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Deciphering genital anatomy of rare, delicate and precious specimens: first study of two type specimens of mayflies using micro-computed X-ray tomography (Ephemeroptera; Heptageniidae)

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Abstract

The use of non-invasive techniques to study a wide array of zoological specimens has been increasing considerably during the 21st century. Among these techniques, micro-computed X-ray tomography (µ-CT) is gaining much attention. This method may allow access to hardly visible and internal structures of valuable specimens (e.g. type specimens) through virtual dissections. We studied two type specimens of Ephemeroptera belonging to the family Heptageniidae using µ-CT; the male lectotype of *Epeorella borneonia* Ulmer, 1939 (pinned specimen) and the male holotype of *Rhithrogeniella ornata* Ulmer, 1939 (specimen in ethanol). These specimens are the only male adults known in their respective genera; hence a detailed description of their genitalia could reveal important taxonomic and phylogenetic information. We present here the first-ever µ-CT study of mayfly type specimens, and confirm that male genitalia of *R. ornata* lack titillators, whereas those of *E. borneonia* possess a pair of titillators which were concealed within the penis lobes.

Introduction

Micro-computed X-ray tomography (hereafter µ-CT) is a relatively new technique which allows creation of virtual cross-sections of an object that can be used to rebuild a virtual model (3D model) without destroying the specimen and with a high resolution (pixel size about 0.5µm) (Friedrich *et al.* 2014). Although the pioneer µ-CT work in insect morphology was published at the beginning of the 21st century (Hörnschemeyer *et al.* 2002), this technique has only recently been presented as a valuable tool to solve taxonomic problems (e.g. Faulwetter *et al.* 2013; Michalik *et al*. 2013; Ruch *et al.* 2014; Simonsen & Kitching 2014). For example, a search in Web of Science™ conducted early December 2015, with keywords 'micro-CT' AND 'taxonomy' gave only 38 papers, more than half of them (20) published since 2014. One of the most promising applications of µ-CT in zoological research, is the possibility to virtually study rare and precious specimens such as type material (Simonsen & Kitching 2014). Moreover, the use of µ-CT technology seems to be harmless for DNA integrity (Hall *et al.* 2015).

Mayflies (Ephemeroptera) are a small order of primitive winged insects, whose adults are generally weakly chitinized and very delicate (Sartori & Brittain 2015). Studies of mayfly morphology through µ-CT has been applied recently on male genitalia (e.g. Alba-Tercedor & Sainz-Cantero Caparros 2010), compound eyes (Alba-Tercedor 2016 this volume), larval mouthpart structures (e.g. Staniczek 2015 and in prep.) and egg organization (Alba-Tercedor & Sanchez Almazo 2014). One of us (MS) recently revised Georg Ulmer's Heptageniidae collection from Southeast Asia. This included revisions of the genera *Epeorella* Ulmer, 1939 and *Rhithrogeniella* Ulmer, 1939 (Sartori 2014a, 2014b). Both genera are still poorly known, and the male lectotype of *Epeorella borneonia* Ulmer, 1939 and the male holotype of *Rhithrogeniella ornata* Ulmer, 1939, are the only specimens of this sex known so far for both genera. In their re-description, Sartori (2014a, 2014b)

