Arthropod Structure & Development 45 (2016) 301–306

Contents lists available at ScienceDirect

Arthropod Structure & Development

journal homepage: www.elsevier.com/locate/asd

Mud and silk in the dark: A new type of millipede moulting chamber and first observations on the maturation moult in the order Callipodida

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ARTICLE INFO

Article history:
Received 18 March 2016
Accepted 8 April 2016
Available online 22 April 2016

Keywords:
Postembryonic development
Anamorphosis
Cave
Spinneret

ABSTRACT

The postembryonic development of millipedes includes a series of stadia separated by moults, a process known as anamorphosis. The moulting process and especially the moulting into maturity, i.e., with fully developed copulatory organs, remains unknown for most millipede species. We have kept specimens of *Lusitanipus alternans* (Verhoeff, 1893) in the laboratory for one year and studied its moulting process, including the first study of the maturation moult in the order Callipodida. Unlike the typical silk cocoon reported for other callipodidans, this species builds a new type of solid moulting chamber, using the available substrate reinforced by a silken web. We present the detailed ultrastructure of the moulting chamber and silk. It takes five days to build the moulting chamber and between 29 (female) and 35 (male) days to shed the exuviae. The male maturation moult is preceded by an evagination of a gonopodal sac between the 6th and 7th body rings, in which the gonopods are developed. Females evaginated completely their vulval sacs, retracting them after shedding the exuviae. Vulval sac size seems to increase with the progressive reduction of the second pair of legs.

1. Introduction

Millipedes (Diplopoda) play a very important role as detritivores in many terrestrial ecosystems (David and Handa, 2010). They take active parts in nutrient cycles by fragmenting, decomposing and transporting organic material, as well in the dispersal of propagules of bacteria, fungi and arbuscular mycorrhiza through the soil (Snyder and Hendrix, 2008; Rabatin and Stinner, 1988).

However, millipedes also use the substrate in more active ways. Thus, some millipedes construct individual capsules ("oothecae") for their eggs (e.g., Aouti, 1980), or build "nests" for numerous eggs, using fecal pellets and/or undigested substrate (Minelli and Michalik, 2015).

The postembryonic development of millipedes includes a series of stadia separated by moults. At each moult new segments are added, at least in the beginning of the postembryonic development, a process known as anamorphosis (Enghoff et al., 1993). The moulting process and especially the moulting into maturity remains unknown for most millipede species (Minelli, 2015). Observation of moults is in some cases complicated by the fact that the millipede builds a moulting chamber of substrate (e.g., Adis et al., 2000) and/or by threads produced from spinnerets in their posterior end (telson) (Enghoff et al., 1993; Enghoff and Akkari, 2011; Minelli, 2015; Shear, 2008). Spinnerets are regarded as an ancestral trait of eugnathan millipedes, which has subsequently been lost in the "typical" cylindrical millipedes, Juliformia (Brewer and Bond, 2013; Minelli, 2015).

The composition of the secretions from millipede spinnerets is unknown. Only Adis et al. (2000) reported that threads produced by spinnerets of some polydesmidan millipedes apparently consist of acid mucopolysaccharides. In spite of the lack of knowledge on the composition of these secretions, we shall use the term silk like previous authors referring to secretions from millipede spinnerets.

The order Callipodida contains medium-sized to large millipedes distributed in warm-temperate zones of the northern

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http://dx.doi.org/10.1016/j.asd.2016.04.001
1467-8039/

Fig. 1. *Lusitanipus alternans* building the moulting chamber with muddy substrate and silk.

hemisphere. Adult males of Callipodida have one of the two leg-pairs on body ring seven transformed into gonopods; in late juvenile stadia of males, this leg-pair is strongly reduced. In adult females of many Callipodida, including *Lusitanipus alternans* (Verhoeff, 1893), the second pair of legs is strongly reduced (Reboleira and Enghoff, 2015). Callipodidan millipedes have well-developed spinnerets, and the construction of silk cocoons for moulting have been reported for *Acanthopetalum carinatum* (Brandt, 1840), *Abacion texense* (Loomis, 1937), *Callipus foetidissimus* (Savi, 1819) and *Prolysioptealum scabratum* (L. Koch, 1867) (Fanzago, 1874; Nguyen Duy-Jacquemin, 1979; Enghoff and Akkari, 2011).

We have kept 30 specimens of the endemic Portuguese callipodidan *L. alternans* for one year, simulating environmental conditions in the laboratory, and studied its moulting process, as well as its moulting chambers, for the first time.

2. Material and methods

Specimens of *L. alternans* were collected in caves in central Portugal (Reboleira and Enghoff, 2014) and transported in cold dark chambers to the Natural History Museum of Denmark, University of Copenhagen where they were kept in a dark chamber for one year at 17 ± 0.2 °C.

The millipedes were acclimatized for 4 weeks in Petri dishes with water-saturated gypsum and were subsequently installed in plastic boxes (19 × 12 × 5 cm) or in Petri dishes (diameter 7 cm) with autoclaved natural sediment (cave clay). They were fed every week with organic vegetables. High humidity was maintained by adding water to the substrate weekly.

Specimens were observed periodically out of their dark chamber. In most cases the moulting chamber was built adjacent to the walls of the containers, allowing observation of the moulting process without opening it. In other cases the moulting chambers were carefully opened for observation and closed immediately.

Microphotographs were taken using a Leica digital camera M205A mounted on a stereomicroscope Leica DFC 420. Images were processed with a Leica Application Suite program and final stacking made using Zerena software. For scanning electron microscopy (SEM), parts of the specimens and moulting chambers were critical point dried in a Tousimis Autosamdi 815, serie A. Other samples were transferred to 96% ethanol then to acetone, and air-dried. The dried specimens were mounted on aluminium stubs, coated with platinum/palladium and studied in a JEOL JSM-6335F scanning electron microscope. SEM Images were processed with Adobe Photoshop CS6.

Material is deposited in the Natural History Museum of Denmark, Zoological Museum, University of Copenhagen (ZMUC).

3. Results

Among the 30 specimens kept, only four moulted. They all moulted only once during one year. The first millipede built a moulting chamber exactly four months after being installed in laboratory conditions (Fig. 1). Building and closing the chamber took five days altogether.

The moulting chamber is built of the natural substrate, which is reinforced with a mesh of silken web conferring stability to the construction (Fig. 2). The natural sediment is previously processed by the mouthparts while building the chamber. We could not observe if the building material had passed through the millipede's gut – we only saw millipedes manipulating substrate with their mouthparts and first legs. Inside the moulting chamber there is a very thin layer of white silk, only visible trough the microscope, between the animal and the chamber wall.

The silk used in this construction is produced by the spinnerets that are located on the telson of the millipede (Fig. 3). The threads have a diameter between 1.5 and 2.4 μm (Fig. 2B). The moulting chamber constitutes a solid shelter with microscopic pores around 25 μm diameter (Fig. 2C), which enable gas exchange with the outside.

During the acclimatization process a juvenile specimen began to produce a moulting chamber with gypsum from the floor of the Petri dish, clear evidence that *L. alternans* will produce the moulting chamber with the available substrate.

After closing the moulting chamber, the millipedes were wound into a spiral with the head in the centre (Fig. 4). Table 1 summarises our observations on the duration of the moulting process.

A subadult male remained in a lethargic state for 35 days before moulting into a mature male with fully developed copulatory organs, gonopods (Fig. 4A). During this period we observed