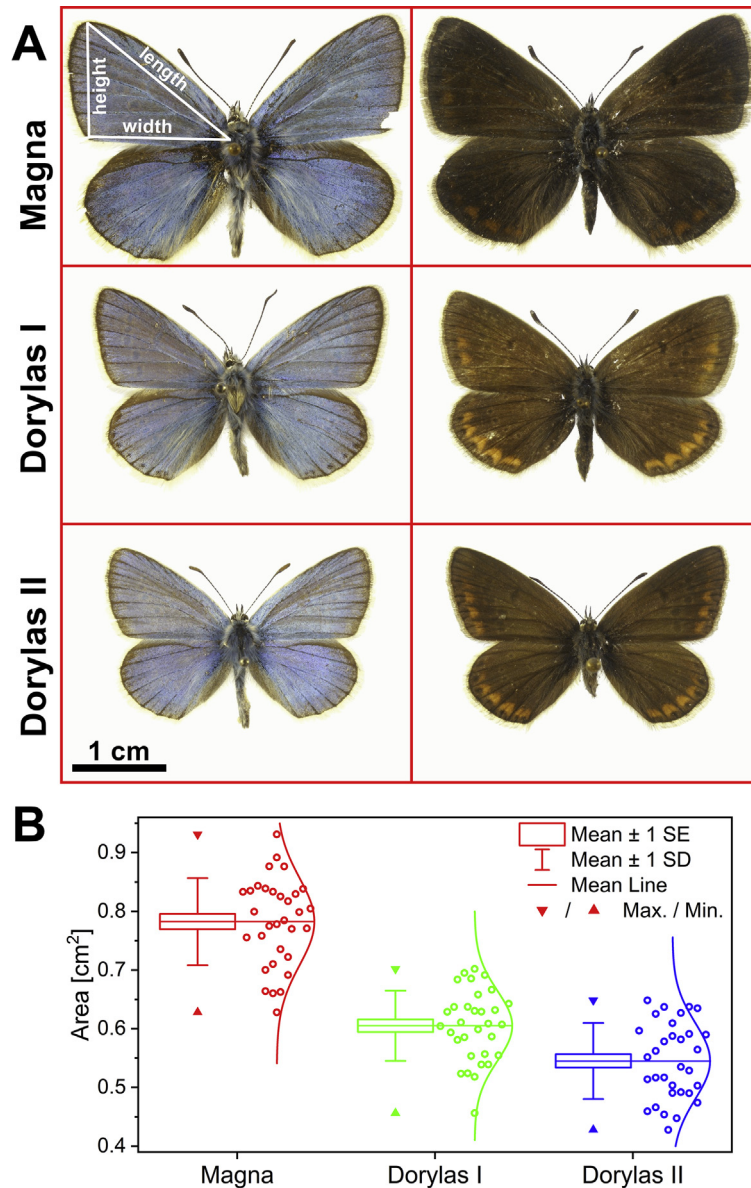
SEM images were taken using a LEO 1540 XB electron microscope (Carl Zeiss AG, Jena, Germany). Wing pieces were cut and mounted on a sample holder with double-sided conductive tape; single scales were also placed on conductive tape. To ensure that the original structure of the wing scales was preserved, no other treatment was applied. For TEM, a standard sample preparation procedure was performed: after fixing and dehydration, a few millimeters of wing pieces were embedded in Spurr's resin (SPI Supplies, West Chester, PA, USA) and slices of 70 nm were cut with an ultramicrotome. For better contrast, the sections were stained with uranyl acetate and lead citrate, and then inspected using a CM20 TEM apparatus (Philips, Eindhoven, Netherlands) operating at an accelerating voltage of 200 keV.