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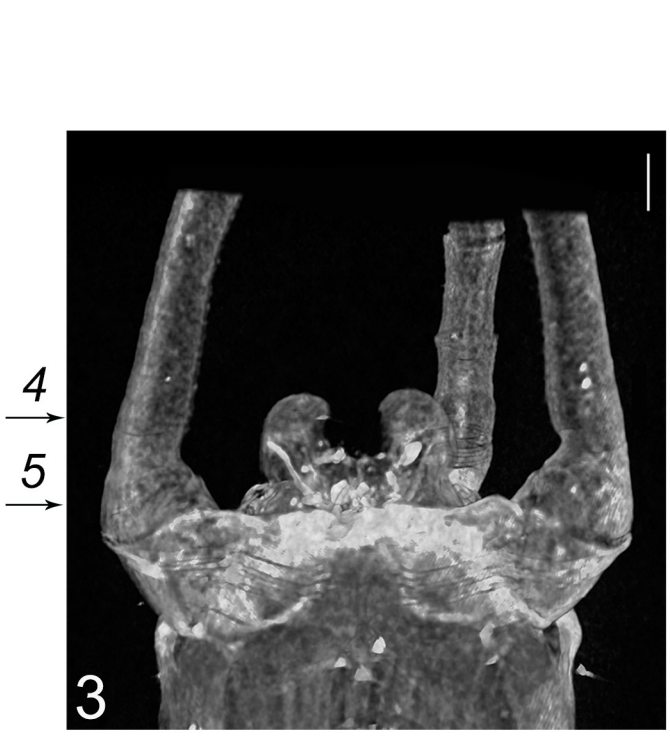
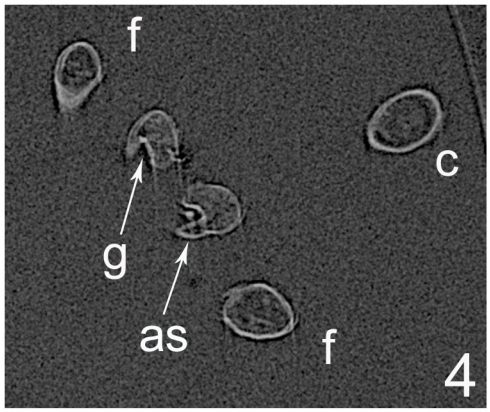
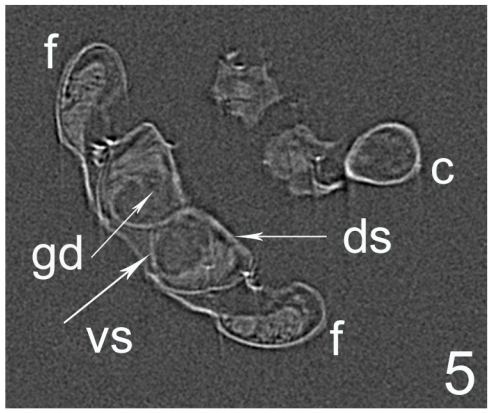
mentioned that the exact structure of the genitalia, in particular the presence or absence of sclerotized titillators, could not be ascertained because the two specimens were too valuable to be dissected.

Here, we present µ-CT analyses of these two type specimens for the first time. Both specimens are deposited in the collections of the Zoologisches Museum (CeNaK), Hamburg University, Germany.





