UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
INSTITUTO DE BIOCIÊNCIAS – RIO CLARO

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS (ZOOLOGIA)

# OVIPOSIÇÃO E MORFOFISIOLOGIA DA ESPERMATECA E GLÂNDULAS COLETÉRICAS EM DUAS ESPÉCIES DE CUPINS PRAGA

IAGO BUENO DA SILVA

Rio Claro – SP
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**IAGO BUENO DA SILVA**

Tese apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutor em Ciências Biológicas (Zoologia)
Orientadora: Dra. Ana Maria Costa Leonardo

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## CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: Oviposição e morfofisiologia da espermateca e glândulas coletéricas em duas espécies de cupins praga

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Rio Claro, 15 de dezembro de 2023

Às Nairs de minha vida, Nair Bueno e Nair Rosa (*in memorian*)

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*Se manter de pé contra o que vier*

*Vencer os medos,* ***mostrar a que veio***

*Ter o foco ali e sempre seguir rumo à vitória*

*Dead Fish – Vitória*

## RESUMO

Em cupins, a fundação da colônia precede o primeiro ciclo reprodutivo, caracterizado pela cópula do casal real e oviposição, seguidos por um período de inatividade. Durante esse período (*lag phase*), o casal real investe energia no cuidado parental. A dinâmica de oviposição já foi amplamente estudada em espécies de cupins de regiões temperadas, onde o ciclo de oviposição e a *lag phase* são bem definidos e persistem por meses. O sucesso na oviposição não depende apenas da atuação dos ovários, mas também de outras estruturas do sistema reprodutor, como a espermateca e as glândulas coletéricas, que atuam na estocagem de espermatozoides e oviposição, respectivamente. A dinâmica de oviposição em espécies de cupins distribuídas na região Neotropical é pouco estudada se comparada a de espécies de regiões temperadas. Da mesma forma, a espermateca e as glândulas coletéricas são pouco estudadas nesse grupo em relação aos himenópteros eussociais (abelhas e formigas) e outros Dictyoptera (baratas e louva-a-deus), respectivamente. A presente tese avaliou a dinâmica de oviposição e a morfofisiologia da espermateca e glândulas coletéricas nos cupins pragas *Coptotermes gestroi* e *Cryptotermes brevis*. Os resultados das investigações se distribuem em quatro artigos publicados, divididos em quatro capítulos. O capítulo 1 detalha a oviposição nas duas espécies por um período de nove meses, destacando a ocorrência de *lag phase* em *C. brevis*, enquanto as rainhas de *C. gestroi* ovipositam continuamente. Os capítulos 2 e 3 abordam, respectivamente, as alterações morfofisiológicas e morfométricas da espermateca e glândulas coletéricas em rainhas de diferentes idades e status reprodutivos de *C. gestroi*. O capítulo 4 apresenta uma abordagem similar, mas em *C. brevis*, destacando a importância da cópula e oviposição nessas alterações. As dinâmicas de oviposição apresentadas pelas duas espécies configuram diferentes estratégias reprodutivas adotadas após a fundação da colônia. Conforme as rainhas copulam, ovipositam e envelhecem, alterações morfofisiológicas e morfométricas ocorrem na espermateca e glândulas coletéricas, modificações essas importantes para a iteroparidade em cupins, onde o casal real apresentam repetitivos eventos de reprodução ao longo do ciclo de vida da colônia.



**Palavras-chave:** Reprodução, ciclos reprodutivos, Isoptera, *Coptotermes gestroi*, *Cryptotermes brevis*

## ABSTRACT

Termite colony foundation precedes the first reproductive cycle, in which the royal couple copulate and lay the first batch of eggs, followed by several months of reproductive inactivity. During this period, referred to as the lag *phase*, the royal couple invests energy towards parental care. Oviposition dynamics has been well studied among termite species from temperate areas, in which the first oviposition cycle and the *lag phase* last some months. The success of the oviposition does not rely only on the development of the ovaries, but also on the functionality of structures related to the reproductive system such as the spermatheca and colleterial glands, whose function is to store spermatozoa and secrete contents related to the oviposition, respectively. The oviposition dynamic has been poorly studied within termite species distributed in the Neotropical region. Similarly, information regarding the spermatheca and the colleterial glands is scarce for termites when compared to eusocial Hymenoptera (bees and antes) and other Dictyoptera (cockroaches and mantises). The main goal of this thesis was to evaluate the oviposition dynamic, as well as the morphophysiology of the spermatheca and colleterial glands in the two major termite pest species, *Coptotermes gestroi* and *Cryptotermes brevis*. The scientific results are distributed into four published articles, divided into four chapters. The first chapter provides a detailed analysis of the oviposition dynamics in these species during a 9-month period, highlighting the occurrence of a *lag phase* in *C. brevis*, whereas *C. gestroi* queens showed an uninterrupted oviposition. The chapters 2 and 3 detail, respectively, the morphophysiological and morphometrical changes of the spermatheca and colleterial glands between females of *C. gestroi* of different ages and reproductive status. The chapter 4 provides a similar approach in *C. brevis*, emphasizing the importance of the copulation and oviposition in such changes. The distinct oviposition dynamics of the sampled species reflect different reproductive strategies post-colony foundation. As the queens are inseminated, lay eggs, and age, important morphophysiological and morphometric changes take place in the spermatheca and colleterial glands. Such modifications are important for the iteroparity in termites, in which the royal couple goes through several reproductive events during the colony lifecycle.



**Keywords:** Reproduction, reproductive cycles, Isoptera, *Coptotermes gestroi*, *Cryptotermes brevis*

**SUMÁRIO**

# *Introdução*

## 1. INTRODUÇÃO GERAL

Os cupins abrangem insetos hemimetábolos eussociais pertencentes à ordem Blattaria, infraordem Isoptera (Inward *et al*., 2007; Krishna *et al*., 2013). Além disso, formam um grupo monofilético com baratas e louva-a-deus denominado Dictyoptera (Legendre et *al*., 2015). Atualmente, são reconhecidas 2972 espécies de cupins viventes, distribuídas em nove famílias e 312 gêneros. Dessas famílias, quatro ocorrem no Brasil (Kalotermitidae, Serritermitidae, Rhinotermitidae e Termitidae) (Constantino, 2023). A família Termitidae possui radiação mais recente e é também a mais diversa, compreendendo aproximadamente 70% das espécies (Engel *et al*., 2009). As famílias Kalotermitidae e Rhinotermitidae, por outro lado, recebem destaque pelo potencial invasivo e de se tornarem pragas, especialmente a última, a qual abrange espécies de cupins subterrâneos (Evans, 2021).

