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Characterizaton of the morphology of *Osmia* genus cocoons and hairs by nonlinear laser microscopy

Aleksander Kovačević
Institute of Physics
University of Belgrade
Belgrade, Serbia
aleksander.kovacevic@ipb.ac.rs

Ana Kovačević
Center for Multidisciplinary Studies and Faculty of Biology
University of Belgrade
Belgrade, Serbia

***Abstract*—Insects are the major pollinators of most plants. Within pollinating insects, all families of bees are the most recognized as one of the most efficient pollinators. Their number decreased in last several decades with some species becoming endangered. Due to the ability to produce honey, the most attraction is paid to honeybee. However, there are other bees as important pollinators, like solitary bees (family Megachilidae), and more research would give better insight not only into their commercial significance, but into their protection and support as well, which would lead to the protection of biodiversity. Two important structures of bees are hairs – for collecting and transporting of pollen, and cocoons – as a larvae protection in their earliest stage of growth. Using innovative methods from optics and photonics in entomology research, like laser microscopy with ultrafast beams, offers advantages such as optical sectioning (excitation of low volumes) and excitation by longer wavelength, which preserves biological tissues. Within the genus *Osmia* (mason bees), morphology of the cocoon wall has been describes, with the hair microstructure as well.**

***Keywords*—solitary bees, cocoons, hairs, laser-induced fluorescence, two-photon absorption**

I. Introduction

The transfer of pollen from the anther to the stigma enables fertilization of a plant. Pollinating agents can be water, wind, animals – such as insects, birds, and bats – and plants. Noticed pollinator decline is explained by concepts including possible reasons, like exposure to pathogens, parasites and pesticides, habitat destruction, climate change and others.

Although the existence of pollinator decline can be difficult to determine, a number of possible reasons for the theoretical concept have been proposed, such as exposure to pathogens, parasites, and pesticides; habitat destruction; climate change; market forces; intra- and interspecific competition with native and invasive species; and genetic alterations.

Bees are the most recognized pollinators and their adaptation to pollination includes shape (morphology) and electrostatic charge and in some bees specialized structure for transporting – a scopa. Most attractive bees are honeybees (family: Apidae), due to their strong pollinating ability, and also because of the possibility to harvest honey they produce, as well as to use beeswax, propolis, royal jelly and bee venom. However, other bees, like solitary bees (family: Megachilidae), are also significantly important plat pollinators.

Megachilidae could be found on all continents except Antarctica. There are around 4000 species, with 442 in Europe [1]. Species of the family are one of the most efficient pollinators in the world, just to mention genus *Megachile* and genus *Osmia*. Some exceptional important pollinators of domestic plants are: *Megachile rotundata* (Fabricius, 1787) [2], *M. pugnata* (Say, 1837) [3], *Osmia bicornis*, *O. cornuta*, *O. cornifrons* (Radoszkowski, 1887), and *O. lignaria* (Say, 1837) [4-8].

Osmia is present on all continents except South America, Australia and Antarctica [9]. These solitary bees are also called mason bees due to the way of how they make nests [10], where they make chambers where they lay their eggs [11]. They have four development stages, egg-larva-pupa-adult. Larva spins cocoon and becomes pupa, while adults mature during autumn and winter hibernation.

Pollination by *Osmia* is efficient due to the anatomy and behavior. Foraging starts in early spring on Rosaceae (apple, pear, apricot, almond, strawberry, ...), even in bad weather making them better pollinators than honeybees [12-17].

Important elements of bee structures are hairs for pollen collection and transport, and cocoons for larvae protection. Body hairs cover almost all the body surface of most of the bees. Research in bee body hairs is important because different morphology corresponds to the different roles. Most known bee hair role is in collecting pollen. Besides abdomen, thorax and head, hairs could be found on eyes, mandibules, and a pair of hind legs, where they form specialized structures for pollen collecting: corbicula (Apidae) or scopa (almost all solitary bees) [11, 18]. If the length and the density of hairs is compared for all bees, *Osmia* spp. could be called “the hairiest bees” [19]. Hair morphology could be specialized in order tocollect pollen more efficiently [20-21], for thermoregulation [22], as mechanoreceptors and chemoreceptors [23-24] and for oil collection from flowers [11, 18]. Silk is a protein polymer present in large number of land Arthropoda, and in Hymenoptera it evolved in the direction of protecting juvenile individuals [25]. Larvae of all Megachilidae, and so of *Osmia*, form cocoons [26]. In Megachilidae, cocoons are mainly consisted of two or three layers. One of the most important roles is the protection against desication [26-27].

