Final Scientific Report

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Due to the reorganizing decided by the Hungarian Academy of Sciences (HAS) as of 1st January 2015 the MFA was transferred from the HAS Research Centre for Natural Sciences to the HAS Centre for Energy Research. Unfortunately, the financing for the present project for the year 2015 was received by the HAS CER only in November 2015, therefore in the first year, the financial resources for carrying out the planned work were extremely limited. This made impossible to carry out the work as planned.

1. Introduction

By the strict definition, a photonic crystal is a periodic nanoarchitecture in 3D of two non-absorbing media of different refractive indexes, which has such a period of the alternation of the two media, that in a certain wavelength range (forbidden gap) of the electromagnetic waves, no propagation is possible in any direction in the structure. On the other hand, in the past decade numerous papers reported not fully ordered photonic nanoarchitectures of biologic origin. These quasiordered, or disordered nanoarchitectures do not possess a full photonic band gap, but only a partial one. Still they are used by the living beings very efficiently in sexual communication and/or cryptic behavior. Moreover, often these nanoarchitectures are tuned to the conditions of the habitat.

We intended to reveal the differences of the structures and optical properties of related species living in different habitats and of not related species living in the same, or similar habitat. In this way from the point of view of physics we hope to relate the structural modification of a certain photonic nanoarchitecture with the ways in which it reflects in the modifications of the optical properties. On the other hand, from the point of view of biology we intended to reveal to what extent the conditions of a certain habitat induce photonic nanoarchitectures with similar/different structures and similar/different optical properties.

An additional research direction that got emphasis during our work is the stability-variability of the two kinds of colors: structural and pigment based, both in time and geographically. Associated to this, we also investigated the stress resistance of these two different colors.

2. Investigation of the structural color of two Lycaenid species living in the same type of habitat.

Structural coloration variability was investigated in two Blue butterfly species common in Hungary. The males of *Polyommatus icarus* (Common blue) and *Plebejus argus* (Silver-studded blue) individuals use their blue wing coloration for conspecific recognition. Despite living in the same type of habitat these two species display difference in prezygotic mating strategy: the males of *P. icarus* are patrolling, while *P. argus* males have sedentary behaviour. Therefore, the species-specific photonic nanoarchitecture which is the source of the structural coloration may have been subjected to different evolutionary effects. Despite the increasing interest for photonic nanoarchitectures of biologic origin, the studies focusing on the biologic variability of structural coloration when examining statistically relevant numbers of individuals from the same species, were lacking. The structural coloration of the four wings of 25-25 individuals captured in the same location, and in the same period (in total 100 samples for each species) were measured and compared. To be able to decide the most suitable ways of measuring the complex optical objects (the nanostructured wing scales with typical dimensions of 50 x 100 μm^2 form a kind of a "mosaic" on the wing surface), different measuring methodologies were used: a) a setup measuring primarily the normal reflectance; b) a setup using integration sphere measuring all the light reflected under any angle. Clearly the integrated measurement provides more reliable and operator independent data. On the other hand, usually it is not possible to dismember 50 museum samples for measurements. It was useful to see that, although with a somewhat larger scattering, but the normal incidence measurements yielded data consistent with the more rigorous integration sphere measurements. Significant differences were found in the near UV wavelength region which is perceptible by these polyommatine butterflies but invisible for human observers, but the peak position of the structural color of the species living in the same habitat is almost coincident. Difference in the intensity of structural coloration was also observed which is in accordance with the mating strategy of these insects: the experimentally brighter *P. icarus* males' patrolling behaviour requires more conspicuous wing coloration than the *P. argus* males' perching mating strategy where less intense blue colour was measured. The paper was published in Plos ONE [8].

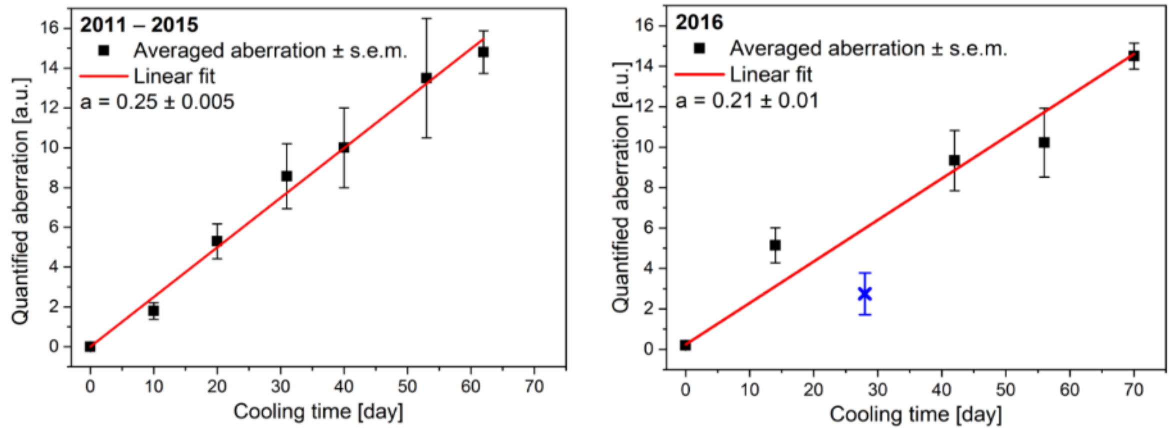
3. Rearing butterflies and the investigation of the stress-stability of the structural color and of the pigment-based pattern