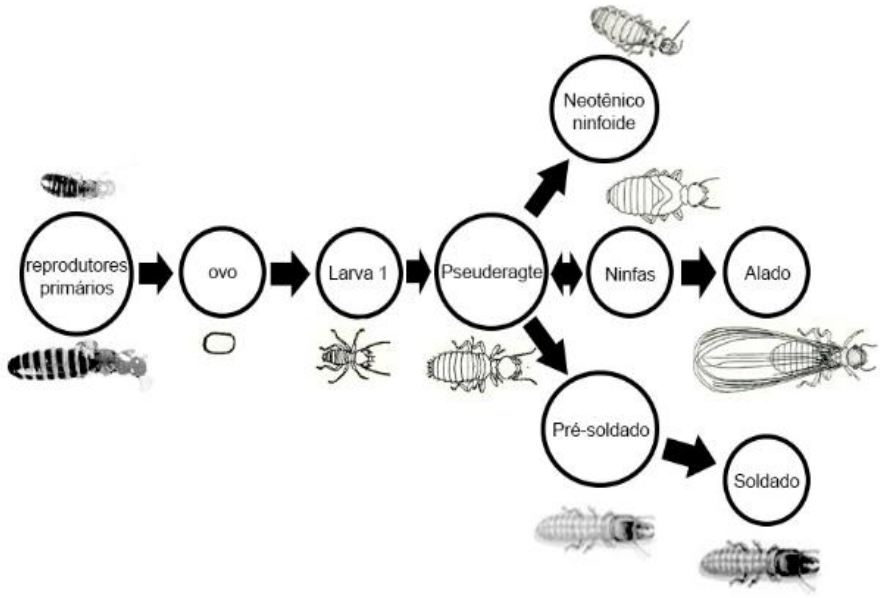
Juntamente com as formigas, cupins são dominantes em termos de biomassa em ambientes terrestres tropicais, sendo que a biomassa seca combinada desses dois grupos de insetos é de aproximadamente 120 Mt de carbono, superior àquela atribuída a todos os outros artrópodes terrestres juntos (Tuma *et al.*, 2020). A radiação evolutiva e sucesso ecológico dos cupins são atribuídos à capacidade de explorar diferentes fontes de celulose, possibilitada por associações com a microbiota intestinal, assim como à sua complexa organização social, caracterizada pela divisão reprodutiva de tarefas, envolvendo castas com distintas adaptações morfológicas e fisiológicas para tal (Korb; Thorne, 2017; Engel, 2019; Chouvenc *et al.*, 2021).

As colônias de cupins são formadas por castas pertencentes a duas linhagens: áptera e reprodutiva. A primeira é composta por operários e soldados, sendo os primeiros indivíduos responsáveis pelas diferentes tarefas da colônia, como construção, forrageamento e cuidado com ovos e imaturos, ao passo que os soldados são responsáveis pela defesa. A linhagem reprodutiva, por sua vez, é composta por ninfas, reprodutores alados e o casal real (rei e rainha), os últimos sendo responsáveis pela cópula, fertilização e postura de ovos, originando todos os indivíduos da colônia. Os reprodutores alados, também denominados imagos, deixam os ninhos natais nas épocas de revoada, a fim de formarem pares e fundarem um novo ninho, onde se tornarão reis e rainhas primários ou imaginais (Roisin, 2000). Em algumas espécies de cupins, é comum a formação de reprodutores neotênicos ou secundários. Esses reprodutores recebem essa denominação pois são indivíduos imaturos que desenvolveram seu sistema reprodutor, tornando-se reprodutores suplementares, quando os reprodutores primários ainda estão presentes nas colônias; ou de substituição, quando o rei ou a rainha (ou ambos) morre (Myles,

1999). Os reprodutores neotênicos podem se diferenciar a partir de ninfas, sendo denominados ninfoides, ou de operários, sendo então referidos como ergatoides (Figuras 1.1 e 1.2) (Myles, 1999; Haifig *et al*., 2016; da Silva *et al*., 2019).

A diferenciação de castas em cupins pode se dar através do desenvolvimento linear ou bifurcado. Na diferenciação linear (presente em famílias como Kalotermitidae e Archotermopsidae), existe um estágio imaturo tardio totipotente, que é denominado falso operário ("pseudergate"). Esta casta pode sofrer mudas estacionárias ou se diferenciar em ninfas, as quais sofrem mudas sucessivas até se tornarem reprodutores alados ou neotênicos (Figura 1.1). As ninfas, no entanto, também podem sofrer mudas regressivas, voltando ao estágio de pseudergate (Roisin; Korb, 2011). Assim, o desenvolvimento de castas em espécies que adotam a diferenciação linear é plástico e uma muda irreversível ocorre somente em estágios tardios do desenvolvimento.