Besides optical microscopy, nonlinear laser microscopy is used as an innovative tool in entomological research offering

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monitoring of small and tiny objects. The advantages are in better penetration within the sample with less damaging; moreover, due to excitation fluorescence, some samples do not need dyeing and it is possible to scan the sample layer by layer making a 3D image [28].The term “nonlinear laser microscopy” is used in order to avoid long and complex term which would include lasaer beam scanning, two-photon excitation fluorescence and confocal scanning. In entomology, chitine autofluorescense enables the investigation of insects structures [29-31].

In this work, three European solitary bees are investigated by nonlinear laser microscopy: *Osmia cornuta* (Latreille, 1803), *O. bicornis* (Linnaeus, 1758) and *O. caerulescens* (Linnaeus 1758).

II. Materials and Methods

For this research, cocoons have been collected from controlled populations in Belgrade vicinity during 2020 and 2021. A total of 20 cocoons and adults of *O. cornuta* (10 males, 10 females), 20 of *O. bicornis* (10 males, 10 females) and 6 of *O. caerulescens* (3 males, 3 females) have been processed.

Cocoons have been stored in glass jars in the refrigerator (+5°C) until opening. At the side of the head, cocoon top has been cut, the bee sex has been determined by the head hair color (black – female, white – male) and each bee has been pulled out by a tweezer and stored in separate jar. Dorsal and ventral side of cocoons have been cut to ~1 mm^2 pieces. Layers and sublayers have also been separated by tweezers.

Bees have been freezed (-20°C) for 24h, warmed to room temperature and relaxed in a chamber for 48h. By entomological needles, they have been attached to polystyrene substrates and let dry. Hairs from heads (frons and clypeus), dorsal abdomen (S2-S4) and ventrall abdomen (T3-T5) have been collected by tweezers. For microscopic monitoring, ~4 µl of distilled water has been used for hairs in order to reduce the temperature during fluorescence and to prevent melting.

Setup is based on a modified Zeiss optical microscope. A pump laser pumps a fs laser (a source of a fs beam). Fs beam passes through a scanner to a beam expander and is focused by an objective to the sample generating fluorescence. A fluorescence signal from the sample is separated from a laser beam by a dichroic filter. During optical microscopy, the visual signal from the sample has been sent to the camera and used for recording and positioning of the sample. During two-photon excitation fluorescence, the fluorescence signal is sent to the photomultiplier.

Pump laser is Coherent Verdi V12 (532 nm, CW, 9.3 W), fs beam source is Coherent Mira 900F (730 nm, pulsed ~150 fs, 76 MHz), while microscope body is a Carl Zeiss (Jena) microscope – modified, reconstructed and upgraded in the Institute of Physics Belgrade. Used objectives are Panachromat 40x/0.65inf/0.17-A, 12.5x/0.25inf/-A and 40x/1.0DIS VIS-IR inf-0. Added are scanner, beam expander, photomultiplier, fluorescence filter (400-700 nm), Canon EOS 100D camera, and PC with software made in the Institute of Physics Belgrade to control the system and for image analysis. Also are added a refrigerator (+5 °C) and a freezer (-20 °C) for biological samples.

During optical microscopy, Canon EOS 100D camera captured images obtained by using objectives Panachromat 40x / 0.65 inf / 0.17-A, Panachromat 12,5 x / 0,25 inf /-A, and Zeiss 40x / 1.0 DIS VIS-IR inf / 0 with oil immersion.

In the regime of two-photon excitation fluorescence at 730 nm, a 400-700 nm filter separated excitation beam from the fluorescence signal. Monitoring have been used with the Zeiss 40x / 1.0 DIS VIS-IR inf / 0 ovjective with iol immersion. Fs beam power at the entrance of the microscope has been varied in the range 16-48 mW.

III. Results

Cocoons of all three *Osmia* bees have been analyzed by nonlinear laser microscopy.

In *O. cornuta* and *O. bicornis*, the outer layer of their cocoons is a sparse web of thin silk threads within which some admixtures, such as feces or polen grains, are infrequently distrubuted. In *O. caerulescens*, the outer layer consists of two sub-layers. The outer sub-layer is – as in *O. cornuta* and *O. bicornis* – a sparse web of thin silk threads. On the other hand, the inner sub-layer of the outer layer consists of densely distributed silk threads which form a thin silk film. The threads in the inner sub-layer of *O. caerulescens* are thicker and darker if compared to the threads of the outer sub-layer. In Fig. 1, the outer layer of *O. cornuta* is presented.