### 2.6. Spectroscopic measurements

For optical reflectance spectral investigation, we used a modular fiber optic spectrophotometer (Avantes Avaspec-HS1024 TEC, Apeldoorn, Netherlands) and a halogen - deuterium (Avantes AvaLight-DH-S-BAL) light source. All spectra were recorded against a white standard reference WS-2 (Avantes). For the measurement of the reflected specular component, we used a normal incidence probe, which combined the incoming illuminating fibers and outgoing detecting fibers.

### 2.7. Modeling and statistical analysis

The photonic nanoarchitectures of wing scales were modeled as chitin multilayers using the transfer matrix method (Pendry and MacKinnon, 1992; Yeh, 2005). The thickness of each layer in the



**Fig. 1.** Imagines of *P. dorylas* and the results of wing size measurements. (A) The left column shows males while the right column shows females. Upper row = univoltine *P. dorylas* (*Magna*) (Eastern Carpathians); middle row = first generation of bivoltine *P. dorylas* (*Dorylas I*) (Pannonian region); lower row = second generation of *P. dorylas* (*Dorylas II*) (Pannonian region). On the left forewing of a *Magna* male it is indicated in white how measurements were taken. (B) Area statistics for mountainous *Magna*, lowland *Dorylas I*, and *Dorylas II*. The boxes denote the standard error of the mean, while the bars show the standard deviation of the mean. Maximum and minimum values are shown as triangles. One-way ANOVA and post hoc Tukey's test evaluation showed significant differences between the three groups ( $p < 0.05$ ).

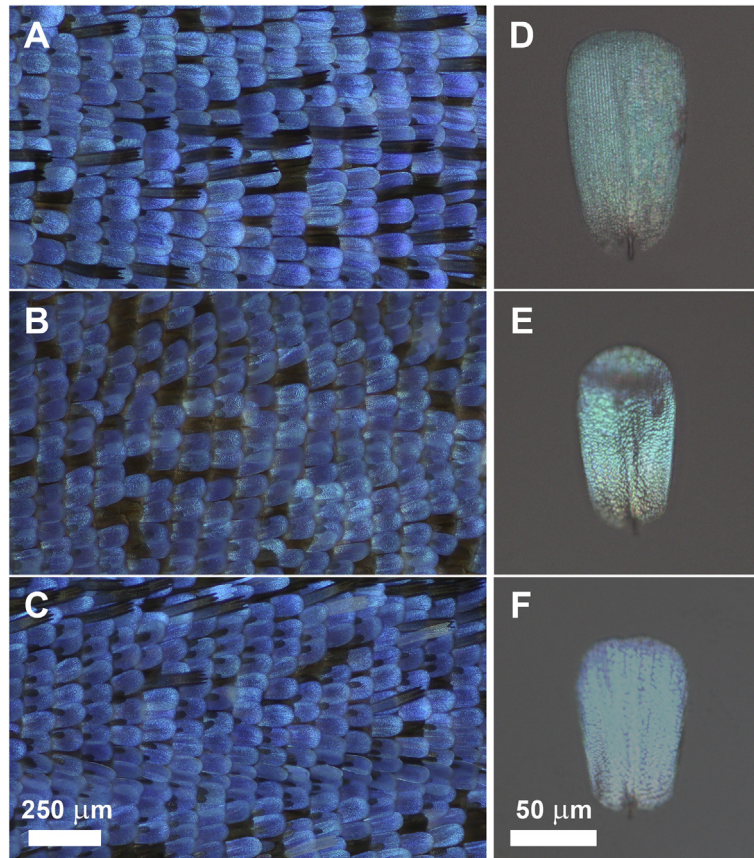
nanostructures was measured in cross-sectional TEM images using multiple points for both *Dorylas* and *Magna* specimens. These values were then averaged and used as an input in simulations where the reflectance spectra for normally incident light were calculated. The statistical analysis was carried out using Origin 2018 (Originlab, Radnor, PA, USA) software. The raw data of the wing size parameters were evaluated using One-way ANOVA supplemented with post hoc Tukey's test.