**Figura 1.1 -** Padrão de desenvolvimento linear de castas em cupins.

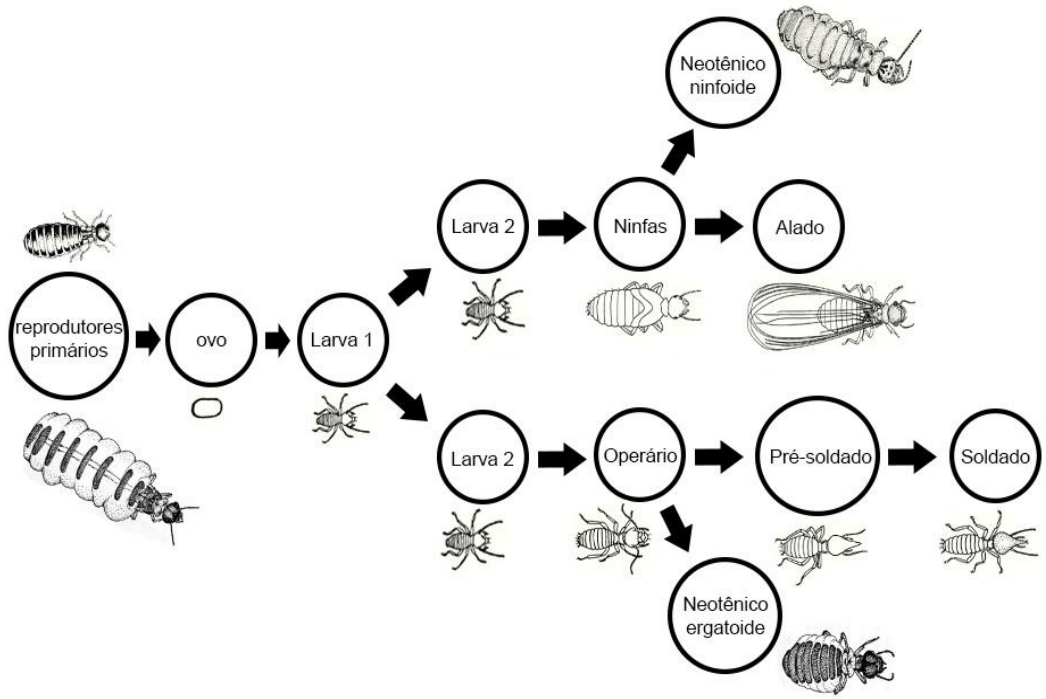



A larva (estágio imaturo) se diferencia em um instar larval tardio, conhecido como "pseudergate". Este indivíduo é totipotente, podendo se diferenciar em outras castas ou sofrer mudas estacionárias. Fonte: Adaptado de Grassé (1986).

No desenvolvimento bifurcado (comumente em Termitidae e na maioria das espécies de Rhinotermitidae), ocorre uma separação precoce e irreversível entre as linhagens áptera e reprodutiva (Figura 1.2), mediada por uma expressão gênica diferencial e ação hormonal, e tida como uma condição derivada em cupins. (Roisin; Korb, 2011). Nesse padrão de desenvolvimento, a maior parte da prole sofre uma muda irreversível para a linhagem áptera e estéril, composta por operários verdadeiros e os soldados (Roisin, 2000). Em contrapartida, uma

parte menor da prole se diferencia na linhagem reprodutiva, a qual é igualmente irreversível (Maekawa *et al*., 2008).

**Figura 1.2** - Padrão de desenvolvimento bifurcado de castas




As linhagens reprodutiva (ninfas e alados) e áptera (operários e soldados) se diferenciam de modo irreversível e precoce. Os operários são verdadeiros, podendo sofrer muda terminal para soldado ou para um reprodutor neotênico ergatoide. Fonte: aptado de Grassé (1986).

### 1.1 Estrutura reprodutiva e oviposição em cupins

De uma forma geral, as colônias de cupins são fundadas por um casal real, o qual permanece junto no ninho, com o rei inseminando a rainha periodicamente (Weesner, 1960; Raina *et al*., 2007). O rei e a rainha são provenientes de reprodutores alados, sendo, portanto, denominados reprodutores primários, os quais monopolizam a reprodução dentro das colônias (Nutting, 1969). No entanto, diversas espécies de cupins, especialmente aquelas não pertencentes à família Termitidae, possuem capacidade de desenvolver reprodutores secundários, os quais podem se originar de diferentes castas e atuar como reprodutores suplementares, na presença do casal real, ou reprodutores de substituição, na ausência do rei e/ou da rainha (Myles, 1999; Haifig *et al*., 2016; da Silva *et al*., 2019).

A fundação de colônias em cupins precede diferentes estágios de desenvolvimento, sendo o primeiro denominado “estágio incipiente”, o qual dura aproximadamente sete meses (Chouvenc; Su, 2014). Nesse estágio, ocorre o primeiro ciclo reprodutivo, onde a rainha é inseminada e põe os primeiros ovos. O primeiro ciclo de oviposição em cupins já foi amplamente estudado em espécies subterrâneas (Rhinotermitidae) distribuídas em regiões temperadas, onde a postura de ovos dura de 30 a 70 dias e é seguida por um período de inatividade reprodutiva, denominada *lag phase*, que dura de quatro a seis meses (Tabela 1). Esse período é caracterizado pela atrofia dos testículos e ovários do rei e da rainha, respectivamente (Raina *et al.*, 2003), decorrente do investimento de energia e recursos no cuidado dos ovos e das larvas, as quais são dependentes dos reprodutores (Maekawa *et al*., 2010). Em espécies de madeira seca (Kalotermitidae), a dinâmica relacionada aos ciclos de oviposição e de inatividade reprodutiva são menos conhecidas e restritas a poucas espécies. Amostragens de *Cryptotermes brevis* em regiões subtropicais, por exemplo, indicaram que os reprodutores cessam a reprodução após seis meses da fundação da colônia, retomando essa atividade após três meses de *lag phase* (McMahan, 1962), provavelmente após a diferenciação de larvas dependentes em pseudergates.

Outros fatores que podem impactar e interromper a produção de ovos em cupins incluem as flutuações climáticas, especialmente em regiões temperadas, onde as rainhas cessam completamente a oviposição durante os meses mais frios (Weesner, 1969; Nozaki; Matsuura, 2021). No entanto, amostragens de espécies de Termitidae na região Neotropical indicam a ocorrência de ovos e larvas durante todo o ano, sugerindo que as variações climáticas nesta região são mais amenas e não causam interrupção na produção de ovos em colônias de cupins (Fernandes *et al*., 1997; Torales; Coronel, 2004; Laffont, 2012; Annoni *et al*., 2013).

Conforme as larvas vão mudando para operários e ultrapassam um limiar numérico, ocorre uma transição irreversível de cuidado parental para aloparental, no qual o cuidado com a prole é feito pelos operários enquanto o casal real se limita às tarefas reprodutivas (Chouvenc; Su, 2017). Sugere-se que a transição para o cuidado aloparental foi fundamental para a evolução da eussocialidade e estabelecimento de divisão reprodutiva de tarefas observadas em colônias de insetos sociais (Chouvenc, 2022). Diante desse cenário, no qual o rei e a rainha investem energia e recursos somente na reprodução, ocorre um progressivo desenvolvimento de seus aparelhos reprodutores, caracterizado, especialmente, pelo aumento e funcionalidade dos testículos e dos ovários, respectivamente. Tal desenvolvimento resulta na maior produção de espermatozoides e ovos (Raina *et al.*, 2003; Laranjo *et al.*, 2019).

