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Giant spermatozoa and a huge spermatheca: A case of coevolution of male and female reproductive organs in the ground louse *Zorotypus impolitus* (Insecta, Zoraptera)

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ABSTRACT

The male and female genital apparatus of the recently discovered ground louse *Zorotypus impolitus* were examined using light and electron microscopy. The rounded testes and a large seminal vesicle are connected with a complex of four accessory glands by a long tapering ejaculatory duct. Two accessory glands have the same whitish coloration, whereas the third one is pale blue, and the elongated and cylindrical fourth one translucent. The sperm are the largest known in Hexapoda, 3 mm long and 3 μm wide, with a volume of ca. 21,000 μm^3; the ratio between the diameter of the axoneme and the width of the main body of the sperm ranges between 1:10 and 1:13. The exceptional width of the spermatozoa is due to an extreme enlargement of the mitochondrial derivatives and accessory bodies. A single sperm is contained in a small globular spermatophore (100 μm). The highly unusual external transfer correlates with an atypical mating behavior. The male produces several to many spermatophores during the mating process. As in other zorapterans the ovaries are panoistic and the eggs bear two micropyles. An exceptionally large apical spermathecal receptacle is present; it is connected with the vagina by a long spermathecal duct, which varies structurally along its course. A correlation between the sperm size and the size of the spermatheca is likely. Ultrastructural features of different species support two strikingly different models of male and female reproductive apparatus in the small order Zoraptera. This is in stark contrast to the extreme uniformity of their external morphology. It is likely that sexual selection played a decisive role in the evolution of the reproductive system.

1. Introduction

Zoraptera (ground lice or angel insects) are a very small group of neopteran insects. Their systematic position is intensively discussed. They were discovered at the beginning of the last century by Silvestri (1913) and received little attention for a long time, mainly due to their inconspicuous appearance, cryptic lifestyle, and presumed rarity. Zorapterans are small (less than 4 mm) and fast-moving insects and difficult to recognize in the field. They are mainly found under bark and in rotting logs in subtropical and tropical areas (Grimaldi and Engel, 2005). With only 39 extant species presently described they belong to the smallest insect orders (Rafael et al., 2008; Mashimo et al., 2013).

Studies on Zoraptera cover descriptions of new extant and fossil species (e.g., Karny, 1922; Gurney, 1938; Engel, 2000; Engel and Grimaldi, 2002; Rafael and Engel, 2006; Terry and Whiting, 2012; Mashimo et al., 2013), the morphology and phylogenetic placement (Rasnitsyn, 1998; Beutel & Weide, 2005; Terry and Whiting, 2005; Friedrich and Beutel, 2008; Yoshizawa, 2007, 2011; Wang et al., 2013; Letsch and Simon, 2013), and the mating behavior of several species (Choe, 1994a, 1994b, 1995; Dallai et al., 2013). Detailed studies on the morphology of the male and female reproductive systems, including ultrastructural characterizations of eggs and spermatozoa, were available for only two species, *Zorotypus caudelli* (Dallai et al., 2011, 2012a; Mashimo et al., 2011) and *Zorotypus hubbardi* (Hünefeld, 2007; Dallai et al., 2012b). Based on evidence presented in these studies it was suggested that Zoraptera

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are an example for coevolution of the male and female reproductive organs, an evolutionary process also documented in other insect orders (e.g., Dybas and Dybas, 1981; Miller and Pitnick, 2002).

The species *Zorotypus impolitus* is particularly interesting in its mating behavior, as males and females do not perform the characteristic copula reported in other zorapterans, but conduct a highly unusual external sperm transfer. Males repeatedly deposit a tiny spermatophore on the female abdomen, each of them containing only a single giant spermatozoon. This method of sperm transfer represents a unique and remarkable condition for pterygote insects (Dallai et al., 2013). Considering this, a detailed knowledge of the involved structures appeared desirable. The primary aim of this study was therefore to examine and document the male and female reproductive organs in detail, including eggs and spermatozoa. The morphological findings are discussed with respect to their functional and evolutionary implications.

2. Materials and methods

2.1. Source and culture of specimens

Male and female specimens of *Z. impolitus* were collected at Genting Highlands, Peninsular Malaysia in the spring 2011 and 2012. The insects were kept at room temperature (18–22 °C) in small glass containers with hardened plaster of Paris at the bottom. They were fed with specimens of the springtail *Folsomia candida* and small amounts of yeast. Water was regularly sprinkled on the bottom of the containers to maintain humid rearing conditions.

2.2. Histology and light microscopy (LM)

Adult specimens were dissected under an Olympus SZX12 stereo light microscope in sodium phosphate buffer to which 3% sucrose was added (PB, 0.1 M, pH 7.2). Male and female reproductive systems were isolated and photographed *in toto*. The material was then transferred to 2.5% glutaraldehyde buffered in PB overnight at 4 °C. After careful rinsing in PB, samples were post-fixed in 1% osmium tetroxide for about 1 h at 4 °C, washed several times in PB, dehydrated in a graded ethanol series, and finally embedded in Epon–Araldite resin. Part of the testes and ejaculatory ducts were treated with tannic acid following the method described by Dallai and Afzelius (1990).