### 3. Results

#### 3.1. Wing surface areas

According to our measurements, the wing surface area of *Magna* and *Dorylas I-II* could be readily discriminated using the methods

we applied herein. The *Magna* forewing surface area was characteristically 0.63–0.93 cm<sup>2</sup> with mean area of  $0.78 \pm 0.013$  cm<sup>2</sup>, the *Dorylas I* had a wing surface area of 0.46–0.70 cm<sup>2</sup>, with mean area of  $0.61 \pm 0.011$  cm<sup>2</sup>. The range of the *Dorylas II* was 0.43–0.65 cm<sup>2</sup>, with a mean of  $0.55 \pm 0.011$  cm<sup>2</sup> (Fig. 1B). Neither *Dorylas I* nor *Dorylas II* reached a wing surface area of 0.7 cm<sup>2</sup>; their typical range was 0.42–0.70 cm<sup>2</sup>. Therefore, in quantitative terms, *Magna* and *Dorylas I-II* wing surface areas differed characteristically: this was also evident when using the naked eye to examine typical imagines (see Fig. 1A, in which each cell of the table is similarly sized) and when were analyzed using One-way ANOVA followed by Tukey's test which showed significant differences between all three groups of samples ( $n_{\text{total}} = 96$ ,  $p < 0.05$ ). Consequently, it appears that there is also a small, but significant difference in wing size between the two generations, as the first generation (*Dorylas I*) was 1.11 times



**Fig. 2.** Optical microscopic images of *P. dorylas* male forewing surfaces taken in the medial area below the cubital vein, along with individual scales from the same area. A and D = *Magna*, B and E = *Dorylas I*, C and F = *Dorylas II*.

larger than *Dorylas II*, on average, although the ratio of the mean forewing area between *Magna* and *Dorylas I* was 1.3.

### 3.2. Scale sizes and numbers

According to our observations on wing surface areas, the separate wing scale sizes were analyzed (Fig. 2). The dimensions (length and width) of the color-generating cover scales on *Magna* were found to be different from those in the two generations of *Dorylas I-II*. The average dimensions of *Magna* scales were 122 by 70  $\mu\text{m}$  ( $n = 24$ ), whilst those of *Dorylas I-II* were 105 by 59  $\mu\text{m}$  ( $n = 29$ ). Using One-way ANOVA we obtained significant difference ( $p < 0.001$ ) between the scale size of *Magna* and *Dorylas I-II* groups (Fig. 3B and C). According to our manual count, the number of color-generating scales in the examined area (1.96  $\text{mm}^2$ ) for *Magna* was  $443 \pm 22$ , but was  $519 \pm 12$  for *Dorylas I* and  $532 \pm 20$  for *Dorylas II* ( $n = 15$ ,  $p < 0.009$ ). This shows that the same physical area was covered by a smaller number of larger-sized scales in *Magna* specimens (Fig. 3A).

### 3.3. Nanomorphology and spectral properties

SEM and TEM images demonstrated that the nanomorphology of the bivoltine *Dorylas* and the univoltine *Magna* cannot be distinguished qualitatively at the micron level (Fig. 4), which is probably a general phenomenon for the subtribe (Bálint et al., 2007, 2012). Our measurements suggest that the nanoarchitectures occurring in the lumen of the scales were also identical in quantitative terms. The spectra of *Magna*, *Dorylas I*, and *Dorylas II* males

