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# Testicular development and modes of apoptosis during spermatogenesis in various castes of the termite *Reticulitermes labralis* (Isoptera:Rhinotermitidae)

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ABSTRACT

The separation of reproductive and non-reproductive roles based on caste differentiation is the most prominent characteristic of termites. However, little is known about the mechanism of male reproductive division that underlies caste differentiation. In the present study, testicular development and stage-specific apoptotic patterns were investigated and compared during spermatogenesis in reproductives, workers and soldiers of the termite *Reticulitermes labralis*. The results showed that male workers were divided into two types, the workers with spermatozoa ($W_S$) and the workers without spermatozoa ($W_N$). Spermatogenesis in $W_N$ and soldiers arrested at the spermatocyte stage. Moreover, there were significant differences in testicular size and spermatogenesis among the various castes. The mode of apoptosis in late instar $W_S$ was similar to the reproductives, as demonstrated by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) analysis. First, the majority of apoptotic cells were spermatogonia, and the spermatogonia of both late instar $W_S$ and reproductives exhibited lower apoptotic rates compared with late instar $W_N$ and soldiers. Second, the spermatocytes and spermatids showed very little apoptosis in the late instar $W_S$ and reproductives, and no TUNEL signal was detected in any of the examined spermatozoa. Our findings suggest that the male workers undergo a basal developmental schema comprising two undifferentiated larval instars, followed by a bifurcated development into either (i) the sexual lineage, in which the workers are able to provide normal spermatozoa to queens, or (ii) the neuter lineage, in which the male workers lose reproductive options. The level of testicular development may explain the significant discrepancies in reproductive capacity among the reproductives, workers and soldiers and reveal the reproductive division in male workers. These differences are controlled by apoptosis during early spermatogenesis.



## 1. Introduction

All existing termites are eusocial insects. Generally, eusociality is defined by the (i) overlap of generations, (ii) cooperative brood care, and (iii) reproductive division of labour. The latter is the key feature of eusocial insects (Michener, 1969). The reproductive division among the different castes of a termite colony is primarily triggered by environmental stimuli, allowing reproductives, workers, and soldiers to develop within a colony with essentially the same genetic background (Korb et al., 2009). In addition, genetic diversity may be related to the differentiation of intraspecific groups (Frati et al., 1992). Generally, reproductives lay eggs, and sterile workers and soldiers care for younger siblings and provide colony defence, respectively (Korb, 2015). Lower termites are characterized by a unique flexibility in development. In the genus *Reticulitermes* within Rhinotermitidae, the workers can develop in three ways: (i) via moults into higher instar workers, (ii) via two successive moults into soldiers, or (iii) via one moult into apterous neotenic reproductives that develop in the absence of or at a great distance from the primary reproductive to provide for continued or additional growth of the colony (Korb and Hartfelder, 2008; Korb et al., 2009). In addition, it was reported that the male workers of *Reticulitermes speratus* can develop into inconspicuous reproductive males without undergoing a moult and without exhibiting any significant change in morphology (Fujita and Watanabe, 2010). The reproductive plasticity of the workers gives colonies tremendous

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flexibility in response to environmental change and the deterioration of the nest and food resources. Dominant individuals typically have well-developed gonads (Geva et al., 2005). In many social species of Hymenoptera, such as ants, wasps and honeybees, reproductive differentiation is controlled by gonadal activity (Koudji and Doumandji, 2008; Okada et al., 2010). In the termite *R. speratus*, the testis size of the workers increases from approximately 0.2 mm–0.4 mm during development into neotenic reproductives (Fujita and Watanabe, 2010). Therefore, gonadal development in the termite would appear to be the most significant biological process that leads to the formation of caste-specific differences in tasks and status.

Spermatogenesis is a complex process of proliferation and differentiation, transforming spermatogonia into mature spermatozoa. In insects, this process involves spermatogonial proliferation by repeated mitotic divisions, resulting in a group of primary spermatocytes. These spermatocytes undergo two successive meiotic divisions and give rise to spermatids, which, after deep morphological changes (spermiogenesis), form sperm bundles (Dallai, 2014). Functional spermatogenesis requires an intrinsic regulation, which is managed by a conserved genetic program that underlies the development of germ cells, ensuring sperm viability. The lack of an efficient control mechanism may lead to spermatogenetic alterations, which, in turn, can result in the production of damaged spermatozoa (Dias et al., 2013, 2015). Aberrant spermatogenesis in the two species *Stenognathellus denisi* and *Sminthurides aquaticus* (Collembola) is described, in which the secondary spermatocytes with a small size and reduced cytoplasm are not able to perform the second meiotic division and degenerate (Dallai et al., 2004). The number of sperm produced can be affected by environmental conditions, such as food deprivation, a condition capable of determining a significant reduction in the number of sperm produced by the moths *Plodia interpunctella* and by the dipterans *Sarcophaga stercoraria* and *Drosophila melanogaster* (Gage and Cook, 1994; Hellriegel and Blanckenhorn, 2002; Amitin and Pitnick, 2007). In addition, the number of sperm also depends on the size of both sperm and testis (Pitnick et al., 2009). However, there have been no studies to examine the characteristics of spermatogenesis in workers and soldiers of termites.

