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Neotenic formation in laboratory colonies of the termite *Coptotermes gestroi* after orphaning

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Abstract

The termite *Coptotermes gestroi* (Wasmann 1896) (Rhinotermitidae: Coptotermitinae) is an exotic species in Brazil and information concerning its reproductive developmental biology is scarce. We induced the formation of neotenics in laboratory colonies through orphaning experiments. Orphaning experiments were conducted in three-year old colonies of *C. gestroi* kept under laboratory conditions. After three months, eight nymphoid neotenics were observed in one colony after queen removal. Histological analysis showed that these neotenics were non-functional. The results suggest that these individuals may have arisen from the first nymphal instar (N1) or from an early N1 instar after one or two larval moults. Neotenics also were recorded on two incipient colonies of *C. gestroi* that lost the queen naturally.

Keywords: subterranean termites, castes, nymphoid reproductives, queen replacement, orphaning

Introduction

Termites are hemimetabolous insects with colonies constituted of reproductive and immature individuals. The plasticity of the caste system in termite societies is essential for neotenic reproductive formation, mainly in lower termites. Neotenic reproductives are individuals with juvenile characters that may replace the primary reproductives (king and queen) in a colony (Noirot 1969; Roisin 2000). According to Thorne (1996) the term neotenic reproductive refers to any reproductive termite that is not derived from an alate. These individuals can develop from a variety of instars and from individuals with or without wing buds (Thorne 1997).

The subterranean termite *C. gestroi* is a native species of Asia, but it was accidentally introduced into the southeast region of Brazil from marine cargo, probably at the beginning of the 20th century (Araujo 1958). This species has been misidentified as *C. havilandi* (Kirton & Brown, 2003) and is responsible for extensive damage in urban areas of São Paulo State (Lelis 1999; Costa-Leonardo *et al*. 1999). *C. gestroi* form large field colonies, which produce all-year-round nymphs and non-functional neotenics, even in the presence of the imaginal pair (Costa-Leonardo *et al*. 1999; Costa-Leonardo 2002; Costa-Leonardo & Arab, 2004). Furthermore, functional neotenics were also found in colonies of this species (Lelis 1999). Functional neotenics are neotenics that have mature gonads and, sometimes, the neotenic females develop physogastry (Lenz & Barrett 1982; Lelis 1999); however, the physogastry of neotenics is less developed than that of the primary queens. On the other hand, the knowledge of the fecundity of functional neotenics is limited. The incidence of neotenics varies greatly among *Coptotermes* species, being mainly of the nymphoid type (Myles 1999). Thus, in order to understand some aspects of the secondary reproduction in *Coptotermes gestroi*, we followed the neotenic induction in laboratory colonies after orphaning.

Materials and Methods

Male and female alates of *Coptotermes gestroi* were collected after dispersal flights in August in the city of Rio Claro, São Paulo State, Brazil (22º 23' S, 47º 32' W). Individuals were sexed and each couple was placed in a Petri dish (6 cm diameter) containing sawdust as food source. One hundred couples were used to set up the colonies. After five months, the new-formed incipient colonies were transferred to 250 ml plastic containers filled with decayed sawdust of *Pinus* sp. and *Eucalyptus* sp. moistened with distilled water. Sawdust and water were added periodically during the study period. After three years, the colonies were dismantled and the neotenics were collected and subjected to morphometric analysis. We measured the head width and the first wing bud length because these body parts are the most affected by growth at each molt in the imaginal pathway of *C. gestroi* (Barsotti 2001). The number of antennal segments, body color, fresh weight, body length, posterior tibia length, and the presence of body injuries in these individuals were also recorded. The measurements were performed according to Roonwal (1969). The data were compared to those obtained for nymphal instars mentioned in early studies (Costa-

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Leonardo *et al*. 1999; Barsotti 2001) with the purpose of establishing the developmental origin of the neotenics.

