Concealed Androconial Scales in Metalmark Butterflies (Lepidoptera: Riodinidae): New Insights from Confocal Laser Scanning Microscopy[1]

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Abstract Confocal scanning microscopy is widely used in the biological sciences—from tissue protein expression to the study of fossil amber inclusions. However, it is rarely used in insect taxonomy despite its unique feature for the examination of structural detail in small samples. In this study, we use confocal laser scanning microscopy (CLSM) to image certain poorly studied male secondary sexual organs, namely androconial scales, in a group of riodinid butterflies (Lepidoptera: Riodinidae). This group shows cryptic and mimetic morphology, so pheromones are thought to play an important role in mate choice. We found significant interspecific structural differences in androconial patches. The use of CLSM provides a simple, nondestructive and highly effective method to examine complex morphological characters, which could be applied to other structures and taxa across Lepidoptera and other insects.

Key Words concealed androconial scales, confocoal laser scanning microscopy, Riodinidae, Lepidoptera

Confocal microcopy was conceived in the 1950s by Marvin Minsky at Harvard University, but it was not until 1987 that it was commercialized and more broadly applied (Amos and White 2003). It requires a powerful and mobile light source, a laser, and sufficient computer power to stack resulting captions into a single high-resolution image (Lee et al. 2009). Advancements in technology enabled the more widespread use of confocal laser scanning microscopy (CLSM) during the 1990s, and it has been used in various areas of the biological sciences, mainly in molecular and cellular biology. Conventional fluorescence microscopy is limited due to the need to focus at a particular distance, with out-of-focus depths creating a background haze that prevents the clear visualization of three-dimensional structural details. As a consequence, conventional fluorescence microscopy is best suited to studies of thin or flat cells (Amos and White 2003). Scanning electron microscopy (SEM) is also widely used to study insect structures (Friedrich et al. 2014, Schawaroch et al. 2005) but suffers from various drawbacks, discussed in detail below. With modern confocal microscopes, the light beams focus on several

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focal planes of the specimen. Multiple image captions are taken and three-dimensional images can then be created using image software. CLSM allows three-dimensional morphological characters to be studied in detail.

CLSM has been rarely applied to insect taxonomy. It has been used to illustrate and describe the morphology of other arthropods, notably microscopic copepods. However, extensive staining was required (Brooker et al. 2012). Unlike in copepods, the insect cuticle does not require labelling with fluorescent dyes, as it contains naturally autofluorescent compounds like resilin (Michels and Gorb 2012) that are easily excited with ultraviolet or visible light (Klaus et al. 2003). When working with historically valuable specimens, such as type specimens or individuals from rare species, a noninvasive, nondestructive technique is critical to reduce potential collateral damage (Schawaroch et al. 2005). Thus, CLSM is ideal for the study of cryptic insect characters that require three-dimensional visualization.

Here we examine the enigmatic concealed androconial scales (CAS) of four metalmark butterfly species in the genus *Mesenopsis* (Lepidoptera: Riodinidae). Androconial organs are specialized structures, such as hairs or scales, which are thought to be involved in the dissemination of male pheromones to influence the behavior of females (Hall and Harvey 2002). Hall and Harvey (2002) were the first to survey the different types of androconial organs across the Riodinidae and reported that 25% of the family's species had some kind of androconial organ. Androconial organs are highly diverse in morphology and can be found on wings, on abdomens, or as external appendages in different species. CAS were first described by Harvey (1987) and are highly modified structures that have evolved independently at least three times in the Riodinidae. All 13 genera of the tribe Symmachiini, which includes our study genus, *Mesenopsis*, have CAS along the anterior margin of tergites 4–7 (Hall and Harvey 2002). In *Mesenopsis*, CAS are found in tergites 4 and 5 and concealed beneath the posterior margins of the tergite preceding them (Hall and Harvey 2002; Fig. 1). Only one species of the genus *Mesenopsis*, *Mesenopsis bryaxis* (Hewitson), was studied by Hall and Harvey (2002) with SEM and, although CAS structural variation was apparent when compared to closely related genera, they could not conclude if the same would be observed among other members of the genus.