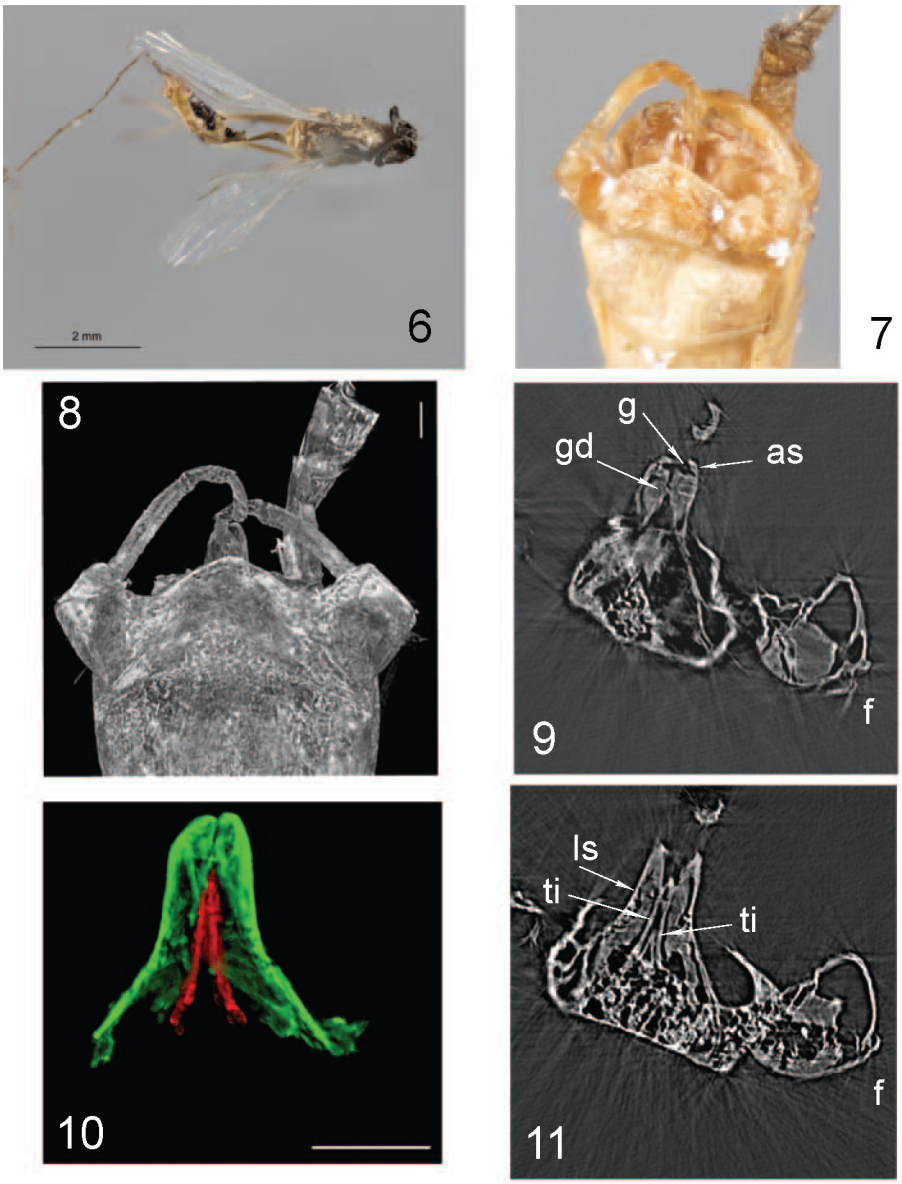


FIGURES 1–5. *Rhithrogeniella ornata* Ulmer, 1939, holotype. **FIGURE 1.** Specimen in lateral view; **FIGURE 2.** Ventral view of the genitalia; **FIGURE 3.** µ-CT volume rendered reconstruction of the genitalia in ventral view, arrows and numbers refer to the transversal slides; **FIGURE 4.** µ-CT transversal section (slide 656) of the genitalia at apex; **FIGURE 5.** µ-CT transversal section (slide 593) of the genitalia at base; abbreviations: as: apical sclerite; c: cercus; ds: dorsal sclerite; f: forceps (gonopod); g: gonopore; gd: gonoduct.

Material and methods

The holotype of *Rh. ornata* is a specimen stored in ethanol and originating from Indonesia, Java, Buitenzorg, [Bogor], collected in July 1932 by Dr. Lieftinck (Fig. 1). The lectotype of *E. borneonia* is a pinned specimen coming from Borneo, Nanga Serawei, collected between 12 and 18 November 1924 by Prof. Dr. H. Winckler (Fig. 6).

The specimens were scanned at Ernst-Moritz-Arndt Universität Greifswald using an Xradia MicroXCT-200 X-ray imaging system (Carl Zeiss X-ray Microscopy Inc., Pleasanton, USA), at 40kV and 200µA, with an exposure time ranging from 28 to 30 seconds, and with a pixel size between 1.06–1.68µm. Before analysis, the wet specimen of *Rh. ornata* was transferred into an alcoholic iodine solution (1% iodine in pure ethanol) in order to enhance the contrast (Friedrich *et al.* 2014), and scanned in a plastic tube. The pinned *E. borneonia* was simply fixed on the support with the needle. For both specimens, only the distal abdominal segments VIII-X were scanned. The obtained data were processed using the 3D analysis software AMIRA v. 5.4.2 (Visage Imaging, Berlin, Germany). Virtual reconstruction of the genitalia was performed by delineation in each section (segmentation) and a smooth surface was computed using the surface editor.