Orphaning experiment

Twenty colonies of *Coptotermes gestroi* were set up from an imago couple collected during the swarming season in Rio Claro city. These colonies were first placed in Petri dishes (6 cm of diameter) and transferred twice to larger containers. Three years later, the queen was removed from ten of these young colonies and the king from the other ten remaining colonies. All the orphaned colonies contained eggs, larvae, workers, soldiers and both primary reproductives at the time of orphaning. To minimize disturbance, the first examination of the orphaned colonies was performed after three months. Afterward, the colonies were examined after 5, 8, and 12 months following the removal of the primary reproductives. Neotenics were subjected to the same morphometric analysis described previously. Mann-Whitney U test (p=0.05) (Sokal & Rohlf 1995) was performed to analyze median differences of head width and wing bud length between neotenics obtained in the laboratory colonies (described above) that lost the queen naturally (control group) and from orphaning experiments.

Histological analysis

Histological analysis was used to determine reproductive functionality of the neotenics individuals. The neotenics obtained from both natural and orphaned colonies were fixed in FAA fluid (formalin: acetic acid: alcohol =1:1:3) and embedded in JB4 resin. The material was sectioned and stained with hematoxylin-eosin and toluidine blue. The sections were then analyzed and photographed under a Zeiss light microscope. The results were compared to those obtained for primary queens from laboratory colonies.

Results

Nymphoid neotenics were obtained naturally only in two colonies (A and B) of *C. gestroi* each formed from a single pair of alates and kept under laboratory conditions for three years. These colonies contained workers, soldiers, and the king, however the primary queens were absent. The neotenics collected were all females, showing a thin body, a slightly yellow color, and 14 antennal segments. Ten neotenics were obtained from colony A, with a fresh weight ranging from 3.1 – 4.4 mg, head width ranging from 0.94 – 1.01 mm, wing bud ranging from 0.31 – 0.47 mm in length, and hind tibia ranging from 0.78 – 0.94 mm in length. In colony B we found only one neotenic that showed smaller wing buds (Table 1). All the neotenics found in colonies A and B had slightly yellow pigmentation and lacked compound eyes. Some of these individuals were missing some antennal segments, legs, and had injuries at the genital plates. The other 98 colonies did not produce neotenics and the royal pair was present.

Neotenics were also observed three months after removal of the primary reproductives in young colonies of *C. gestroi*. The histological analysis showed that the female neotenics were non-functional because the ovaries of these individual showed only primary oocytes (Fig.1A) and the spermatheca were always empty (Fig. 1B). Male neotenics showed slight developed testicles with indistinct lobes (Costa-Leonardo unpublished). Conversely, ovaries of queens of laboratory colonies showed ovarioles containing both primary and terminal oocytes, and spermatheca containing spermatozoa. Non-functional neotenics were recorded in eight queen-orphaned colonies. The number of neotenic females ranged from 1 to 28 and from 2 to 8 for neotenic males (Table 2). Colony 4 was the only one showing non-functional neotenics of both sexes (Table 2). The highest number of these individuals was recorded 8 months after queen orphaning. The number of non-functional neotenics decreased after twelve months of queen removal. All the individuals showed 14 antennal segments and a white or light yellow body color (Table 2). Some of the individuals had injuries on their body. On the other hand, only one non-functional male neotenic was observed in one of the king-orphaned colonies. This individual had a fresh weight of 3.8 mg, a head width of 0.94 mm, a body length of 3.98 mm, a wing bud length of 0.16 mm, and a hind tibia length of 0.70 mm. Other individuals observed at the time of orphaning included the king or queen, larvae, workers, and soldiers. Neotenics were not registered when larvae were not present in the colonies at the time of orphaning (Table 2).