Preliminary studies of specimens in this genus and their organs by the authors led us to note important variation in CAS structures in *Mesenopsis*. We, therefore, decided to study this group in greater detail. The aims of this study were to (a) describe, for the first time, the morphology and ultrastructure of CAS in the genus *Mesenopsis* and (b) define a protocol for studying three-dimensional microstructures using CLSM in historically valuable Lepidoptera specimens.

Materials and Methods

Confocal images were acquired with a Nikon A1-Si laser-scanning confocal microscope using a 40× oil-immersion objective (Numerical Aperture = 1.3). Images were recorded with pixel dimensions between 0.08 and 0.31 μm. Autofluorescence of the scales was excited with the following laser lines: 405-

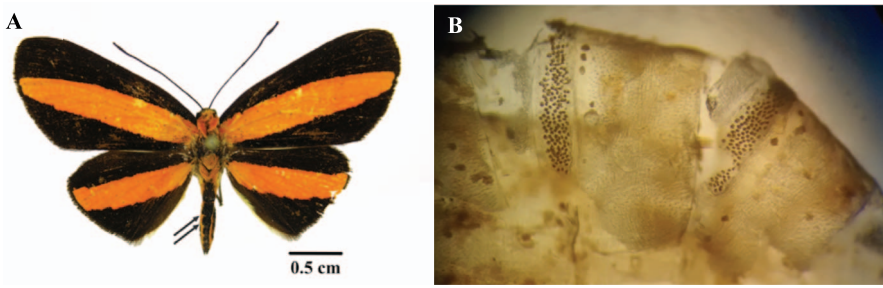

Fig. 1. *Mesenopsis* and concealed androconial scales (CAS). (A) *Mesenopsis melanochlora* with arrows indicating the approximate location of tergites 4 and 5 where CAS are found. (B) Light microscope detail of tergites 4 and 5 in *M. melanochlora* where bands of CAS can be seen during dissection of abdomen.

nm line of 100-mW cube laser (Coherent Inc., Santa Clara, CA), 488-nm line of 50-mW sapphire laser (Coherent Inc.), 561-nm line of 50-mW sapphire laser (Coherent Inc.), and 640-nm line of 40-mW cube laser (Coherent Inc.). Autofluorescence signal was collected with four photomultiplier detectors with the following wavelength emission windows: 425–475 nm for the 405-nm laser, 500–550 nm for the 488-nm laser, 570–620 nm for the 561-nm laser, and 675–725 nm for the 640-nm laser. The specimens were visualized using a 25.5-μm (1.2 airy units) confocal pinhole and a number of z-stacks (typically between 145 and 165) with optical thickness between 200 and 300 nm each were acquired. The fluorescence signal from each z-stack was then projected onto a maximum projection image and used to generate a three-dimensional model of the specimen using a Nikon NIS-Elements software (www.nis-elements.com).

The methodology described was applied to four specimens representing different species in the genus *Mesenopsis* as follows: *Mesenopsis melanochlora* (Godman and Salvin), *Mesenopsis albivitta* (Lathy), *Mesenopsis briseis* (Godmand and Salvin), and *M. bryaxis* (Hewitson). The study specimens used are held at the Natural History Museum (London) where most of the types of this genus are found. Specimens from this genus are rare in collections, so we were only able to find suitable male specimens for these four species out of the six described in the genus. We adapted the standard dissecting protocol for Lepidoptera to a gentler approach that prevents the androconial scales from detaching from the cuticle. We placed the abdomens in 10% potassium hydroxide (KOH) solution overnight at room temperature. After this stage, the sclerotized genitalia were removed and soaked in KOH for longer when needed for further examination.