The detailed evaluation of the *Polyommatus icarus* butterflies resulted from our own rearing experiments under controlled conditions, revealed very remarkable results which were published in the Nature family journal Scientific reports ([22] Krisztián Kertész, Gábor Piszter, Zsolt Endre Horváth, Zsolt Bálint & László Péter Biró, ***Changes in structural and pigmentary colours in response to cold stress in Polyommatus icarus butterflies***, Scientific Reports 7 (2017) 1118; DOI:10.1038/s41598-017-01273-7). While numerous papers have investigated the effects of thermal stress on the pigmentary colors of butterfly wings, such studies regarding structural colors are mostly lacking, despite the important role they play in sexual communication. To gain insight into the possible differences between the responses of the two kinds of coloration, we investigated the effects of prolonged cold stress (cooling at 5 °C for up to 62 days) on the pupae of *Polyommatus icarus* butterflies. To carry out this experiment, we had to produce ourselves the pupae subjected to prolonged cooling as the time elapsed from the pupation to the starting of the cooling proved to be critical. The wing surfaces colored by photonic crystal-type nanoarchitectures (dorsal) and by pigments (ventral) showed markedly different behavior. The ventral wing surfaces exhibited stress responses proportional in magnitude to the duration of cooling and showed the same trend for all individuals, irrespective of their sex. On the dorsal wing surface of the males, with blue structural coloration, a smaller magnitude response was found with much more pronounced individual variations, possibly revealing hidden genetic variations. Despite the typical, pigmented brown color of the dorsal wing surface of the females, all cooled females exhibited a certain degree of blue coloration. UV-VIS spectroscopy, optical microscopy, and scanning and transmission electron microscopy were used to evaluate the magnitude and character of the changes induced by the prolonged cold stress. We found that the blue coloration of the females is produced by scales with similar optical properties and similar nanoarchitecture as the blue scales of the males. In the same time, in the case of the dorsal wing surface of the males the major effect was the disordering and mixing of the ordered layers of blue cover scales (upper layer) and of the brown ground scales (lover layer). The results were presented as plenary, invited and oral talks in several international conferences, too [15, 20, 21, 23]. This work also generated a remarkable media attention in Hungary: the main findings were presented on the homepage of the Hungarian Academy of Sciences, in the Index web portal, in a scientific broadcast of the Kossuth Radio, and in two TV presentations (for details see: http://www.nanotechnology.hu/magyarul/2017/2017_07_13_lepke_mta.pdf

http://www.nanotechnology.hu/magyarul/2017/2017_07_23_lepke_szonda.mp3

https://www.youtube.com/watch?v=4-vXy0WtrTk&feature=youtu.be)

Before the termination of the project, we checked ourselves the reproducibility of our result. We found a remarkable reproducibility in the modifications induced by the prolonged cooling in the pigment-based pattern on the dorsal wing surfaces (Fig. 1).



(a) (b)

Fig. 1. Quantified aberration of the pigment-based pattern on the ventral wing surfaces of *Polyommatus icarus* butterflies. (a) Earlier results reported in [22]; (b) Results of the set of cooling experiments to check the reproducibility. The vertical bars on each data point indicate the magnitude of the phenotypic differences between the individuals in a certain group cooled for a given duration. (s.e.m = standard error of the mean)

This work was carried out on a very large number of individuals. The experiment was conducted with more than 200 freshly formed pupae, for each cooling time were assigned 30 pupae, to allow statistical evaluations, too.