The fresh weight of the neotenics from orphaned colonies ranged from 2.4 to 5.5 mg and the mean wing bud length appeared to be smaller for the individuals of colonies examined after three months of orphaning (Table 3). Nevertheless, some colonies showed neotenics with different wing bud length (Fig. 2). Comparison of head width and wing bud length between neotenics obtained naturally (after queen death) and those from orphaning experiments showed that the median head-width was significantly larger for neotenics obtained naturally (Mann-Whitney U-test, U= 68.00, p< 0.0001); however, there were no differences of wing bud length medians between these individuals (Mann-Whitney U-test, U= 446.00, p= 0.6722). Analysis of the head width and wing bud length obtained from nymphs of field colonies revealed that there were six instars in the nymph developmental pathway of *C. gestroi*. During nymph

Table 1. Mean ± SE of the measurements (mm) and fresh weight (mg) of *Coptotermes gestroi* neotenics found in two laboratory colonies after the queen's death.

Colony	N*	Fresh weight	Head width	Body length	Wing bud length	Hind tibia length
A	10	3.7 ± 0.46	0.99 ± 0.04	4.34 ± 0.22	0.40 ± 0.12	0.83 ± 0.07
B	1	3.5	0.94	4.29	0.16	0.78

N: number of neotenics; *: all the neotenics were females.

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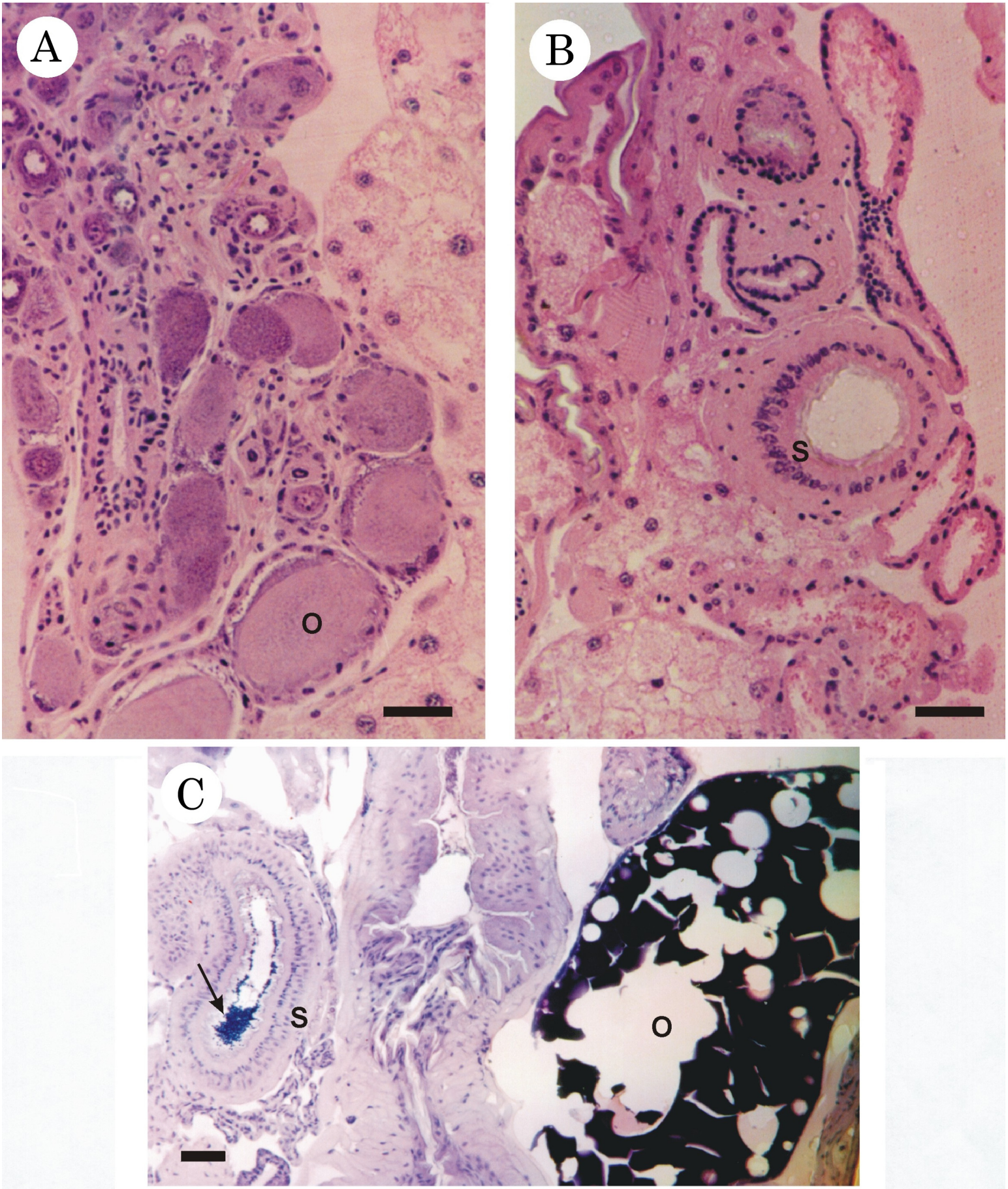