The abdomen was then cleaned with a soft brush to remove all the scales other than CAS, which tend to stay firmly attached to the tergites if the above soaking protocol is followed (Fig. 1B). The resulting material was stored unstained in glycerol. The tergites of interest were then extenuated manually to facilitate

visualization of CAS using CLSM (Fig. 1B). A trial protocol showed that abdomens were best stored in glycerol, and not water, to be correctly visualized by CLSM. Props were initially used to allow for three-dimensional structures to maintain their shape. However, the resulting slide was too thick to visualize the scales with CLSM and could not reach optimal resolution at 40× with immersion oil. Therefore, we stored the unstained abdomens flat on a glass slide with glycerol. After 2 weeks of ambient temperature drying, the slides were examined and successfully photographed under CLSM. We used freely available NIS Elements Viewer software to process the images that resulted from CLSM. By creating three-dimesional volume-rendering videos we were able to study the ultrastructure of the CAS.

Results

Slide-mounted dissections of unstained abdomens in glycerol were successfully visualized with CLSM and the ultrastructure of CAS in four species of *Mesenopsis* was imaged for the first time. The visualization of 145 focal planes per image enabled us to describe the structures found within and surrounding CAS (Fig. 2).

The transversal images obtained at different focal planes show that CAS are extremely convex scales made up of thin, longitudinally ridged tissue, in contrast with the spongy tissue found in other groups' androconial organs. CLSM images reveal important intrageneric differences in CAS (Fig. 3). *Mesenopsis melanochlora* and *M. albivitta* (Fig. 3A, B) share a similar convex cave-like structure, whereas *M. bryaxis* (Fig. 3D) presents a medial folding in the dorsal facet of the androconial scale. In contrast, the CAS of *M. briseis* seem to be shorter in length, although sharing a similar cave-like morphology. Sockets are found at the base of the setae holding each androconial scale, which are visualized here for the first time (Fig. 3C), and can be observed in all species when extracting the lower focal planes of the stacked images. Three-dimensional rendering confirmed that the scales' convex shape and the trichomes surrounding them are characteristics conserved among the species (Fig. 4).

Discussion

CLSM in conjunction with the protocol described above enabled an incomparable visualization of a cryptic insect character. Most previous examples of its usage relate to the imaging of Diptera genitalia (Klaus et al. 2003) and various other small three-dimensional structures that had been overlooked before the use of CLSM (Schawaroch et al. 2005). To our knowledge, this is the first time that the technique has been applied to butterfly structures. CLSM presents several advantages when compared to the more commonly used SEM. First, the low-risk preparation protocol allows for the use of highly valuable historical specimens, potentially including species type specimens, because the abdomen and genitalia can be preserved undamaged. This is particularly important for groups such as Riodinidae butterflies that are rare in nature and represented by very few individuals in collections. Second, the freely available and user-friendly software, NIS Elements Viewer, can