The phenotypic changes induced by prolonged cooling (2 – 12 weeks at 5 °C in the dark) of freshly formed *Polyommatus icarus* pupae were investigated. Cooling halted the imaginal development of pupae collected shortly after transformation from the larval stage. After cooling, the pupae were allowed to continue their developmental cycle. The wings of the eclosed specimens were investigated by optical microscopy, scanning electron microscopy, UV-VIS spectroscopy and microspectroscopy. The eclosed adults presented phenotypic alterations that reproduced results that we published previously for smaller groups of individuals remarkably well; these changes included i) a linear increase in the magnitude of quantified deviation from normal pigment-based ventral wing patterns with increasing cooling time (Fig. 1); ii) slight alteration of the blue coloration of males; and iii) an increasing number of blue scales on the dorsal wing surface of females with increasing cooling time (Fig. 2). Several independent factors, including disordering of regular scale rows in males, the number of blue scales in females, eclosion probability and the probability of defect-free eclosion, showed that the cooling time can be divided into three periods: 0 – 4 weeks, 4 – 8 weeks, and 8 – 12 weeks, each of which is characterized by specific changes. The shift from brown female scales to first blue scales with a female-specific shape and then to blue scales with a male-specific shape with longer cooling times suggests slow decomposition of a substance governing scale formation.

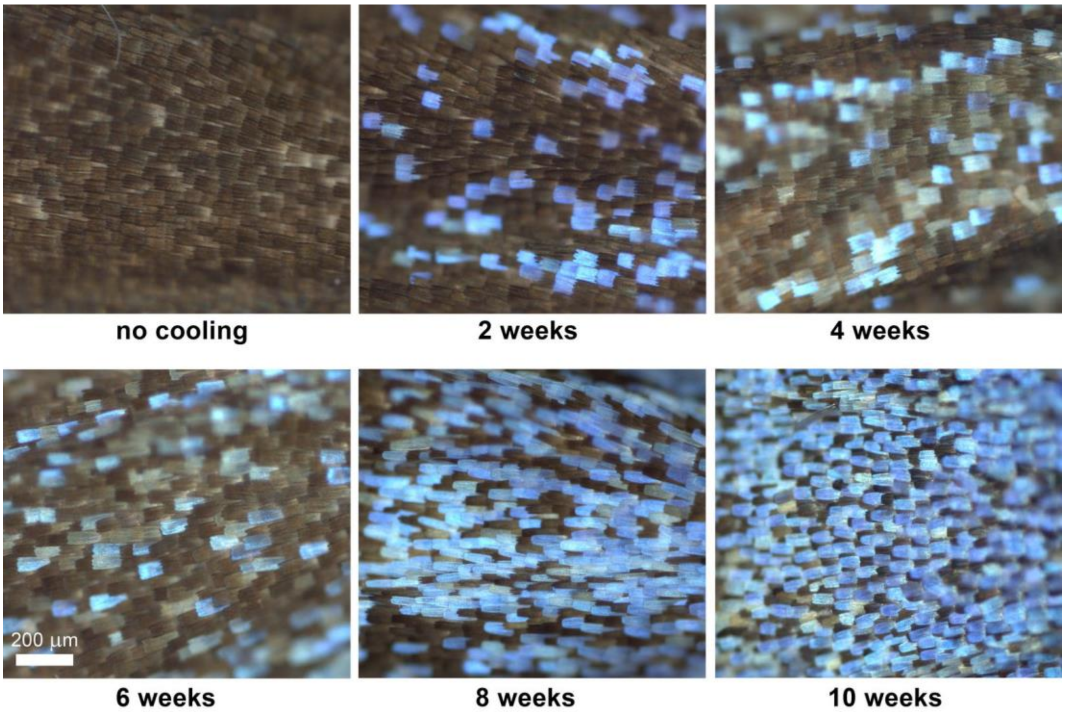


Fig. 2. Changes in the wing scales of female *P. icarus* butterflies with increasing cooling times, as seen under an optical microscope.

The paper is under review in Plos ONE [44].

4. Stability in time of the blue color of the male *Polyommatus icarus* butterflies and biogeographic patterns in this color

The males of Lycaenid butterflies often exhibit a conspicuous blue coloration generated by photonic nanoarchitectures on their dorsal wing surfaces. Using UV-VIS spectroscopy, and the Spectroboard – an instrument we developed earlier to allow the rigorous and nondestructive examination of museum exemplars - we investigated the spatio-temporal variations of this coloration for male *Polyommatus icarus* butterflies, considering an interval of more than 100 years and a geographical range spanning Europe (West) and Asia (East). The blue coloration in Hungary was found to be very stable both within a year (3 broods typical in Hungary) and within the period of 100 years (more than 300 generations). East-West geographic variation was investigated among 314 male *P. icarus* butterflies. In agreement with earlier genetic and