**Tabela 1 -** Dinâmica de oviposição em colônias incipientes de cupins subterrâneos (Rhinotermitidae) de regiões temperadas.

| Espécie | Primeiro ciclo de oviposição | Período de inatividade | Referência |
|---|---|---|---|
| *Coptotermes formosanus* | 60-70 dias | Aprox. 4 meses | (Raina *et al*., 2003) |
| *Reticulitermes speratus* | Aprox. 75 dias | Aprox. 5 meses | (Maekawa *et al*., 2010) |
| *Reticulitermes lucifugus* | 30 dias | 4-6 meses | (Nutting, 1969) |
| *Reticulitermes flavipes* | 30-60 dias | 4-5 meses | (Janowiecki, 2012) |
| *Reticulitermes flavipes* | 30-60 dias | Pelo menos 6 meses | (Brossette *et al*., 2017) |
| *Reticulitermes grassei* | 30-60 dias | 5-6 meses | (Brossette *et al*., 2017) |
| *Reticulitermes urbis* | 30-60 dias | - | (Ghesini; Marini, 2009) |

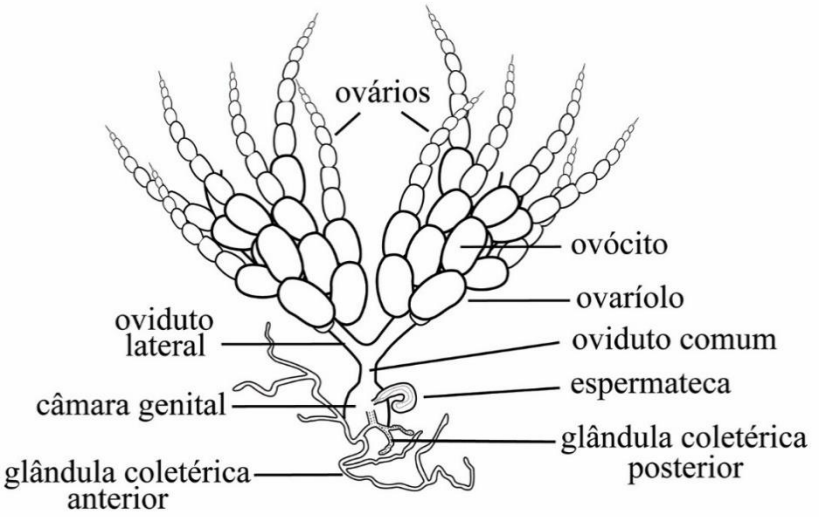
Fonte: Elaborado pelo autor

O desenvolvimento progressivo dos ovários das rainhas resulta em uma hipertrofia abdominal, denominada fisogastria (Grassé, 1982). Durante esse processo, diversas modificações ocorrem em outras partes do corpo, incluindo o corpo gorduroso, cutícula, sistema traqueal e hemolinfa (Bordereau, 1982; Han; Bordereau, 1982; Costa-Leonardo *et al*., 2013; Nozaki; Matsuura, 2019). O desenvolvimento da fisogastria depende do número de operários disponíveis, não só para cuidar dos ovos e das larvas dependentes, mas também para suprir as demandas energéticas da rainha. A alta oferta de nutrientes resulta na maior síntese de hormônio juvenil nesta reprodutora, impactando positivamente a produção de ovos (Sieber; Leuthold, 1982).

Os ovários das fêmeas de cupins são pareados e denominados panoísticos, devido à ausência de células nutridoras ou trofócitos (Wheeler, 2009a). Cada ovário contém diversos ovaríolos, que variam em quantidade e maturação de acordo com o status reprodutivo da rainha, e se abrem em um oviduto lateral. Os dois ovidutos laterais, então, se unem em um oviduto comum, o qual termina na câmara genital (Costa-Leonardo *et al.*, 2022) (Figura 1.3). A

produção de ovos configura o aspecto mais importante da biologia reprodutiva de fêmeas de insetos (Lange, 2009). No entanto, uma oviposição bem-sucedida não depende só do desenvolvimento e funcionamento dos ovários, mas também da maturação de outros órgãos associados ao sistema reprodutor, os quais devem atuar de maneira coordenada com a produção de ovos (Lange, 2009). A cópula e consequente transferência de espermatozoides, por exemplo, age como um gatilho para a secreção de miotropina, hormônio que estimula as contrações musculares do oviduto, dando início ao processo de ovulação (Klowden, 2009). Durante esse processo, os ovos são liberados dos ovaríolos e adentram os ovidutos, sítios de transporte e também de fertilização (Shen *et al*., 2021). Dentre esses órgãos, destacam-se a(s) espermateca(s), especializada(s) na estocagem de espermatozoides (Simmons, 2013), e as glândulas coletéricas ou glândulas acessórias das fêmeas, as quais auxiliam no processo de oviposição (Gillott, 2002).

**Figura 1.3 -** Esquema do aparelho reprodutor em uma rainha de *Coptotermes gestroi*




Notar a localização espermateca, de forma recurvada, e das glândulas coletéricas, de forma tubular. Produzido pelo autor. Fonte: Elaborado pelo autor.

## 1.2. Espermateca: função, distribuição e estrutura

Esse órgão permite que fêmeas estoquem espermatozoides após a cópula e antes da fertilização dos ovos, o que resulta em uma série de vantagens (Raina *et al*., 2007; McLay; Greco, 2011; Orr; Zuk, 2012). Essas incluem a viabilidade de espermatozoides por longos períodos, especialmente em espécies que copulam durante um curto intervalo de tempo, a separação temporal entre cópula e fertilização, possibilidade de fertilização dos ovos na ausência de machos, e redução de cópulas adicionais (Baer, 2005; Baer *et al*., 2009, 2016).

Adicionalmente, a espermateca permite que as fêmeas dos insetos controlem a paternidade, principalmente em espécies poliândricas, onde mais de um macho pode copular com a mesma fêmea (Snow; Andrade, 2005). Assim, a fertilização facultativa de ovos facilita a seleção de descendentes viáveis, por meio de uma seleção sexual pós-cópula (Klowden; Chambers, 2004).

A espermateca está presente em todas as ordens de insetos e, como possui origem epidérmica, é revestida internamente por cutícula (Engelmann, 1970). Na maioria das espécies, existe uma única espermateca, considerada uma característica ancestral para os insetos, mas alguns grupos podem apresentar várias espermatecas, caso de representantes da ordem Dermaptera (Kamimura, 2007). Em relação à sua estrutura, a espermateca pode possuir um reservatório, glândula espermatecal, um duto e musculatura associada (Pascini; Martins, 2017a). O reservatório espermatecal está diretamente associado com a estocagem e manutenção dos espermatozoides após a fertilização. Além disso, essa região pode ser dividida em câmaras, além de apresentar variação morfológica e de tamanho entre as espécies e entre fêmeas de diferentes idades (Martins *et al.*, 2005; Viscuso *et al.*, 2015; Pascini; Martins, 2017). O reservatório é revestido internamente por uma cutícula, cuja espessura varia, criando uma barreira física entre o ambiente onde os espermatozoides são estocados e o meio externo (Pascini; Martins, 2017).