Figure 1. A. Longitudinal section of the ovary of female neotenic obtained after queen orphaning. Note only primary oocytes (O) in the ovarioles. B. Longitudinal section of the female neotenic abdomen showing the spermatheca (S) with the empty lumen. C. Longitudinal section of a three-years old queen abdomen showing the spermatheca (S) containing spermatozoa (arrow) and a large terminal oocyte (O).

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Table 2. Sex and number of neotinics found in young colonies of *Coptotermes gestroi* after queen removal

Colony	Time after orphaning (months)	N		Body colour
		Male	Female	
1	3	0	0	
2		8	0	White
3	5	0	1	White
4		2	1	
5		0	22	
6	8	0	11	White
7		0	28	Yellow
8	12	0	15	Yellow
9		0	0	
10		0	0	

N: number of neotenics. All the individuals showed 14 antennal segments and lack of eyes.

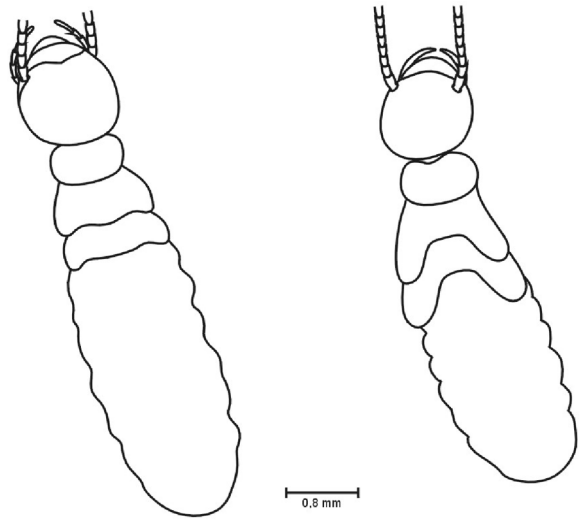

Figure 2. Neotenics from colony 7 opened eight months after queen removal. Observe the different wing bud lengths among the individuals.

differentiation, both the head width and wing bud length increased (Fig. 3). The plot of head width versus wing bud length also showed non-functional neotenics as a cluster of points close to the first nymphal instar (N1), suggesting that these individuals may have originated from N1 or any precursor of this instar (Fig. 3).

Discussion

Colonies of *C. gestroi* can readily replace primary reproductives with neotenics, which developed from nymphs as deduced from the presence of wing buds. Larvae available at the time of orphaning could possibly develop into nymphs and hence provide a supply of neotenics, as has been reported for *Coptotermes acinaciformis* (Lenz *et al*. 1988). Non-functional neotenics obtained in this study presented juvenile morphological characters, such as

Table 3. Range and mean ± SE (in parentheses) of the measurements (mm) and fresh weight (mg) of the neotenics that arising from queen orphaned colonies.