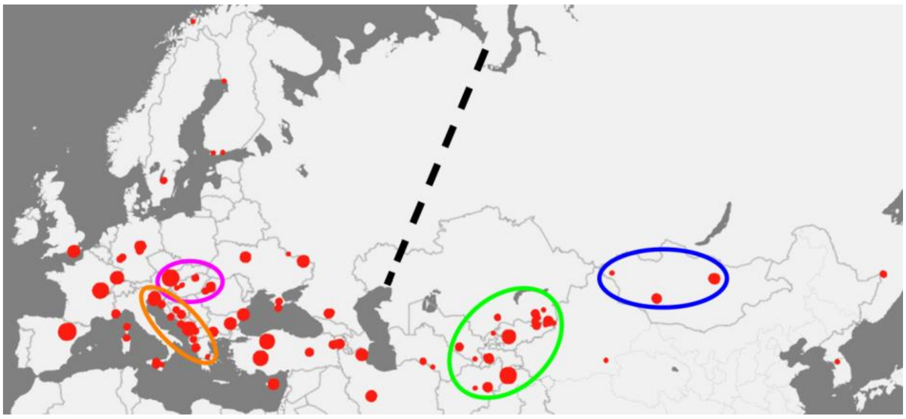


Fig. 3. The geographical source locations of the samples analyzed are indicated by red circles. The size of a circle is proportional to the number of the samples from that location. Four test regions were selected and are encircled on the map: two from Europe (Central East European Plane (magenta), Adriatic Coast (orange)) and two from Asia (Mongolian Steppe (blue) and Central Asia (green)). The broken black line approximately marks the location of the Ural Mountains, separating Europe and Asia.

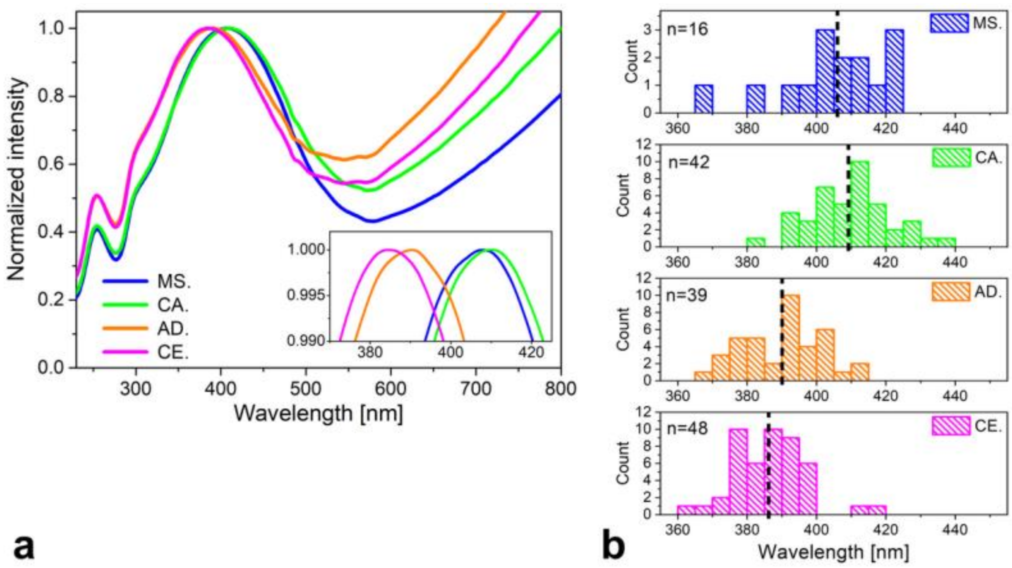


Fig. 4. Spectra of butterflies from the four regions marked in Fig. 3. a) Spectra averaged over all of the individuals and all of the measurements for a given region. Strong overlap in the spectral position of the blue reflectance maximum of the European (magenta and orange) and Asian (blue and green) samples is apparent; inset: magnified view of the blue peak region. b) Histograms of the spectral position of the blue reflectance maximum of the samples from the four regions; the difference in the spectral position of the blue reflectance maximum in a) is fully supported by the histograms, which show the spectral position of the averaged spectra of the individual samples. The numerical value in the left upper corner of each histogram indicates the number of individual samples for that histogram.

morphometric studies, it was found that the western males are not divided in distinct lineages. Clear differences in coloration were found between the western and eastern populations (Fig. 3), with a transition in the region of Turkey. These differences were tentatively attributed to bottleneck effects during past glaciations. The necessity of DNA based investigations was emphasized by these results. The results were published in the Nature family journal Scientific Reports [52].