Além de isolar os espermatozoides fisicamente, evitando danos mecânicos, contato com a hemolinfa e ação de radicais livres, o reservatório espermatecal fornece um ambiente com pH apropriado, baixos níveis de oxigênio, nutrientes, compostos bactericidas e antioxidantes (Dallai, 1975; Prokupek *et al*., 2008; Rogers *et al*., 2008; Al-Lawati *et al*., 2009; King *et al*., 2011; Gotoh *et al*., 2017). A secreção de tais compostos pode ocorrer de duas maneiras: pela atividade das glândulas espermatecais ou do próprio epitélio do reservatório. As glândulas epermatecais variam em número entre os grupos de insetos, assim como a quantidade de células secretoras que as compõem. Adicionalmente, as glândulas estão geralmente conectadas ao reservatório espermatecal por meio de ductos glandulares, secretando compostos dentro do lúmen (Hartmann; Loher, 1999; Pascini; Martins, 2017). Na presença de glândulas espermatecais, o epitélio da espermateca executa apenas uma função de transporte (Schoeters; Billen, 2000). No entanto, na ausência dessas glândulas, o epitélio espermatecal é responsável pela síntese e secreção de compostos em direção ao lúmen, conforme reportado em representantes da superordem Dictyoptera, além das ordens Zoraptera e Mecoptera (Lawson; Thomas, 1970; Raina *et al*., 2007; Winnick *et al.*, 2009; Dallai *et al*., 2012; Yang; Hua, 2021). Em ambos os casos, as unidades secretoras são compostas por células de classe III (unidades

bicelulares), compreendendo uma célula secretora, responsável pela síntese de compostos, e uma célula com canal cuticular responsável pelo transporte da secreção até o lúmen (Noirot; Quennedey, 1974, 1991).

O duto espermatecal geralmente compreende uma estrutura curta e revestida internamente por cutícula, que conecta diretamente o reservatório à câmara genital. No entanto, sua função não se limita a simplesmente conectar essas regiões (Pascini; Martins, 2017). Em algumas espécies de abelhas e formigas, por exemplo, o duto atua na seleção de espermatozoides que deverão ser estocados por meio de uma constrição entre o duto e o reservatório, forçando a passagem individual dos gametas (Oppelt; Heinze, 2007). Já no gafanhoto *Gomphocerus rufus* (Acrididae), as células epiteliais do duto secretam enzimas que dissolvem o espermatóforo transferido pelo macho durante a cópula, liberando o conteúdo seminal contido na referida estrutura (Hartmann; Loher, 1999). Uma função amplamente atribuída ao duto espermatecal é a de transportar espermatozoides, por meio da contração de diferentes fibras musculares (Pascini; Martins, 2017). A musculatura da espermateca é composta por fibras longitudinais e circulares, as quais podem revestir todo o órgão, incluindo reservatório e ducto, ou regiões específicas (Martins; Serrão, 2002; Bailey; Nuhardiyati, 2005). A sobreposição dessas fibras ao redor do duto espermatecal de várias espécies de insetos resulta na formação de uma zona de constrição, denominada bomba espermatecal, a qual é diretamente relacionada ao transporte de secreção e gametas em direção ao lúmen da espermateca ou a câmara genital (Martin; Serrão, 2002; Pascini *et al.*, 2012).

### 1.2.1. Espermateca nos cupins

A reprodução em cupins difere daquela observada nos Hymenoptera eussociais, onde a rainha copula por um curto período de tempo com um ou vários machos, e estoca espermatozoides por décadas a fim de fertilizar os ovos (Baer *et al*., 2009; Den Boer *et al.*, 2009). Dessa forma, estima-se que rainhas da formiga cortadeira *Atta colombica* estoquem centenas de milhões de espermatozoides após o voo nupcial (Baer; Boomsma, 2006). Nos cupins, a cópula ocorre múltiplas vezes ao longo da vida dos reprodutores e supõe-se que a capacidade de estocagem da espermateca seja limitada quando comparada a abelhas eussociais e formigas (Hartke; Baer, 2011). Estudos quantitativos acerca da estocagem de espermatozoides em rainhas de cupins são escassos, mas estima-se que rainhas primárias de *Coptotermes formosanus* possam armazenar até 14 mil espermatozoides durante o primeiro ciclo reprodutivo (Raina *et al*., 2007). Tal estocagem persiste, em menor número, durante o

período de inatividade reprodutiva ou mesmo após a remoção dos reis (Weesner, 1956; Raina *et al.*, 2007). No entanto, o status reprodutivo das rainhas, assim como a espécie amostrada, podem influenciar na quantidade de espermatozoides estocados.

A espermateca dos cupins se dispõe como uma bolsa recurvada, cujo tamanho e anatomia variam entre as diferentes famílias (Hartke; Baer, 2011). Adicionalmente, glândulas espermatecais nunca foram descritas em Isoptera, de modo que somente o epitélio da espermateca parece atuar na secreção de compostos (Noirot, 1969; Grassé, 1982). Costa-Leonardo e Patrício (2005) mostraram que a espermateca apresenta forma de um dedo recurvado nas famílias Termitidae, Serritermitidae, Rhinotermitidae e Kalotermitidae. Tal conformação corresponde a duas regiões distintas, uma distal e outra proximal, além de um duto espermatecal (Raina *et al.*, 2007). Por outro lado, a espermateca possui forma de "guarda-chuva" em *Zootermopsis* (Archotermopsidae) e se dispõe dividida em ramos em *Hodotermes mossambicus* (Hodotermitidae), apresentando expansões saculares nas pontas (Geyer, 1951; Costa-Leonardo; Patrício, 2005). Poucas abordagens anatômicas e histológicas contemplaram a espermateca de rainhas de cupins (Dean; Gold, 2004; Costa-Leonardo; Patrício, 2005; Raina *et al*., 2007; Ye *et al.*, 2009), o que resultou em escassas e fragmentadas informações sobre a referida estrutura. Além disso, comparações entre espermatecas de rainhas de diferentes idades e status reprodutivo são praticamente inexistentes, de modo que as características morfofisiológicas pré- e pós-cópula permanecem pouco conhecidas nesses insetos.