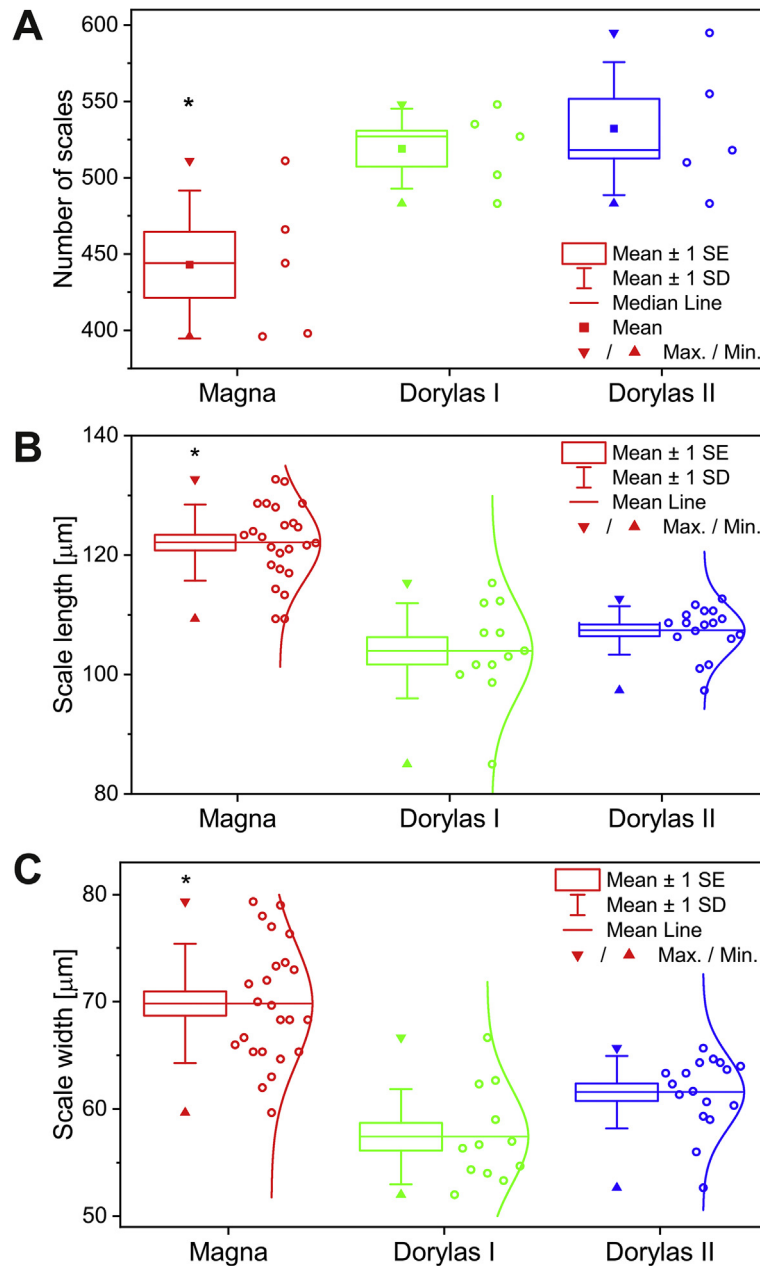
were identical in terms of the maximal blue reflectance, when plotted normalized to this peak (Fig. 5). There were no notable differences observed, and individual variations fell within the normal range displayed by other closely related species (Piszter et al., 2016) and showed a typical polyommata pattern (Bálint et al., 2007). All specimens produced a conspicuous peak within the visible blue range with very strong reflectivity at a wavelength of 440 nm. As already reported for *P. icarus*, older museum specimens exhibit a certain degree of melanin decomposition, which is manifested by an increased reflectance (black curve) on the red side of the spectrum (Kertész et al., 2019). It is worth emphasizing that for freshly captured individuals, the spectral curves of *Magna* and *Dorylas II* overlapped perfectly.

## 4. Discussion

### 4.1. Wing surface areas

The most characteristic difference that we could detect between *Magna*, *Dorylas I*, and *Dorylas II* was the wing surface area. On average, the ratio of wing area in *Magna* relative to the wing area of *Dorylas I* was 1.3 and 1.44 with respect to the wing area of *Dorylas II*. The ratio of wing surface areas in *Dorylas I* to *Dorylas II* was 1.11.