Semi-thin sections (1 μm) were cut with a Reichert Ultracut E ultramicrotome, mounted on glass slides, stained lightly with 1% toluidine blue. Free spermatozoa obtained from the seminal vesicle were also used for light microscopy observations. In both cases, samples were viewed under a Leica DMRB interference contrast microscope equipped with an AxioCam digital photocamera (Carl Zeiss). For light microscopic observations of the egg micropyles fixed portions of the chorion were mounted in a polyvinyl-lactophenol medium, Heinz liquid, and observed under a biological microscope Nikon OPTIPHOT.

2.3. Electron microscopy (SEM and TEM)

For SEM preparations of the egg micropyles, fixed eggshells were dehydrated in a graded ethanol series, critical point dried, coated with gold, and then observed under a TOPCON SM-300 SEM. For TEM observations, samples were processed as described above for histological preparations. Ultrathin sections (60 nm) were obtained with a Reichert Ultracut E ultramicrotome equipped with diamond knives, mounted upon copper grids, stained with uranyl acetate and lead citrate, and then observed with a Philips CM 10 transmission electron microscope operating at an accelerating voltage of 80 kV.

3. Results

3.1. The structure of the male reproductive system

The paired ovoid-elliptic testes of males of *Z. impolitus* vary in size depending on the stage of sexual maturity. They are larger in young adult males, about 500 μm long and 300–350 μm wide (Fig. 1A). In older males, the two testes often differ distinctly in size. An unusual condition observed in some cases is that one of the testes appears strongly reduced. The testis follicles are always indistinct. Each testis is connected to an unusually large seminal vesicle (700 μm long, 350–400 μm wide) through very short deferent ducts which are indistinct (Fig. 1A). An elongated (800–1200 μm) cylindrical ejaculatory duct (diameter from 100 μm proximally to 30 μm distally) originates from the seminal vesicle and connects it with an accessory gland complex (Fig. 1A, B). Four types of accessory glands are present (Fig. 1A, B): two are whitish, closely adhering to each other, and of equal size (400 μm long and 100 μm wide); a third pale-blue gland (500 μm long and 70–100 μm wide) is associated with the whitish glands; the fourth gland is translucent, cylindrical and elongated (1000 μm long and 50 μm wide), and located opposite to the pale-blue gland. Posteriorly the accessory gland complex opens into a duct which is connected to the external genitalia posteriorly (Fig. 1B).

3.1.1. The spermiogenesis

The testes of *Z. impolitus* do not contain cysts of germ cells at different stages of maturation. The entire lumen appears filled with elongating spermatids, which appear sectioned at different levels in Fig. 2A (inset).

At an early stage of spermatid differentiation, the nucleus is large and elliptic, with a granular appearance and surrounded by microtubules, some cisterns of rough endoplasmic reticulum, and many electron-transparent vesicles (Fig. 2A, B). The acrosome is extremely large and irregularly shaped (about 12–15 μm wide), with a dense and compact content (Fig. 2A, B). Mitochondria fuse to form a large “Nebenkern” (Fig. 2A); at a later stage this structure forms two very large derivatives (8 × 5 μm) with a uniform, moderately dense matrix; cristae are still recognizable along their inner membrane (Fig. 2C). In some sections, mitochondrial derivatives show small areas with a more electron-dense appearance, which are surrounded by bundles of membranes (Fig. 2D). Toward the posterior region of the spermatids (only 3.5–2.5 μm in diameter), the derivatives appear kidney-shaped in cross-section (about 1.5 × 0.5 μm) and the matrix is dense (Fig. 3B). At this stage, sperm flagella with a large electron-transparent derivative on one side, and with a smaller and much denser derivative on the opposite side, can be observed quite frequently. Between the two mitochondria a cross-sectioned axoneme is visible in Figs. 2B, 3C and D. In a longitudinal section, these derivatives show an irregular shape (Fig. 3A).

When the process of spermiogenesis is almost completed, the shape of the nucleus is cylindrical (4.5–5 μm in diameter). The acrosome is as large as the nucleus in cross-section and both organelles are surrounded by a layer of microtubules (Fig. 2E and inset). In the flagellum the two mitochondrial derivatives appear equal in size and shape, with a dense matrix that shows a thin layer of pale material along their inner face (Fig. 3B–D). In addition to the mitochondrial derivatives, two accessory bodies are visible on both sides of the axoneme; in cross-section, the entire complex appears pagoda-shaped; each accessory body has a trapezoid shape and appears divided into two parts by a septum (Figs. 3D, 4A). The flagellar components described above are surrounded by two closely adherent narrow cisterns; the outer one envelopes the accessory body complex, whereas the inner