### 1.3. Glândulas coletéricas: função, distribuição e estrutura

As glândulas coletéricas, também denominadas glândulas acessórias das fêmeas, ou mesmo glândulas de cimento, são estruturas de origem epidérmica ou mesodérmica e frequentemente associadas a síntese de compostos que recobrem ou auxiliam na fixação dos ovos dos insetos ao substrato (Gillott, 2002). De acordo com estudos em diferentes ordens de insetos, as principais funções atribuídas a essa cobertura dos ovos são prevenir o ataque de predadores, parasitas, microrganismos, e evitar a dessecação dos ovos quando expostos ao ambiente (Gillott, 2002). Além dessa proteção física, compostos químicos podem ser incorporados à superfície dos ovos durante o processo de oviposição, como por exemplo peptídeos bactericidas (Marchini *et al*., 1997). Já em alguns besouros, as glândulas coletéricas servem de sítio para endossimbiontes celulares, os quais fornecem vitaminas e aminoácidos ao inseto hospedeiro. Durante a oviposição, os endossimbiontes são incorporados ao revestimento dos ovos e transferidos às larvas recém-emergidas por meio do consumo da casca

(Schwemmler; Gassner, 1989). Outras funções incluem a síntese de compostos que lubrificam os ovidutos (Sturm, 2011) e, em casos especiais, a síntese de veneno em Hymenoptera aculeados (Gnatzy *et al.*, 2018).

De forma similar à espermateca, as glândulas coletéricas possuem origem epidérmica na grande maioria dos insetos, ainda que alguns grupos apresentam glândulas coletéricas diferenciadas de tecidos mesodérmicos (Gillott, 2002). As glândulas estão localizadas no final do abdômen, geralmente se abrindo no 9º esternito. No entanto, a ocorrência dessas glândulas varia entre os grupos de insetos, estando ausentes em algumas ordens, caso de Ephemeroptera, Plecoptera, Psocoptera, além de grande parte de Coleoptera e Hemiptera (Matsuda, 1976). Quando ocorrem, as glândulas coletéricas são, em sua maioria, pareadas e desembocam próximas à espermateca. Algumas exceções incluem túbulos multipareados, os quais se fundem próximo à câmara genital (Gillott, 2002). Na ausência dessas estruturas, as paredes do oviduto possuem aspecto glandular e desempenham função secretora (Chapman, 1998). De uma forma geral, a estrutura das glândulas coletéricas compreende túbulos ramificados, onde a atividade secretora ocorre. Adicionalmente, as glândulas variam em tamanho e desenvolvimento entre si e de acordo com o status reprodutivo das fêmeas (Stay; Roth, 1962; Sturm; Pohlhammer, 2000; Gillott, 2002). Na região basal, próxima à abertura para a câmara genital, as glândulas são envolvidas por diferentes camadas de músculos, tanto circulares quanto longitudinais (Weesner, 1969), cuja função é controlar a liberação de conteúdo proveniente da atividade secretora (Lococo; Huebner, 1980; Sturm, 2008). As células das glândulas coletéricas pertencem à classe I, onde um único epitélio de células epidérmicas secretoras se dispõe próximo à cutícula; ou à classe III, onde uma unidade bicelular é responsável por secretar e transportar o conteúdo até o lúmen glandular por meio de canais cuticulares (Gillott, 2002; Courrent *et al.*, 2008).

### 1.3.1. Glândulas coletéricas nos cupins

Entre os Isoptera, existem duas glândulas coletéricas, denominadas anterior e posterior, sendo que a estrutura dessas glândulas foi descrita somente em quatro famílias de cupins (Mastotermitidae, Termopsidae, Kalotermitidae e Termitidae) (Grassé, 1982; Soltani-Mazouni; Bordereau, 1987; Courrent *et al*., 2008). Descrições morfológicas realizadas em *Mastotermes darwiniensis* e *Pseudacanthotermes spiniger* indicaram que a glândula anterior é mais desenvolvida e formada por ramificações, enquanto a glândula posterior é reduzida e composta por um único túbulo (Courrent *et al*., 2008). No entanto, a ocorrência de bifurcações nas duas

unidades das glândulas coletéricas já foi constatada para outras espécies, como *Kalotermes flavicollis* e *Zootermopsis nevadensis* (Soltani-Mazouni; Bordereau, 1987; Courrent *et al*., 2008), sugerindo que a estrutura pode variar nos diferentes táxons. A abertura glandular também não segue um padrão, uma vez que as unidades podem se abrir lado-a-lado na câmara genital, ou juntas, por meio de um duto comum (Weesner, 1969; Grassé, 1982; Soltani-Mazouni; Bordereau, 1987; Courrent *et al*., 2008; Costa-Leonardo *et al.*, 2023). Em outros insetos, a presença de um duto comum nas glândulas coletéricas sugere a existência de um sítio onde os compostos secretados por cada unidade são misturados (MA *et al.*, 2013). A região basal de cada glândula, denominada tronco basal, é envolvida por diferentes camadas de fibras musculares estriadas dispostas longitudinal e circurlarmente (Grassé, 1982; Costa-Leonardo *et al.*, 2023).

A função das glândulas coletéricas em cupins não é clara, já que a grande maioria das espécies oviposita individualmente, desprovidos de algum tipo de cobertura (Courrent *et al*., 2008; Wheeler, 2009b). Em outros Dictyoptera, como baratas e louva-a-deus, as glândulas coletéricas produzem a ooteca, uma estrutura característica que aloja os ovos, conferindo proteção adicional contra dessecação e predação. A espécie pimitiva de cupim *M. darwiniensis* (Mastotermitidae) é a única cujas rainhas produzem uma estrutura similar à ooteca, menos desenvolvida e funcional do que aquela presente em baratas (Nalepa; Lenz, 2000). De acordo com Roonwal (1975), na espécie *Odontotermes obesus* (Termitidae), os ovos são depositados em fileiras embebidas por uma matriz, provavelmente produzida pelas glândulas coletéricas. Contudo, aspectos químicos e funcionais dessas glândulas, assim como seu desenvolvimento em fêmeas de diferentes idades e status reprodutivos, permanecem desconhecidos para a grande maioria das espécies.

### 1.4. Espécies invasoras como modelos para estudos reprodutivos em cupins

O prejuízo econômico, em nível global, causado por cupins ultrapassa US$ 40 bilhões todos os anos, e estima-se que 80% desses danos seja causado por espécies subterrâneas (Oi, 2022). Aproximadamente 28 espécies de cupins são consideradas invasoras em diferentes partes do mundo, as quais foram translocadas de suas regiões endêmicas, podendo se tornar pragas de notável capacidade de expansão em novas áreas. A maioria dessas espécies pertence às famílias Rhinotermitidae (cupins subterrâneos) e Kalotermitidae (cupins de madeira seca) (Evans, 2021). No entanto, as espécies subterrâneas correspondem à maior porcentagem de danos causados (Rust; Su, 2012), provavelmente devido à maior fecundidade e taxa de

oviposição de suas rainhas, quando comparados às rainhas de espécies de madeira seca (Roisin; Korb, 2011).