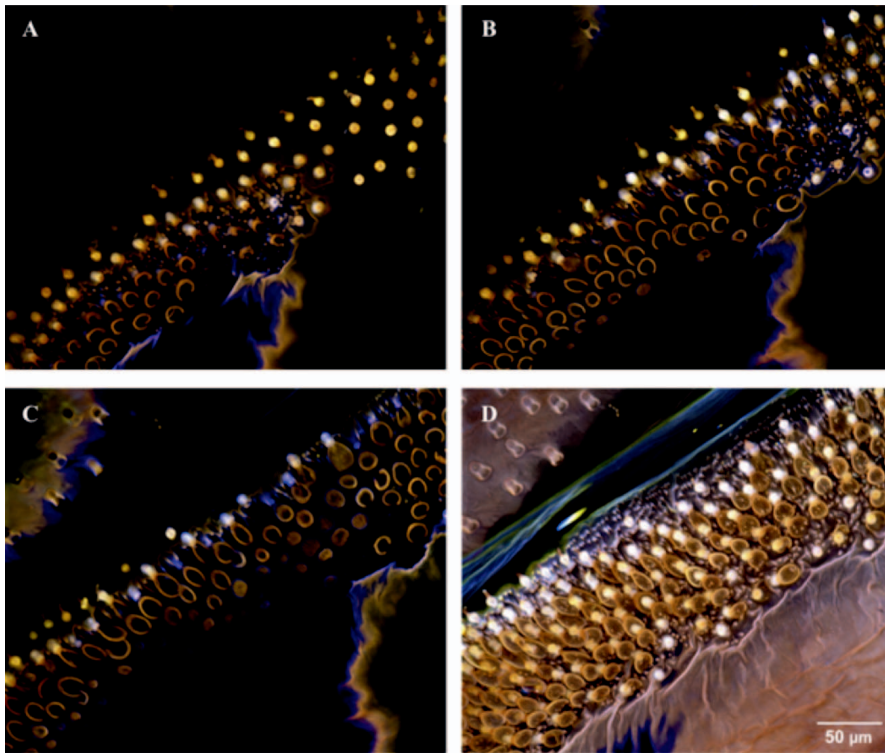

Fig. 2. Images of focal planes of concealed androconial scales in *Mesenopsis melanochlora*. (A–C) Series of images at different focal planes on a dorsal view of a band of concealed androconial scales (CAS) extracted with NIS Elements Viewer with the deepest layer (A), the sockets and setae that hold CAS attached to the tergites can be observed, whereas in higher layers (B, C) cross-sections of the scales can be seen. (D) Three-dimensional reconstruction of CAS band made of 145 images at different focal planes stacked by NIS Elements Viewer. Scale bars all 50 µm.

create images that digitally rotate in three-dimensional space, allowing structures to be studied in detail from all angles (Fig. 4). Third, and perhaps most importantly, CLSM is ideal for the study of the ultrastructure of relatively large three-dimensional characters as it creates images at different focal planes that can be later stacked for a complete three-dimensional view (Fig. 2). Insect external structures are particularly suitable for CLSM due to the natural autofluorescence of their cuticle, which eliminates the use of dyes.

In this study, we described in detail the morphology of CAS and demonstrated that they are morphologically diverse within the *Mesenopsis* genus. Androconial characters should be studied further within the family Riodinidae, as they may