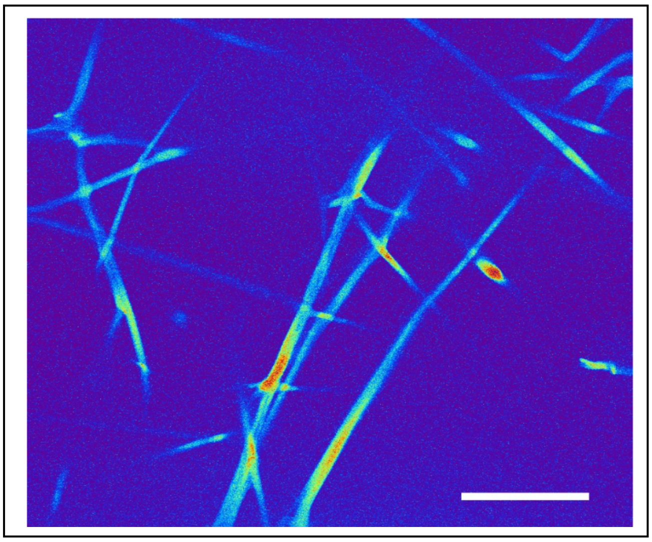

Fig. 1. Silk threads in the outer layer of the cocoon of *Osmia cornuta*; white bar represents 50 µm

In *O. caerulescens*, the middle layer of the cocoon is not present. The cocoon middle layer of *O. cornuta* and *O. bicornis* consists of two sub-layers. The outer sub-layer is a web, denser then the outer layer web, with semi-thick silk threads. The inner sub-layer is a thin silk film. In Fig. 2, the outer sub-layer of the inner layer of *O. bicornis* is presented. A trapped pollen grain is visible as a big oval-shaped object in the middle of the image.

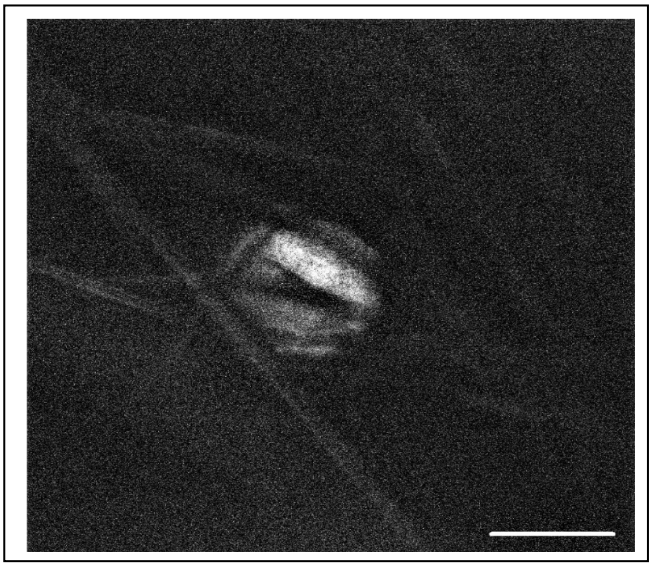

Fig. 2. *Osmia bicornis*, a pollen grain trapped within the outer sub-layer of the cocoon middle layer; white bar represents 50 μm

The inner layer of the cocoon of all three investigated bees is most rigid and most compact of all three layers, and consists of three sub-layers. The outer sub-layer is a web of thick threads, the middle sub-layer is formed from a lot of deposed matter, while the inner sub-layer is a thin silk film. In Fig. 3, the middle sub-layer of the inner layer of *O. caerulescens* is presented. The deposed matter is distributed as a regular structure.

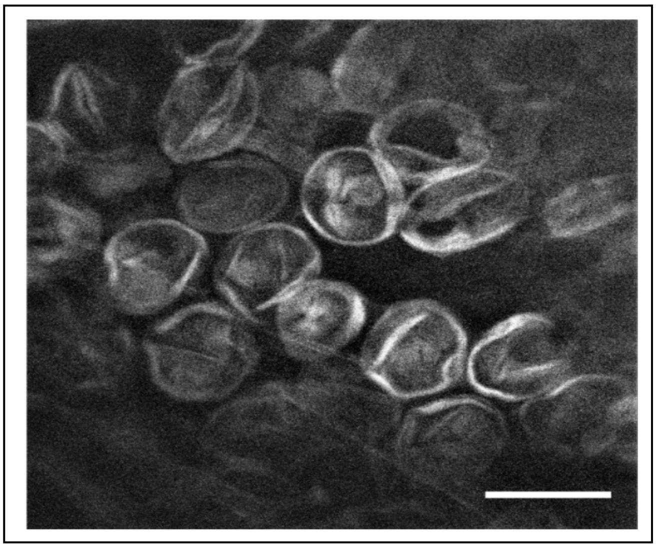

Fig. 3. Middle sub-layer of the cocoon inner layer of *Osmia caerulescens*; white bar represents 50 μm

Increasing the number of layers serves as better protection against predators. For the defense agains water, one layer is sufficient.