During spermatogenesis, the balance between proliferation and cell death signals determines the maturation fate of the germ cells. Apoptosis, also known as programmed cell death, often denotes an active and highly choreographed process of cell suicide and may be triggered by several factors, including intrinsic signals and external stimuli (Baum et al., 2005). Invertebrates and vertebrates display many similarities in the use of apoptosis to eliminate dangerous or damaged cells and regulate cell numbers by removing excess cells during spermatogenesis. Failure of apoptosis to selectively deplete abnormal germ cells earmarked for programmed cell death would lead to abnormal spermatozoa in the ejaculate and male infertility (Sakkas, 2003). In the insect Lepidoptera, the degenerative changes in the germ cells of the testis during pupal diapause are controlled by apoptosis, and apyrene sperm development occurs by the apoptosis of their nuclei (Polanska et al., 2005; Shimoda et al., 2007). One of the best studied aspects of apoptosis in social insects occurs during caste determination in bees (Baum et al., 2005). In the honeybee *Apis mellifera*, an adult queen ovary contains as many as 200 ovarioles, whereas the ovary of a worker has less than 10. This large discrepancy in ovariole number is a result of apoptosis (Capella and Hartfelder, 1998). In the termite *Reticulitermes aculabialis*, our previous studies also indicate that apoptosis during early oogenesis is an efficient and crucial regulatory mechanism affecting the number of oocytes and the reproductive capacity of the reproductives, soldiers and workers (Su et al., 2014). Therefore, the available evidence suggests that apoptosis is not only necessary for the normal development of germ cells in many organisms but also plays a vital role in the caste differentiation in social insects.

Although *Reticulitermes* is an economically important genus and is probably the most studied of all termites, the precise development pattern in workers and soldiers remains uncertain. The separation of reproductive and non-reproductive roles based on caste differentiation is the most prominent characteristic of termites, and the clarification of the mechanism underlying this separation is an important topic in termites. The difference in the physiological detail of testis development among workers, soldiers and reproductives is unclear, although the general morphology of the male reproductive system and sperm of termites have received attention (Sieber and Leuthold, 1982; Riparbelli et al., 2009; Hartke and Baer, 2011). To obtain a better understanding of the development of the testes underlying reproductive division in various castes and to reveal whether each male worker has reproductive flexibility, spermatogenesis and apoptotic patterns were investigated and compared in reproductives, workers and soldiers of the termite *Reticulitermes labralis*.

## 2. Materials and methods

### 2.1. Termites

*R. labralis* colony fragments were collected from the monasteries of Xi'an, China, in May 2011 when the alate adults were swarming. The colonies were comprised of workers, soldiers, and alate reproductives. Newly hatched individuals (larvae) developed into workers (third instar workers) or nymphs (third instar nymphs) through two successive moults, distinguished by the absence or presence of wing pads, respectively. The third instar workers ($W_3$), fourth instar workers ($W_4$) and fifth instar workers ($W_5$) (early instar workers) were identified by antennal segments, the width of the heads and the length of the bodies. Sixth instar workers ($W_6$) (late instar workers, $W_L$) were identified by the presence of 16 or more antennal segments. The male reproductive and late instar worker individuals were discriminated according to the seventh sternite, and the male early instar workers and soldier individuals were discriminated by longitudinal sections. After collection from the field, the abdomens were immediately removed and fixed in Bouin's solution for HE (haematoxylin and eosin) staining. They were also fixed in 4% paraformaldehyde in PBS overnight at 4 °C for terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labelling (TUNEL) analysis.

### 2.2. Measurement of testis development in reproductives, workers and soldiers

The testes of the reproductives and $W_6$ were dissected out in PBS under a stereomicroscope to examine the testis morphology of *R. labralis*. The testicular development of the workers, soldiers and reproductives was evaluated with HE staining. The fixed samples were dehydrated in an ascending ethanol series and embedded in paraffin. Longitudinal sections 6 μm thick were collected on polylysine-coated slides. Deparaffinised and rehydrated sections were stained in HE. Because the testes of *R. aculabialis* had a mostly round shape, the diameter of each testis at its widest point was measured using a digital microscope in accordance with a previous study on *R. speratus* (Shimada and Maekawa, 2010).

### 2.3. Spermatogenesis in various castes

Thin sections through the testes showed different stages of spermatogenesis in the testicular follicles of the reproductives,