FIGURES 6–11. *Epeorella borneonia* Ulmer, 1939. lectotype. **FIGURE 6.** Specimen in dorsal view; **FIGURE 7.** Ventro-lateral view of the genitalia; **FIGURE 8.** µ-CT volume rendered reconstruction of the genitalia in ventral view; **FIGURE 9.** µ-CT longitudinal section (slide 686) of the genitalia through the apex; **FIGURE 10.** µ-CT volume rendered reconstruction of the penis lobes in ventral view, lobes in green, titillators in red; **FIGURE 11.** µ-CT longitudinal section (slide 669) of the genitalia in more ventral position; abbreviations: as: apical sclerite; f: forceps (gonopod); g: gonopore; gd: gonoduct; la: lateral sclerite; ti: titillators

Light microscopy images of the specimens were obtained with a Visionary Digital LK System (Dun Inc., Palmyra, USA) and image stacks were reconstructed and edited using Helicon Focus and Adobe Photoshop CS6 Extended.

Results

The pinned *E. borneonia* moved along the needle during the scan of ca 2–3µm, thus rendering poorer results than could be expected (see in Figs 9–10 the background which looks slightly crumpled due to the movement of the specimen based on a loose mount). The virtual sections of *Rh. ornata* are spoiled with white dots (Fig. 3), meaning that products with a high density were present in the alcoholic iodine solution, possibly iodine crystals.

Detailed analysis of *Rh. ornata* genitalia (Figs 4–5), revealed rather simple genitalia, each of the penis lobes was composed of a ventral, dorso-lateral and apical sclerite, bordering the genital pore, and without accessory structures such as spines or titillators.

Genitalia of *E. borneonia*, although different in shape, also present simple structures with lateral and apical sclerites (Figs 9–10), without apparent spines, but a pair of strongly sclerotized titillators is well visible in the reconstruction (Fig, 11) and are hidden within the penis lobes in the investigated specimen (Fig. 10).

Discussion

The aim of this preliminary study was to evaluate the potential of µ-CT for mayfly morphology, using old, unique, and fragile material. The overall results were satisfactory, despite some problems (see above). Both specimens were unaffected by the treatment and were returned to the ZMH collections without apparent damages.

Our study confirms that *Rh. ornata* male genitalia lack titillators, contrary to what has been proposed by Soldán and Braasch (1986). This is in accordance with our study of this material using optical instruments (Sartori 2014b). *Rhithrogeniella* nymphs are morphologically closer to those of the Holarctic genera *Paracinygmula* Bajkova, 1975 and *Nixe* Flowers, 1980, but the structure of larval gills and the absence of titillators in male imagos allow a clear separation. The combination of having the distal dentiseta of the maxilla simple (Sartori 2014b; Fig.12), primary swimming setae on cerci and terminal filament (Sartori 2014b; Fig. 25) in the nymphs, and the absence of titillators in male imagos is unique among the genera in the subfamily Ecdyonurinae. Only species of *Afronurus* Lestage 1924 lack titillators, but this lineage belong to the tribe Atopopini or Atopopus/fg1 sensu Kluge (2004), based on the multiple branches of the distal dentisetae.

The evidence of the presence of titillators in *E. borneonia* would have not been possible without dissecting the lectotype, an operation highly hazardous with a dry specimen. Thanks to µ-CT technology, we were able to obtain this phylogenetically important information with virtual dissection. *Epeorella* (syn. *Darthus* Webb & McCafferty 2007) was placed in the new tribe Afronurini by Webb & McCafferty (2007), together with genera *Afronurus* and *Parafronurus* Zhou & Braasch 2003. This tribe is highly paraphyletic, because it encompasses taxa with *(Epeorella, Parafronurus)* or without titillators *(Afronurus),* and which distal dentiseta can have multiple branches *(Afronurus)* or bifid as the proximal one *(Epeorella).* Exact phylogenetic position of *Epeorella* within the subfamily Ecdyonurinae still needs to be fixed when more material is available.

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