The detailed analysis of the results showed that under very different climatic conditions, the structural color used in sexual signaling is kept constant. The adaptation to the local climate is achieved by changing the number of generations flying each year (one single in Scandinavia, up to five in South Europe). The differences in European and Asian color are attributed to the lack on gene flow during past glaciations. Our work was the first revealing that differences of structural coloration may indicate gene flow effects. The paper generated media attention in Hungary (see: http://www.nanotechnology.hu/magyarul/2019/2019_02_boglarka/2019_02_22_mta_boglarka.pdf, http://www.nanotechnology.hu/magyarul/2019/2019_02_boglarka/2019_02_22_origo_boglarka.pdf, http://www.nanotechnology.hu/magyarul/2019/2019_02_boglarka/2019_03_02_index_boglarka.pdf)

5. Modeling butterfly scale nanoarchitectures with short range order and linking the structural parameters with the generated color

The colors of butterflies originating from photonic nanostructures, found in the scales can be modeled by solving the Maxwell equations. The color generating scales are composed of a nanostructured chitinous material containing air voids, which causes the structural colors through light interference. We performed optical spectrum simulations utilizing full 3D Maxwell equation calculations on one hand on model structures to reveal the connection between the 3D structure and the optical spectrum, and we applied this knowledge to structures revealed in butterfly scales by electron microscopy. Our simulations showed that different scattering processes determine the spectrum in different wavelength ranges. For large wavelengths (>350 nm) the optical reflection can be well described by a corresponding effective multilayer model and the peak positions are well represented by a simple first-Born approximation. One has to include second order scattering processes inside the layers, however, in order to correctly reproduce the small wavelength side of the spectrum (<350 nm). This means that such details of structure, as the shape of the air voids determine the small wavelength spectrum. The results were published in a book chapter at Springer [51].

6. Investigation of butterflies possessing structural colors on both the dorsal and the ventral wing surfaces

The male *Albulina metallica* butterflies are special in that respect that both the dorsal (blue) and ventral (gold-green) wing surfaces are colored by similarly structured pepper-pot type photonic nanoarchitectures. The difference of colors arises from the different average distance of the holes in the stacked layers building up the photonic nanoarchitecture. We chose this butterfly on one hand because the above detailed properties and because we could modify the color during vapor sensing measurements and in this way, we could compare several different states of the very same structures. On the other hand, the vapor sensing with photonic crystal type nanoarchitectures itself is a phenomenon worth investigation.

Gas/vapor sensors based on photonic band gap-type materials are attractive as they allow a quick optical readout. The photonic nanoarchitectures responsible for the coloration of the wing scales of many butterfly species possessing structural color exhibit chemical selectivity, i.e., give vapor-specific optical response signals. Modeling this complex physical-chemical process is very important to be able to exploit the possibilities of these photonic nanoarchitectures. We performed measurements of the ethanol vapor concentration-dependent reflectance spectra on both wing sides of the male *Albulina metallica* butterfly, which exhibits structural color on both the dorsal (blue) and ventral (gold-green) wing sides. We extended our method of Direct Space Averaging (DSA), developed earlier for the analysis of the SEM images of quasi-ordered photonic nanoarchitectures to the investigation of TEM images, too. Using this, we revealed the details of the photonic nanoarchitecture inside the wing scales of *A. metallica*. On both sides, it is a 1D + 2D structure, a stack of layers, where the layers contain a quasi-ordered arrangement of air voids embedded in chitin. A structural model was built according to the findings revealed by the detailed TEM analysis. Next, we built a parametric simulation model that matched the measured spectra in pristine conditions and after the wing were immersed in air/vapor mixtures of different concentrations. The reflectance spectra were calculated by ab-initio methods by assuming variable amounts of vapor condensed to liquid in the air voids, as well as vapor concentration-dependent swelling of the chitin. From fitting the simulated results to the measured spectra, we found a similar swelling on both wing surfaces, but more liquid was found to concentrate in the smaller air voids for each vapor concentration value measured. These results are in good agreement with our earlier publication in which we demonstrated the capillary condensation of the vapors in the nanovoids and the chemical selectivity of lycaenid butterfly wings used as optical sensors.