Colony	Time after orphaning	FW	HW	BL	WBL	HTL	Sex
2	3 months	2.70 – 3.70 (3.38 ± 0.31)	0.78 – 0.86 (0.85 ± 0.03)	3.74 – 4.06 (3.99 ± 0.18)	0.23 (0.21 ± 0.04)	0.55 – 0.62 (0.55 ± 0.19)	Males
3	5 months	4.1	0.94	4.29	0.47	0.86	Female*
4		4.6	0.94	4.68	0.39	0.86	Female*
		4.00 – 4.70 (4.35 ± 0.49)	0.86 – 1.01 (0.94 ± 0.11)	4.35 – 4.45 (4.41 ± 0.06)	0.23 (0.23 ± 0.00)	0.78 – 0.86 (0.78 ± 0.00)	Males
5		3.10 – 4.70 (3.66 ± 0.39)	0.86 – 0.94 (0.87 ± 0.02)	3.97 – 4.45 (4.13 ± 0.21)	0.23 – 0.47 (0.38 ± 0.06)	0.78 – 0.86 (0.78 ± 0.02)	Females
6	8 months	3.80 – 5.10 (3.49 ± 0.54)	0.86 – 1.01 (0.90 ± 0.05)	3.90 – 4.80 (4.04 ± 0.21)	0.39 – 0.62 (0.47 ± 0.10)	0.78 – 0.86 (0.79 ± 0.02)	Females
7		2.40 – 5.50 (4.53 ± 0.45)	0.86 – 1.01 (0.93 ± 0.04)	3.90 – 4.91 (4.41 ± 0.33)	0.39 – 0.62 (0.51 ± 0.07)	0.78 – 0.86 (0.79 ± 0.03)	
8	12 months	3.50 – 6.20 (4.00 ± 0.07)	0.86 – 1.01 (0.90 ± 0.05)	3.51 – 5.15 (4.25 ± 0.38)	0.16 – 0.47 (0.53 ± 0.02)	0.55 – 0.78 (0.33 ± 0.10)	Females

FW: fresh weight;
HW: head width;
BL: body length;
WBL: wing bud length;
HTL: hind tibia length.
*Only one individual was found in the colony.

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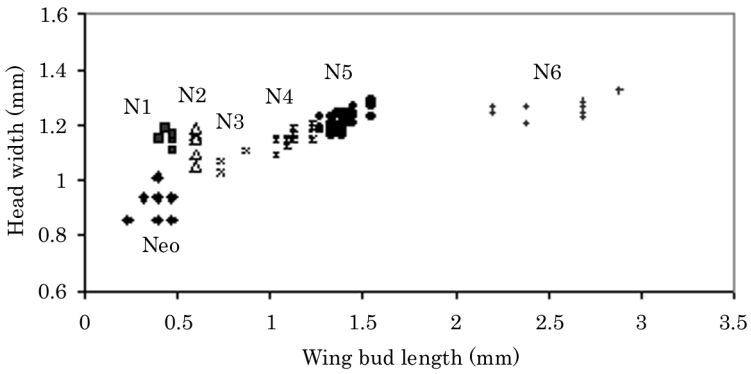

Figure 3. Wing bud length versus head width of neotenics obtained from orphaning experiments and nymphs from field colonies. Neo: neotenics; N1: 1st nymph instar; N2: 2nd nymph instar; N3: 3rd nymph instar; N4: 4th nymph instar; N5: 5th nymph instar; N6: 6th nymph instar.

wing buds and absence of eyes. Conversely, neotenics recorded from field colonies of *C. gestroi* had compound eyes and 15 to 17 antennal segments (Costa-Leonardo *et al*. 1999). These individuals were distinguishable from the other castes by a strong yellow pigmentation (Costa-Leonardo *et al*. 1999), different from the white or light yellow color of the neotenics that appeared after the orphaning manipulations. Neotenics obtained after orphaning in these nymph-less, three year old colonies, appeared to have been induced from precursor larvae that first transformed into N1 nymphs and then, through a second molt, into non-functional nymphoid neotenics. Therefore, because of their premature development, they failed to acquire pigmentation and compound eyes and had fewer antennal segments than the neotenics found in field colonies.