Larger wing surface area can be beneficial in two aspects: (1) in males, a larger wing surface size provides a more intense, and therefore a more visible optical signal, and (2) in females, a larger wing surface of brown coloration can collect more energy for imaginal activity, what is supplemented by oviposition (Biró et al., 2003). These remarks are supported by the fact that the flight



**Fig. 3.** Wing scale number and size results. (A) Number of wing scales counted for *Magna*, *Dorylas I*, and *Dorylas II* on a 1.96 mm<sup>2</sup> area. (B–C) Single wing scale length and width (n = 29). (A–C) The boxes denote the standard error of the mean, while the bars show the standard deviation of the mean. Maximum and minimum values are shown as triangles. One-way ANOVA and post hoc Tukey's test evaluation showed significant differences between the *Magna* and the other two groups (p < 0.001).

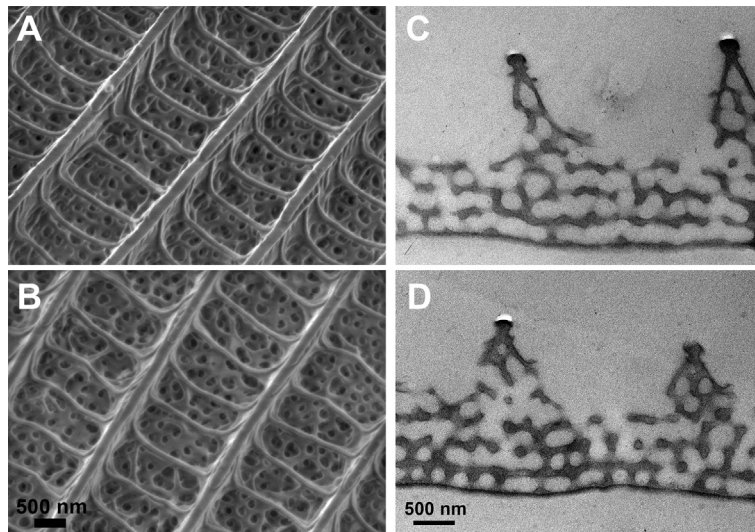
season (Fig. 6) of *Magna* is often interrupted by long rainy and cold periods; therefore, there is considerably less time available for the activity of the butterflies in the Eastern Carpathians than in the Pannonian Lowlands (Fig. 7). The average of daily sunshine hours between 13 May - 30 September in the years 2006–2018 period is  $9.51 \pm 0.68$  in the Budapest region (*Dorylas I-II* population), while  $8.26 \pm 0.73$  in eastern Carpathians (*Magna* population). This lower average of daily sunshine hours correlates with significantly (~25%) lower average temperature: in the Eastern Carpathians the averaged value for the periods 13 May - 30 September in the years 2006–2018 of the daily average temperature is 14.01 °C, whilst in the Budapest region it is 19.46 °C. Consequently, the activity of males in patrolling the habitat must be more intense; their signal therefore appears to be more obvious; also, females need to accumulate energy more efficiently during the short sunny hours as

their activity includes not only feeding and mating but also for searching suitable places for egg-laying.

Female *Magna* individuals are rarely observed, because they spend most of their time in the grass, close to the ground, collecting heat (Bálint, pers. obs.). In the HNHM collection, there are only six female specimens, which is not a sufficient number to statistically prove/disprove whether the females are indeed larger than males. However, on the basis of the available specimens, we are of the opinion that *Magna* females are also larger than those of bivoltine *Dorylas I-II* (Fig. 1A).