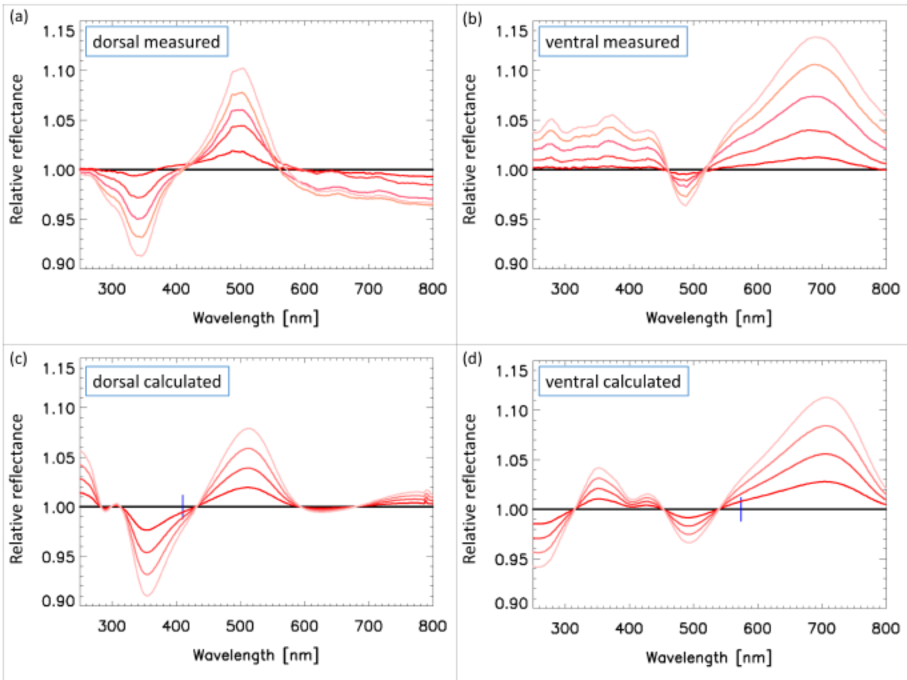


Fig. 5. Comparison of the measured and calculated vapor-dependent dorsal and ventral relative reflectance spectra of the *Albulina metallica* butterfly. (a,b) Measured spectra for 10%, 20%, 30%, 40%, and 50% ethanol vapor concentrations. (c,d) Calculated spectra for 12.5%, 25.0%, 37.5%, and 50.0% ethanol vapor concentrations.

The qualitative agreement of the measured and calculated relative reflectance spectra convincingly show that the structural model is well suited to describe the properties of the nanoarchitecture. The results were published in the journal Nanomaterials [50].

7. Using whole butterfly wings and single scales for chemically selective vapor sensing

Fast, chemically-selective sensing of vapors using an optical readout can be achieved with the photonic nanoarchitectures occurring in the wing scales of butterflies possessing structural color. These nanoarchitectures are built of chitin and air. The *Albulina metallica* butterfly is remarkable as both the dorsal (blue) and ventral (gold-green) cover scales are colored by the same type (pepper-pot) of photonic nanoarchitecture, exhibiting only a short-range order. The vapors of ten different volatiles were tested for sensing on whole wing pieces, very good chemical selectivity was found (Fig. 6). Some of the volatiles were tested on single scales as well, both in reflected and transmitted light. It was shown for the first time that single scales can be used for chemical sensing. Chemically-selective responses were obtained showing that selectivity can be increased by using arrays of sensors (scales), or by stacking several layers of scales on top of each other. The sensing behavior is similar in single scales and on whole wing pieces and is similar in reflected and transmitted light. By immersing single scales in an index-matching fluid for chitin, both the light scattering and the photonic nanoarchitecture were switched off, and the differences in pigment content were revealed. By artificially stacking several layers of blue scales on top of each other, both the intensity of the characteristic photonic signal in air and the magnitude of the vapor sensing response for 50% ethanol vapor in artificial air were increased.

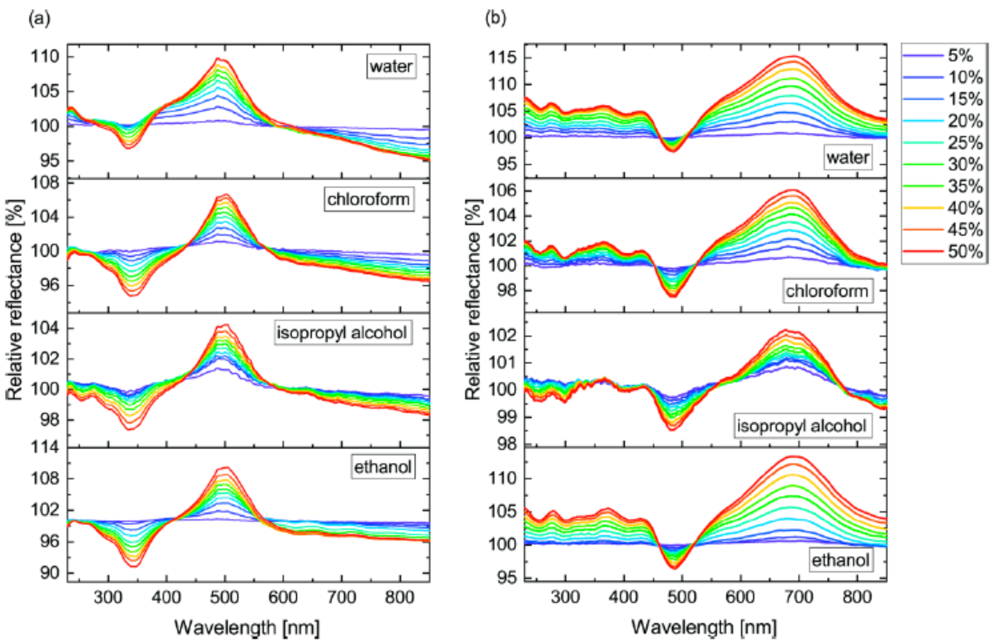


Fig. 6. Averaged relative reflectance spectra of water, chloroform, isopropyl alcohol, and ethanol measured on the (a) blue and (b) gold-green sides of the *Albulina metallica* wing.