Osmia bees are extensively covered with hairs with main role of collecting and transporting pollen grains. The evolution of the relation between bees and plants which they pollinate is best shown in body hairs [32]. Hairs presented on head, abdomen and scopa are investigated. Scopa is a modification on a body of a bee that serves as a pollen-carrying apparatus.

In *O. cornuta*, there are two types of head hairs, one with dense distribution of short branches and the other with sparse distribution of long branches. In *O. bicornis*, there are only had hairs with sparse distrubution of long branches. *O. caerulescens* head hairs have no branches. In all three bees, head hair are 10 to 20 μm thick.

Hairs placed on dorsal part of the abdomen of *O. cornuta* are similar to the head hairs but thicker (20 μm): hairs with short, densely distributed branches and hairs with long, sparsely distributed branches. In *O. bicornis*, abdomen hairs (20 μm thick) are both long and short with dense distribution of both short and semi-long branches. *O. caerulescens* has thickest hairs (40 μm thick) of two types: short hairs with no branching and long hairs with short branches. Since the dorsal abdomen hairs are branched and of various sizes, is is possible that they serve not only for pollen collecting and transporting, but also have a role in thermoregulation and isolation, which is the case with similarly structured thoracal hairs [22].

Only *Osmia* females have scopa hairs. In *O. cornuta* are 20 μm thick, have bulbous root and spiral furrows (Fig. 4).

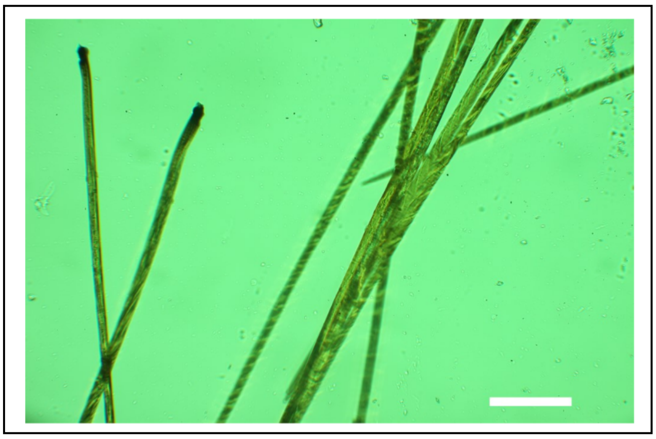

Fig. 4. Optical microscopy image of *Osmia cornuta* scopa hairs; white bar represents 100 μm

O. bicornis scopa hairs are similar to *O. cornuta*: 20 μm thick, with bulbous root and spiral furrows (Fig. 5).

Hairs of *O. caerulescens* scopa are 40 μm thick, have bulbous root and spiral not so prominent furrows [33].

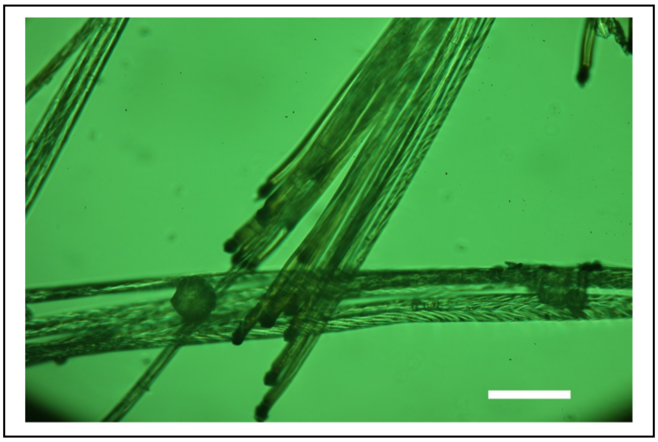

Fig. 5. Optical microscopy image of *Osmia bicornis* scopa hairs; white bar represents 100 μm

IV. Conclusions

Using the method of nonlinear laser microscopy, the investigation and the characterization of the structure and morphology of cocoon and hairs of the taxa within the genus *Osmia* is more precise and easy compared to the investigation by optical microscopy.

Cocoons of *O. cornuta* and *O. bicornis* have three layers. Outer layer consists of a net of silk threads, middle one of a web sub-layer and a thin film sub-layer, and inner layer consists of three sub-layers: a net of silk threads, deposed matter sub-layer, and a thin silk film. Cocoon of *O. caerulescens* has only two layers. The outer layer has a net of silk threads sub-layer and a thin film sub-layer. The inner layer has a net of threads sub-layer, a deposed matter sub-layer and a thin film sub-layer.

Hairs of *O. cornuta* and *O. bicornis* on head and abdomen have branches, and short and long hairs exist. Besides common hairs, on *O. caerulescens* head there are specialized hairs for pollen collecting from nototribical flowers. In all three species, scopa hairs have prominent spiral furrows, which facilitate pollen collecting.

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