#### 4.2. Scale size and optical signals

Simonsen and Kristensen (2003) demonstrated that a larger wing surface area correlated well with longer scales. In order to

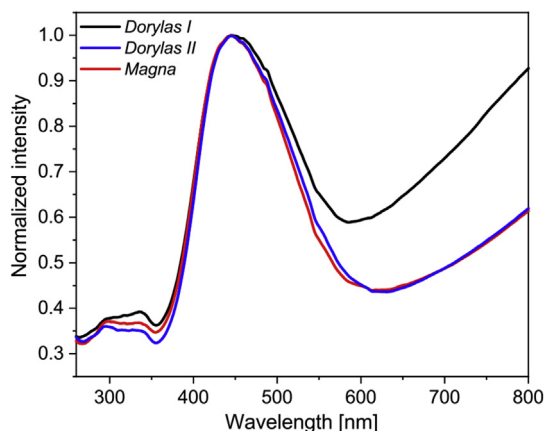


**Fig. 4.** Micrographs of *P. dorylas* scales. A, B = scanning electron micrographs, C, D = cross-sectional transmission electron micrographs. A and C = *Magna*, B and D = *Dorylas I*.

obtain this result, these previous authors assembled and analyzed a large amount of data from a variety of species. The intraspecific variability of scale and wing sizes was not addressed.

As we have shown in the present study, *Magna* scales exhibit different attributes compared to those of *Dorylas I-II*, thus suggesting that the result formulated by Simonsen and Kristensen (2003) was also valid in an intraspecific context. In spite of an increase in scale size in *Magna*, the internal architecture in the lumen of the scales has not changed. This resulted in identical spectral characteristics in all of the populations we investigated (Fig. 5).

Previous studies have shown that optical signals are important factors in prezygotic isolation activities, at least in day-flying Lepidoptera (Kemp and Rutowski, 2011; Imafuku and Kitamura, 2018). These signals are generated by species-specific nanoarchitectures located in the scale lumen; this feature is critical for sexual discrimination.



**Fig. 5.** Mean wing reflectance of univoltine *P. dorylas* (*Magna*;  $n = 10$ ), bivoltine *P. dorylas* first generation (*Dorylas I*;  $n = 9$ ), and second generation (*Dorylas II*;  $n = 8$ ) male specimens measured with an integrating sphere optical setup. The spectra were normalized to the maxima of the reflectance peaks in the blue wavelength region. One can observe that reflectance peaks of the structural blue colors are coincident. The portion of reflectance originating from melanin pigmentation ( $>600$  nm) in *Dorylas I* specimens was higher but there was no difference in the blue structural color.

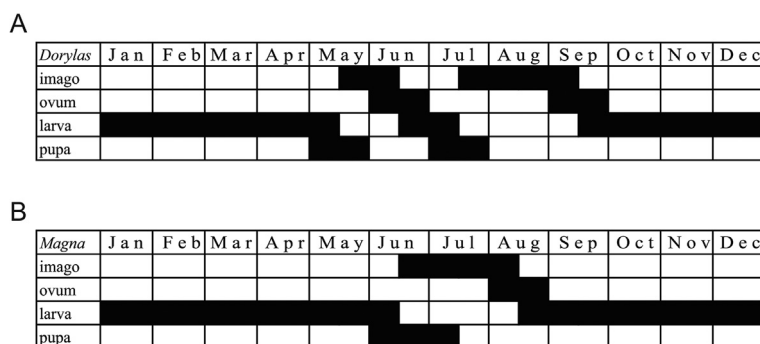
#### 4.3. Nanomorphology and spectral properties

Accordingly, the difference in male wing size does not influence the nanoarchitectures that produce the optical signals in the wing surface. This was clearly evident in our spectral data and further supported by SEM and TEM images (Fig. 4). Therefore, one of the most important species-specific characteristics remains unchanged, despite the alteration of the scale dimensions. Here, it is worth emphasizing that each scale is the product of a single cell (Greenstein, 1972) and the photonic nanoarchitectures are produced during the pupal stage by a complex process of self-assembly (Ghiradella and Butler, 2009; Iwata et al., 2014; Dinwiddie et al., 2014), which has yet to be fully elucidated. In our case, as shown by the length to width ratio of the scales, the scale morphology is affected to a certain extent, but the characteristic dimensions of the color-generating nanoarchitectures are strictly conserved.