The results were published in the journal Sensors [29]. In a subsequent paper (accepted for publication) we showed that the wings of several butterfly species are suitable for detection not only pure vapors, but vapor mixtures, too [46]. We investigated the optical responses the wing scales of several butterfly and moth species when mixtures of different volatile vapors were applied to the surrounding atmosphere. We found that the optical responses for the different vapor mixtures fell between the optical responses of the two pure solvents in all the investigated specimens.

8. Photodecomposition of the melanin (photo-bleaching) in butterfly wings with structural color

Despite the fact that the photobleaching process was demonstrated, as one can see in Fig. 7, we did not succeeded in reveling the molecular level processes in sufficient detail, that would allow the publication of the results in a journal with corresponding impact. At our present level of understanding, we think that, there are several processes taking place in parallel: the photodecomposition of the melanin by the UV component of the solar radiation is enhanced by the temperature of the sample, but in parallel the chitin itself is also suffering a modification which is reversed to a good extent when the sample is stored in dark. On the other hand, after a threshold is exceeded in the photo-alteration process, the changes are not any more reversible, Fig. 8. One may remark from the figure that while the melanin content is monotonically reduced (spectral range of 700 nm), the structural color does not change monotonically (spectral range of 380 nm). We tried to use artificial UV radiation and mechanically pressed, purified chitin pellets of 1 mm thickness to separate the different effects. We learned that chitin itself suffers modifications under UV radiation, which are relaxed in time. Further work is needed to get more insight in these complex processes.

Fig. 7. Male *Polyommatus icarus* butterfly in pristine status (left), and photobleached (right) using solar radiation.

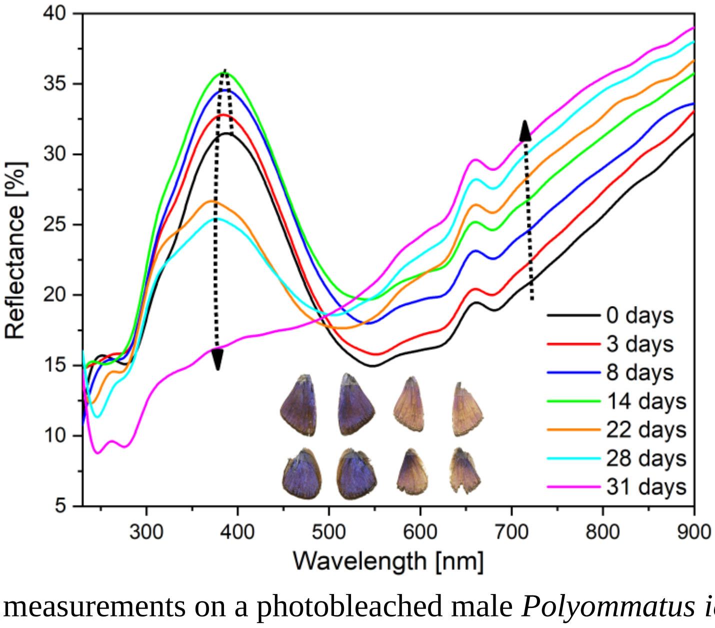


Fig. 8. Reflectance measurements on a photobleached male *Polyommatus icarus* wing. In the bottom inset all four photobleached wings are shown.

9. **Investigation of the effects of wide band absorbers in the visible on the optical properties of disordered photonic structures**

To investigate the effects of wide band absorbers in the visible on the optical properties of artificial, disordered nanoarchitectures produced in the laboratory we used melanin and carbon black suspensions in ethanol. The suspensions exhibited good stability, which allowed the soaking of the artificial photonic nanoarchitectures in these suspensions. The carbon black has a uniform and strong absorption in the UV-VIS range, while the melanin – the same broad band absorber which is found in most butterfly scales - has decreasing absorption from the UV, towards 700 nm. Compact oxide multilayers and photonic nanoarchitectures with air voids were compared. Only a slight redshift of the structural color maxima was observed after absorber deposition for the monolayer of silica nanospheres, the effects reported in the literature were not found.