*Coptotermes gestroi* (Rhinotermitidae) é uma espécie de cupim subterrâneo nativa do Sudeste asiático, caracterizada pelo desenvolvimento bifurcado de castas (Barsotti; Costa-Leonardo, 2005). Adicionalmente, *C. gestroi* constrói ninhos cartonados policálicos classificados como "ninhos múltiplas peças" (Abe, 1987; Korb, 2008), os quais são bem definidos, com grande capacidade de crescimento e separados das fontes de alimento e água (Korb, 2008). Por consequência, uma colônia madura de *C. gestroi* atinge mais de um milhão de indivíduos, sendo a área de forrageamento extremamente vasta (Costa-Leonardo, 2002). As colônias dessa espécie apresentam um casal de reprodutores imaginais, contudo, uma rainha neotênica funcional já foi descrita para *C. gestroi*, inclusive com fisogastria conspícua, característica marcante da rainha imaginal (Lelis, 1999). *C. gestroi* foi introduzido em dezenas de países, incluindo o Brasil (Li et al., 2013), tornando-se uma importante praga estrutural, especialmente no estado de São Paulo, onde causa prejuízos econômicos severos (Fontes; Milano, 2002; Romagnano; Nahuz, 2006).

*Cryptotermes brevis* (Kalotermitidae) é um cupim de madeira seca nativo do Chile e Peru (Scheffrahn *et al*., 2009), o qual apresenta desenvolvimento linear de castas. Diferente de *C. gestroi*¸ essa espécie possui ninhos localizados dentro do próprio alimento, geralmente um pedaço de madeira ou mobiliário, sendo então classificados como "ninhos única peça" (Abe, 1987; Korb, 2008). A limitação de espaço físico e de recursos resulta em um tamanho reduzido da população das colônias, com cerca de 300 indivíduos (Costa-Leonardo, 2002). As rainhas imaginais não apresentam fisogastria verdadeira, possuindo somente um desenrugamento abdominal e, portanto, não possuem volume abdominal e fecundidade similares àqueles observados em rainhas de espécies subterrâneas. O desenvolvimento de rainhas neotênicas é comum nessas espécies, as quais podem apresentar o mesmo grau de fecundidade, ou até mesmo maior, do que o descrito para rainhas imaginais (Noirot, 1969).

Devido ao seu hábito críptico, estudos empíricos com cupins são desafiadores, principalmente se esses envolvem a amostragem de reprodutores. Sendo assim, a formação de colônias e manutenção dessas em condições laboratoriais se mostra uma forma eficaz de estudar diferentes aspectos da biologia reprodutiva desses insetos, incluindo as dinâmicas de oviposição e as modificação no sistema reprodutor entre indivíduos de diferentes idades (Costa-Leonardo; Barsotti; 1998, 2001; Chouvenc, 2019).

A presente tese teve como objetivo geral elucidar aspectos relacionados à oviposição e estocagem de espermatozoides em rainhas das espécies pragas *C. gestroi* e *C. brevis*. Os

objetivos específicos foram i) acompanhar a oviposição de rainhas de colônias incipientes e identificar a ocorrência de *lag phase*; ii) descrever a morfofisiologia e morfometria da espermateca e glândulas coletéricas, além de suas respectivas alterações em rainhas de diferentes idades e status reprodutivos de *C. gestroi* e *C. brevis*; iii) demonstrar a natureza, por meio de testes histoquímicos, da secreção presente na espermateca e glândulas coletéricas de rainhas com diferentes idades das duas espécies. A tese se apresenta na forma de quatro artigos publicados, divididos em quatro capítulos. O capítulo 1 apresenta um estudo comparativo da dinâmica de oviposição em *C. gestroi* e *C. brevis*; os capítulos 2 e 3 apresentam os aspectos morfofisiológicos e morfométricos da espermateca e glândulas coletéricas em rainhas de diferentes idades de *C. gestroi*, respectivamente; enquanto o capítulo 4 detalha as mudanças morfofisiológicas da espermateca e glândulas coletéricas em rainhas de *C. brevis* com diferentes status reprodutivos.

# *Material e métodos*

## 2. MATERIAL E MÉTODOS

**2.1. Cupins:** Neste estudo, foram utilizadas fêmeas virgens e rainhas não-virgens de diferentes idades de *Coptotermes gestroi* (Wasmann, 1896) e *Cryptotermes brevis* (Walker, 1853). Fêmeas virgens foram coletadas durante revoadas das referidas espécies, enquanto rainhas de *C. gestroi* e *C. brevis*, utilizadas para análises referentes ao ciclo reprodutivo e dinâmica de oviposição, foram obtidas de colônias incipientes formadas em laboratório a partir do pareamento de reprodutores alados, conforme descrito no tópico seguinte.

Para estudos morfofisiológicos da espermateca e glândulas coletéricas, rainhas de dois e quatro anos de *C. gestroi* foram obtidas de colônias mantidas em laboratório, enquanto rainhas não-virgens de *C. brevis* foram amostradas de colônias com seis meses e um ano de idade, mantidas sob as mesmas condições. Adicionalmente, rainhas neotênicas ninfoides e uma rainha madura de *C. brevis* também foram incluídas nas análises, sendo essas provenientes de colônias órfãs e de mobiliário, respectivamente.