All nymphal instars of *C. gestroi* have compound eyes, which are white in N1 nymphs and become darker during the nymphal development that precede imagoes (5th and 6th nymph instar) (Barsotti 2001). The non-functional neotenics obtained in this study probably originated from N1 or from an early potential precursor nymph. The variation in wing bud length among the neotenics found in the same colony indicates that these individuals may have had different developmental origins. Neotenics can arise after one or two molts from N4-N6 in the *Reticulitermes* genera (Buchli 1956; Vieau 2001). At the orphaning time, the young laboratory colonies did not contain nymphs, and larvae may have originated an early nymphal instar after one or two molts.

According to Myles (1999), nymphoid and ergatoid neotenics in the Rhinotermitidae may serve either as replacement reproductives, or as supplementary reproductives. Several species of the genus *Coptotermes* are known by produce neotenic reproductives: *C. acinaciformis, C. amani, C. curvignathus, C. formosanus, C. frenchi, C. heimi, C. intermedius, C. lacteus, C. niger, C. sjostedt, and C. vastator* (Lenz & Barrett 1982; Lenz *et al*. 1986; Lenz *et al*. 1988; Lenz & Runko 1993; Myles 1999) and the rate of neotenic development varies greatly among *Coptotermes* species (Lenz *et al*. 1988). All the orphaned colonies with neotenics of *C. gestroi* contained larvae. Larvae in colonies of lower termites are capable of precocious reproductive development (neoteny) at some point of their life cycle (Grassé & Noirot 1960). In this study, neotenic development varied among replicate groups of orphaned colonies. This difference of neotenic production may be attributed to sex, instar, and age within the instar of the larvae present in the colonies (Greenberg & Stuart 1982) at the moment of orphaning.

The sex ratio of neotenics is closer to 1:1 in lower termites (Myles 1999). In *C. gestroi*, neotenics of both sexes are produced at the same time, however, neotenic males were less numerous than females. The data suggest that the differential production of females occurred after queen removal. It is possible that there is gender-specific inhibition in combination with a stronger tendency of females to respond quickly to absence of inhibition, which may induce more female larvae to develop into neotenic reproductives. On the other hand, it is possible that neotenics take more time to appear in king orphaned colonies. The decrease in the number of neotenics registered in the orphaned colonies of *C. gestroi* and the injuries sustained by neotenics indicate that agonistic behavior may act either among neotenics or between neotenics and individuals of other castes. Two alternative hypotheses may explain this; 1) a process of competition among reproductives (Lenz *et al*. 1986; Myles, 1999), or 2) elimination by workers due to limited available resources in the colony. Cannibalism has been reported in Kalotermitidae (Lenz *et al*. 1982) and Termopsidae (Lenz *et al*. 1988). Gender-specific inhibitory mechanisms regulated by semiochemicals of primary reproductives may explain the female biased population of neotenics found in colonies where the queen was removed; however, these compounds have not been yet identified in termites.

Three-year-old, laboratory reared colonies of *C. gestroi* developed non-functional neotenics from N1 precursor larvae within three months after the colony was orphaned. In view of the rapid development of nymphoids in this species, neotenics may be used to build up termite numbers in situations of food abundance or for propagation in areas of accidental introduction, as has been documented for *C. formosanus* (Lenz & Barrett 1982). Little information is available on *C. gestroi* biology and effective management of this species does not yet exist in Brazil. Thus, studies concerning the reproductive biology of *C. gestroi* may be helpful in developing control measures and to determine the colonizing potential of this species.

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