10. **Investigation of biogeographical differences based on DNA microstallites and comparison with the spectral characteristics for *Polyommatus icarus* males.**

A microsatellite is a tract of repetitive DNA in which certain DNA motifs (ranging in length from one to six or more base pairs) are repeated, typically 5–50 times. Microsatellites occur at thousands of locations within an organism's genome. They have a higher mutation rate than other areas of DNA leading to high genetic diversity. Microsatellites have emerged as a popular and versatile marker type for ecological and population genetics applications. Therefore, after our paper reporting biogeographical differences in the structural color of European and Asian male *Polyommatus icarus* butterflies we decided to attempt the correlation of the differences of structural color with genetic characteristics. As a first step, we checked in the available literature if earlier have been reported microsatellites for the species under investigation. As we did not found such data, we had to develop the microsatellites ourselves. This was done by sequencing the genome of the *P. icarus*, followed by the selection of 22 potential microsatellites. Next these microsatellites were checked for variability using non-fluorescent primers from commercial sources. After the variability was confirmed, the much more expensive fluorescent primers were ordered and four European populations were selected for the test: a population from France, two geographically very close populations from Hungary (region of Érd) and a population from the Eastern Carpathians (Romania). From each population individuals were captured on purpose for this investigation and 20 individuals were selected for both spectral and genetic characterization. The spectral measurements revealed that for each of the four investigated populations the coloration of the forewings and the coloration of the hindwings is slightly different (a few nm difference in averages) Fig. 9. One may observe that the two populations sampled in the environs of Érd have almost overlapping averages, while from France to Romania a slight difference may be present, with the Hungarian samples placed between the two extremes. The full evaluation of the DNA microsatellites is still underway, but the preliminary evaluation of the data indicates that no clear genetic difference between the nuclear DNA of the four investigated populations can be found. This is in good agreement with our publishes results [52] indicating that in the European *P. icarus* population there are no characteristic geographic differences in the blue sexual signaling color and in the DNA microsatllites. We plan in the future to extend our investigation to the Asian individuals, too.

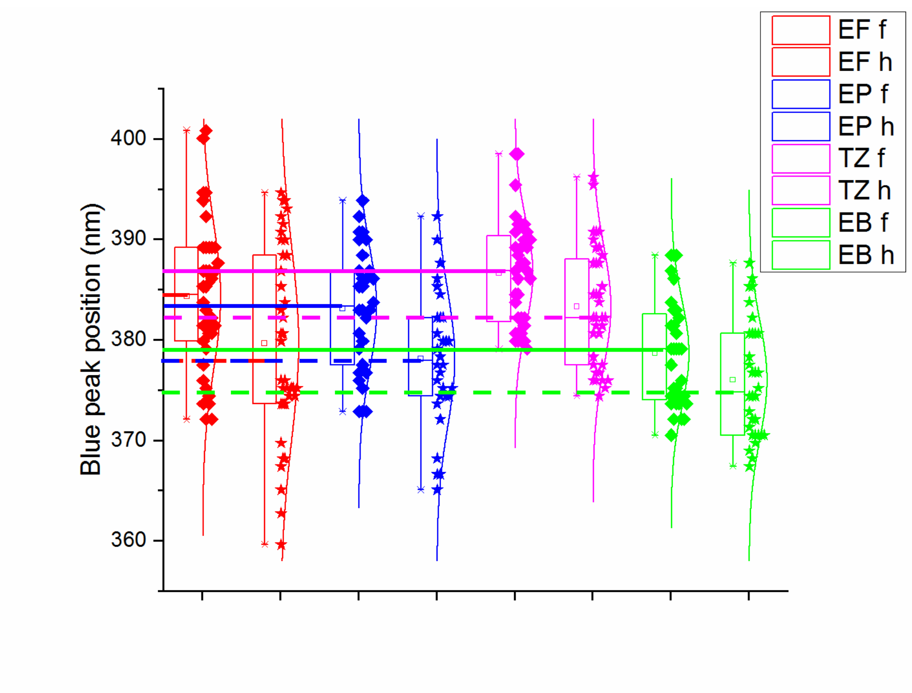


Fig. 9. Spectral characterization of the 80 *P. icarus* males (4 wings x 80 = 320 measurements) from four different geographic locations: red = Érd1 (Hungary); blue = Érd2 (Hungary); pink = France, green = Romania. Diamonds: forewings (f); stars hindwings (h).

Other achievements

A PhD thesis entitled:

"Photonic nanoarchitectures occurring in butterfly scales: investigation and applications"

by Dr. Gábor Piszter

was defended successfully in 2018 at the Physical Doctoral School of the Budapest University of Technology and Economics.

Dr. G. Piszter received the ***Junior Prize of the Academy from the Hungarian Academy of Sciences*** for his work entitled: „Lepkék pikkelyeiben előforduló fotonikus nanoarchitektúrák vizsgálata és alkalmazása" *("Photonic nanoarchitectures occurring in butterfly scales: investigation and applications")*

The results of our work were presented in numerous plenary, invite and oral presentations in international and national scientific conferences (only the plenary, invited and oral presentations are given in the list of publications).