Zoologischer Anzeiger 272 (2018) 20–28

Contents lists available at ScienceDirect

Zoologischer Anzeiger

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

Zoologischer Anzeiger

Morphology of the male reproductive system during post-embryonic development of the termite *Silvestritermes euamignathus* (Isoptera: Termitidae)

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ARTICLE INFO

Article history:
Received 28 March 2017
Received in revised form
28 November 2017
Accepted 28 November 2017

Keywords:
Histochemistry
Reproductive system
Seminal vesicles
Testicles
Vas deferens

ABSTRACT

Despite the recent research on Isoptera reproduction strategies, few studies have focused on the morphophysiology of the reproductive system in Neotropical termite species. The classic literature emphasizes the development of the female reproductive apparatus, generating a knowledge gap on the male reproductive system, including its neotenic differentiation and the evolution of the seminal vesicles. The development of the reproductive system in males of *Silvestritermes euamignathus* was followed from first-instar nymphs until primary kings, with emphasis on the histology of the gonads and seminal vesicles of primary and neotenic kings from field colonies. The maturation of the reproductive system follows post-embryonic development, reaching a peak of development in primary functional kings from field colonies. The testicles are already present in first-instar nymphs and show a progressive development that is completed in primary kings, which are characterized by an enlarged size and greater sperm production. Although spermatogenesis is observed in third-instar nymphs, only in fifth-instar nymphs are spermatozoa present in the testicles and in the seminal vesicles. Spermatozoa are also present in the testicles of neotenic reproductives, derived from different nymphal instars. After the foundation of a new colony and the maturation of the king, there is a significant increase in the reproductive apparatus and the testicular lobes, which mostly occupy the whole posterior abdomen in kings from field colonies. These individuals have a minimum of seven testicular lobes that are completely separated from each other and contain rounded, aflagellated spermatozoa, which are approximately 2–3 μm in diameter. Four-month old kings from laboratory colonies seem to be in a period of reproductive pause, as they display fewer sperm in the seminal vesicles. The seminal vesicles are specialized portions of the vasa deferentia that play a role in storage of spermatozoa. These structures produce a proteinaceous secretion in alates that is not observed in kings. The overall results indicate early maturation of the reproductive system in males of *S. euamignathus*; furthermore, the reproductive status of the different individuals might be linked to the development of the testicles and the production, storage and release of spermatozoa. The lack of a proteinaceous secretion in the seminal vesicles of kings, in comparison to alates, might be related to the maturation of the individuals after colony establishment. The development of the male reproductive system is consistent with queen physogastry, but in more discreet proportions.

1. Introduction

The infraorder Isoptera comprises over 2900 species of termites worldwide, covering nine families according to the classification by Krishna et al. (2013). Approximately 600 termite species are distributed into five families in the Neotropical region, with Termitidae as the dominant family, having more than 420 species in the Neotropics (Constantino, 2017).

The caste system of Termitidae follows a bifurcated pattern, and post-embryonic development can follow one of two lines. In the first line, a first-instar larva emerges from the egg and molts into a second-instar larva that will become either a worker or a presoldier, which will later become a soldier. The individuals that follow this pathway belong to the apterous lineage. In the second line, the first-instar larva molts into a first-instar nymph, which will have successive molting events until become an imago or alate. These

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https://doi.org/10.1016/j.jcz.2017.11.015

individuals, the imago and its predecessors, belong to the imaginal lineage (Noirot, 1969; Roisin, 2000).

Caste differentiation occurs during ontogenetic development and may be influenced by both environmental (Mao and Henderson, 2007) and genetic factors (Hayashi et al., 2007; Matsuura et al., 2009; Kitade et al., 2011). Sometimes, the individuals deviate from their differentiation pathway and generate the so-called neotenic reproductives (Myles, 1999). This occurs either due to changes in the environment, e.g. when the colonies are orphaned (Costa-Leonardo and Barsotti, 1998), or by genetic influences, e.g. asexual queen succession through parthenogenesis (Matsuura et al., 2009; Vargo et al., 2012; Luchetti et al., 2013; Fougeyrollas et al., 2015; Fournier et al., 2016; Matsuura 2017; Fougeyrollas et al., 2017; Hellemans et al., 2017). Neotenic termites may originate from either nymphs or workers, being respectively called nymphoids and ergatoids (Myles, 1999).

Many nymphoid neotenics of both sexes have been widely described in nests of *Silvestritermes euamignathus* (Noirot, 1956; Araújo, 1958; Noirot, 1969; Grassé, 1982; Costa-Leonardo et al., 1996, 1998, 1999; Haifig et al., 2016). This termite species is characterized by building epigeal mounds, very common in Cerrado vegetation (Mathews, 1977; Domingos, 1985). According to Haifig and Costa-Leonardo (2016), the imaginal line of *S. euamignathus* presents one larval and five successive nymphal instars during its post-embryonic development, with nymphoids differentiating from the last three nymphal instars. The neotenics from the same colony may present different morphologies: females with various degrees of physogastry and males with different wing bud sizes and body pigmentation (Costa-Leonardo et al., 1999; Haifig et al., 2016).