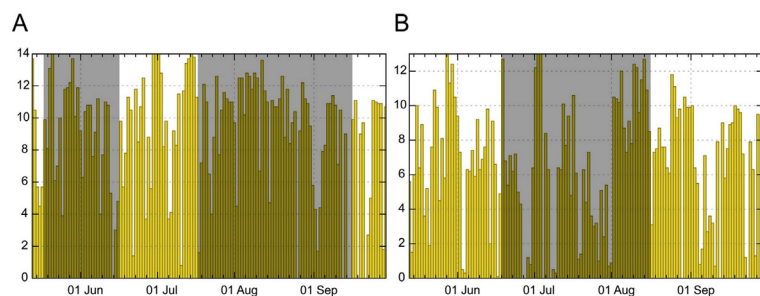
As our simple optical model (supplementary material, Fig. S1) has shown, an alteration of the order of 10 nm in the typical dimension of the scale nanoarchitecture would shift the maximal reflectance by at least 50 nm; this also leads to a simultaneous alteration in morphology at the micron scale. This indicates that the control mechanisms for scale size and shape and for photonic nanoarchitecture size are very likely different.

The body size and tissue size of an animal depend on genetic and environmental factors; however, in the case of *Magna* and *Dorylas I-II*, the same genetic information is expressed under particular environmental conditions. Due to the mountain climate, *Magna* exhibited readily detectable size differences, both macroscopically (wing size) and microscopically (scale size), but rigorously preserved the nanoscale dimension of the color-generating photonic nanoarchitectures.

As a matter of fact, this finding is less surprising if compared to the differences in the cold-stress resistance of sexual signaling colors and pigment-based cryptic patterns in *P. icarus* butterflies. We found that the sexual signaling colors of the males were only mildly, and randomly altered when pupae were subjected to a long duration (up to 60 days) of cooling at 5 °C, while the pigment-based patterns on the ventral wing surfaces were found to change dramatically with the duration of cold stress; the magnitude of the alteration also increased monotonically (Kertész et al., 2017). In further support of this theory, when rearing *P. icarus* butterflies under controlled conditions, we previously obtained individuals



**Fig. 6.** Life history diagrams of *P. dorylas* compiled on the basis of specimens preserved in the Hungarian Natural History Museum. A = bivoltine *Dorylas* population from the Pannonian Lowlands; B = univoltine *Magna* population from the Eastern Carpathians. In the opening columns, the developmental stages are indicated, months are represented in the first rows. The lengthened time for larval development of *Magna* is highly evident.



**Fig. 7.** Sunshine hours during the summer of 2018. Flight periods of the butterflies are indicated by gray blocks. A = bivoltine *Dorylas I-II* in Pannonia (in vicinity of Budapest), B = univoltine *Magna* in Transylvania (Eastern Carpathians). The far less number of hours with sunshine for *Magna* is highly evident (data source: weatheronline.co.uk).

much smaller than those in the wild, resulting in smaller wing surfaces with smaller scales (to be published).

## 5. Summary

Our experiments and observations demonstrate that size of wing surface area and the scales covering the wing membrane are subject to change in polyommata lycaenids. This phenomenon can be attributed for gaining fitness via the control of environmental variables by body size and temperature (Kingsolver and Huey, 2008). In contrast, the color-generating nanoarchitectures producing the male species-specific color located in the scale lumen cannot be altered significantly using variables provided by the environment. Therefore, it can be concluded that the color used for sexual signaling in males is rigorously controlled by the genome of the species and this is crucial for keeping the identity if the organism lives in a community of many closely related and similarly colored and patterned taxa (Bálint et al., 2012).

## Author contribution

ZsB, BLP conceptualization of the project and manuscript writing. GPK, KK, GP measurements. GP, KK data analysis, visualization. ZSEH electron microscopy. BLP, KK funding acquisition. All authors revised the manuscript and read and approved the final version.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.asd.2019.100887>.

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