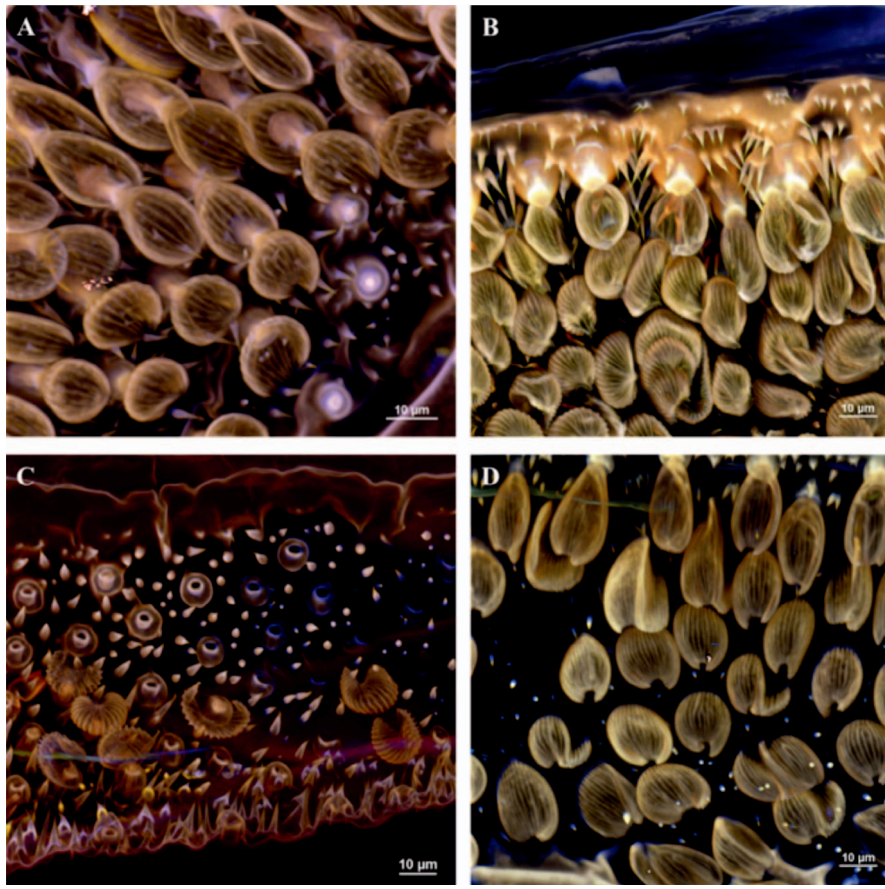

Fig. 3. Three-dimensional reconstructions of four *Mesenopsis* study species.(A) *Mesenopsis melanochlora*, (B) *Mesenopsis albivitta*, and (D) *Mesenopsis bryaxis* viewed from a dorsal-anterior view where the full length of the scales is visible. (C) *Mesenopsis briseis* was imaged more dorsally due to the positioning of the abdomen in the glass slide, and thus only the tips of the scales are visible in this reconstruction and the scale sockets are visible. Scale bars all 10 µm.

provide useful characters for species diagnosis and enable a greater understanding of interspecies differentiation of mimetic species. Scales are likely to be involved in pheromone release during courtship, when males telescope their abdomens "out" and as the tergites are pulled apart, CAS become exposed (Hall and Harvey 2002, Harvey 1987). The structural differences described in this study may have contributed to the speciation of these taxa by altering the mode and tempo of pheromone release during courtship.

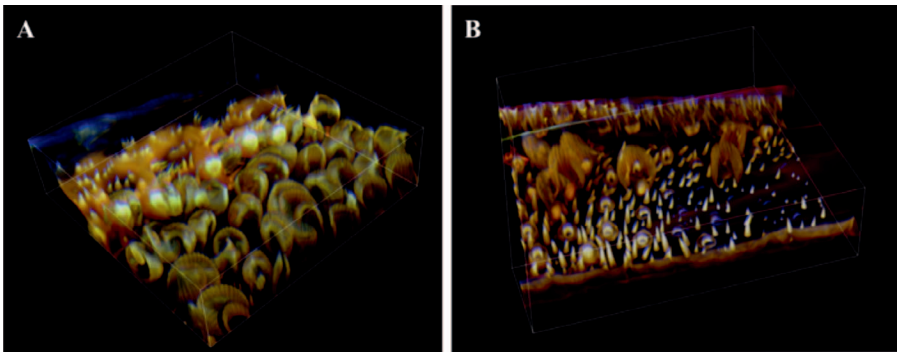

Fig. 4. Three-dimensional volume rendering showing ultrastructure of concealed androconial scales (CAS). Example of CAS cave-like ultrastructure visualized by digitally rotating the volume-rendered image in (A) *Mesenopsis albivitta* and (B) *Mesenopsis briseis*. Dimensions of the three-dimensional objects are (A) width: 112 μm, height: 112 μm, depth: 30 μm and (B) width: 133 μm, height: 133 μm, depth: 36.5 μm.

CLSM has enabled us to visualize in great detail the morphology of structures that have been difficult to interpret in the past. Its advantages over other techniques on visualizing large three-dimensional structures are clear, and it has the potential to become an important tool for studying insect taxonomy. Furthermore, CLSM is particularly useful when working with specimens that have been previously slide-mounted, type material, or taxa rarely found in collections, as is the case with many insect groups.

Acknowledgments

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