The reproductive biology of termites has been widely studied over the last years, as the reproductive mechanism is one of the factors that define the relationships among the castes of a single colony (Hartke and Baer, 2011). On the other hand, the physiological details involved in the production, transference and storage of spermatozoa in termites have received little attention (Dean and Gold, 2004; Ye et al., 2009). The king remains with the queen, and mating occurs intermittently, with oviposition cycles in some species (Raina et al., 2007). According to Hartke and Baer (2011), the ability of termite kings to supply large quantities of viable spermatozoa to the queen, throughout decades, is as important as the fact that some female hymenopterans are capable of storing spermatozoa from their deceased partners for a similar period of time. However, the study of the reproductive system in kings has been neglected when compared to that of queens.

Herein, this study focused on a detailed morphological analysis of the gonads in males of imaginal line, throughout post-embryonic development and its deviations into neotenics, aiming to understand the role of different functional males of *S. euamignathus* in reproduction.

2. Materials and methods

2.1 Termites: In this study, the following males of *Silvestritermes euamignathus* (Silvestri, 1901) were used: alates (collected during swarming); four-month old primary kings (collected from laboratory colonies); primary and nymphoid neotenic kings from field nests. Nymphs of this species were collected directly from nests and classified into five instars (N1 to N5), according to the morphometric analyses performed by Haifig and Costa-Leonardo (2016). The neotenic kings were collected from a field nest headed by 31 females and 29 males and lacking the primary royal couple. The neotenic kings and queens were active in reproduction (functional) because eggs were found in the nest. At least three nymphs of each instar were used for histology, and several alates and kings of different ages were used for dissection (n = 21), for histology (n = 7) and for TEM (n = 3).

2.2 Anatomy of the reproductive system: Twenty-one male alates of *S. euamignathus* were dissected in a saline solution for insects (0.1 M NaCl, 0.1 M Na_2HPO_4, 0.1 M KH_2PO_4), and reproductive organs were isolated under a stereomicroscope. These organs were individually placed on a microscope slide, stained with 1% methylene blue solution, and imaged with the aid of Motic-CAM (Causeway Bay, Hong Kong) camera. Measurements of the length and width of the vas deferens and seminal vesicles were obtained using Motic Image Plus 2.0 ML software.

2.3 Histology and histochemistry: The final abdominal portion of male nymphs of five different instars (N1-N5), male alates, four-month old kings from laboratory colonies and primary and neotenic kings from field colonies of *S. euamignathus* were fixed in FAA (absolute alcohol, acetic acid and formaldehyde, 3:1:1) for approximately 24 h. Later, they were dehydrated in a graded series of ethanol concentrations (70-95%) and transferred to an infiltration resin solution (Leica®). The samples were embedded laterally with Historesin (Leica®) plus catalyzer for polymerization, and 3 μm sections were prepared using a Leica RM2245 microtome. The sections were stained with hematoxylin-eosin and observed under a photomicroscope (Leica DM500/Leica ICC50). Sections of the abdomens of alates and kings were also stained with PAS and xylidine-Ponceau for detection of polysaccharides and total proteins, respectively. Images were recorded with the aid of LAS v4.0 software (Leica Application Suite v4). A quantitative analysis of the testicular area using the five most sagittal sections of the testicles, certified by the lumen of the vas deferens, from different individuals was performed using Image J 1.50i software. Data were logarithmically transformed to achieve both normality and homoscedasticity (Crawley 2007), and analyzed using one-way ANOVA followed by Tukey HSD for multiple comparisons. The analyses were performed in the R program, version 3.3.1 (R Core Team 2016).

2.4 Transmission Electron Microscopy (TEM): The abdomens of male alates were fixed in 2.5% glutaraldehyde, post-fixed in 1% OsO_4, and embedded in Araldite epon resin. The samples were embedded in pure Epon resin and polymerized at 60 °C for 72 h. Ultrathin sections (60–90 nm) were obtained using a Leica Reichert Supernova ultramicrotome and contrasted with uranyl acetate 4% and lead citrate. The material was observed under a Philips CM100 transmission electron microscope (operating at an accelerating voltage of 80 kV) and photographed with a Veleta camera using iTEM software (v. 5.2).

3. Results

The reproductive system of males of *S. euamignathus* consists of two testicles, from which arise two vasa deferentia that end into a single ejaculatory duct (Fig. 1A). Each vas deferens, before reaching the ejaculatory duct, presents a discrete dilation that corresponds to the seminal vesicle. The ejaculatory duct ends in a rudimentary copulatory organ, the penis, which protrudes through the genital pore, located in the intersegmental membrane between the 9th and the 10th abdominal sternites.

A pair of lobed testicles are located laterally to the digestive tube, in the dorsal region of the abdomen, in all individuals. The testicular lobes, vasa deferentia and seminal vesicles are very similar in size and development in fifth-instar nymphs (Fig. 1B) and alates. In neotenic kings and in primary kings from field colonies, testicle development is more conspicuous (Fig. 1C and D), occupying the whole abdomen in primary kings. The testicular lobes of all males are composed of a set of seminiferous tubules, which are joined together by a surrounding capsule, the peritoneal sheath. Spermatogenesis occurs inside the seminiferous tubules that are