### 2.2. Formação de colônias em laboratório

#### 2.2.1. *Coptotermes gestroi*

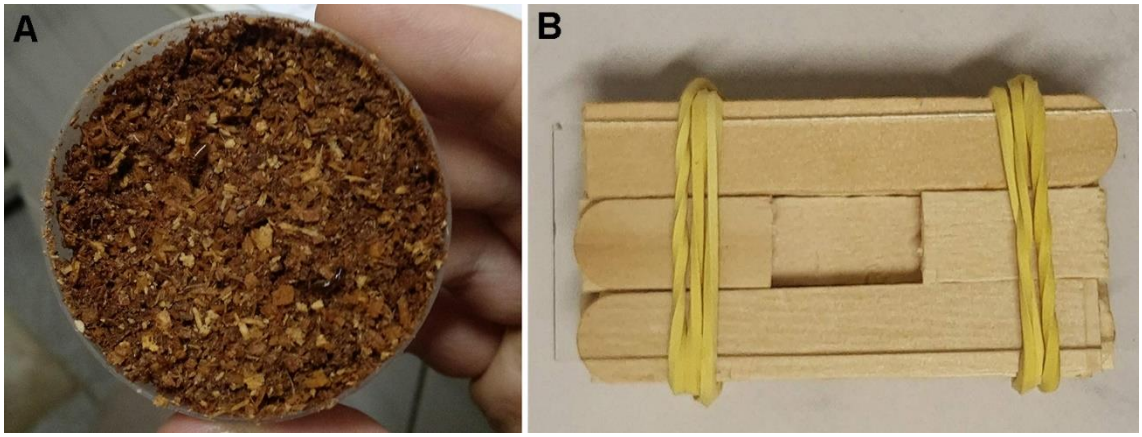
Reprodutores alados de *Coptotermes gestroi* foram coletados em revoadas na cidade de Rio Claro, SP (22°23’ S, 47°32’ W), sexados sob estereomicroscópio Zeiss Stemi SV11, pesados utilizando balança de precisão, pareados e colocados em placas de Petri plásticas de 6 cm de diâmetro. As placas continham serragem de *Pinus* sp. envelhecida e umedecida (Figura 2.1A). Todas as colônias incipientes foram mantidas em sala escura a 25 °C, e monitoradas a fim de acompanhar a postura de ovos e desenvolvimento da prole durante o período incipiente das colônias.

#### 2.2.2. *Cryptotermes brevis*

Reprodutores alados de *Cryptotermes brevis* foram coletados durante a revoada de colônias mantidas no Laboratório de Cupins, no campus da UNESP Rio Claro, SP (22°23’ S, 47°32’ W), sexados com auxílio de estereomicroscópio Zeiss Stemi SV11, pesados utilizando balança de precisão e pareados em colônias construídas com palitos retangulares de *Pinus* sp. Tais colônias consistiram em empilhamentos de 16 palitos compridos (7 cm x 1cm x 0,2 cm) e 4 palitos curtos (2,3 x 1 cm x 0,2 cm) unidos com cola branca atóxica, sendo mantida uma cavidade na região central (2,3 cm x 1 cm x 0,4 cm) de cada empilhamento coberta com uma lâmina de vidro presa por elásticos (Figura 2.1B). Dessa forma, foi possível acompanhar o

desenvolvimento inicial das colônias a fim de avaliar o primeiro ciclo reprodutivo das mesmas. De acordo com Korb & Lenz (2004), uma quantidade de alimento considerada abundante para cupins de madeira seca é de pelo menos 10 $cm^3$ para cada indivíduo. O volume total de madeira dos empilhamentos foi de 24,28 $cm^3$, sendo então abundante para os reprodutores. Pedaços de palitos foram adicionados de acordo com o desenvolvimento inicial das colônias, as quais foram mantidas em sala escura a 25 °C.

**Figura 2.1 -** Colônias incipientes mantidas sob condições laboratoriais.




A. *Coptotermes gestroi*. B. *Cryptotermes brevis*. Fonte: Elaborado pelo autor.

### 2.3. Análise da dinâmica de oviposição em colônias incipientes

#### 2.3.1. *Coptotermes gestroi*

Colônias foram monitoradas semanalmente para verificar a mortalidade, as condições de umidade e disponibilidade de recursos alimentares. No total, cinco colônias de *C. gestroi* foram amostradas em cada um dos seguintes períodos: 2, 10, 20, 30, 50, 80, 100, 120, 150, 180, 200, 220, 250 e 270 dias após o pareamento, totalizando nove meses de experimento (adaptado de RAINA et al., 2003). Em cada amostragem, o censo da população, incluindo os ovos postos, foram contabilizados, e o peso final dos reprodutores foi mensurado utilizando balança de precisão. Antes dos testes estatísticos, todos os dados coletados foram submetidos à análise de normalidade (Shapiro-Wilk) para verificar a distribuição dos mesmos. Na ausência de distribuição normal dos dados, testes não paramétricos foram utilizados. A variação na quantidade de ovos nas referidas amostragens foi comparada utilizando uma análise de variância (ANOVA) seguida pelo teste de Tukey para comparações múltiplas. Ovos e a prole foram fixados em FAA (3 partes de álcool absoluto, 1 parte de ácido acético glacial e 1 parte de formaldeído a 40%) por 24 horas e transferidos para álcool 80%. A fixação do material teve como objetivo identificar os estágios embrionários dos ovos (Seção 4), assim como os estágios

larvais (BARSOTTI; COSTA-LEONARDO, 2005). A variação na massa fresca dos reprodutores durante o período de atividade reprodutiva foi calculada utilizando um teste *t* pareado. Antes dos testes estatísticos, todos os dados coletados foram submetidos à análise de normalidade (Shapiro-Wilk) para verificar a distribuição dos mesmos. Todas as análises foram feitas utilizando o software R (R Core Team, 2020) e os gráficos construídos com auxílio do software GraphPad Prism 9.3.1.

### 2.3.2. *Cryptotermes brevis*

Cinco colônias foram amostradas mensalmente, por um período de nove meses (adaptado de McMAHAN, 1962). Em cada amostragem, as colônias foram desmontadas para avaliar a postura de ovos e a ocorrência de prole. O número de ovos postos durante o período foi comparado utilizando o teste de Kruskal–Wallis seguido pelo teste de Student–Newman–Keuls para comparações múltiplas. Ovos e a prole das colônias foram fixados em FAA por 24 horas e transferidos para álcool 80%. Os ovos foram submetidos à análise de identificação do estágio embrionário (Seção 2.4), enquanto as larvas e pseudergates foram submetidos a análises morfométricas para identificação de seus ínstares (Seção 2.10.1). Similar à *C. gestroi*, a variação na massa fresca dos reprodutores foi avaliada por meio de um teste *t* pareado. As análises e confecção dos gráficos seguiram as etapas descritas para *C. gestroi*.

## 2.4. Identificação dos estágios embrionários

Os ovos obtidos das diferentes colônias incipientes de *C. gestroi* e *C. brevis* foram analisados sob estereomicroscópio Zeiss Discovery V8. Essa metodologia foi um complemento para a dinâmica de oviposição, uma vez que as rainhas poderiam cessar a oviposição ainda que ovos com embriões em estágios finais de desenvolvimento estivessem presentes. Ou seja, a ocorrência de ovos não configura, necessariamente, uma oviposição ativa das rainhas. Assim, os ovos foram classificados em estágios de I a V, de acordo com o desenvolvimento do embrião (MATSUURA; KOBAYASHI, 2007). O estágio I é predominantemente composto por vitelo, passando por estágios de formação da banda germinativa e alongamento do embrião (estágios II e III, respectivamente). Os estágios IV e V caracterizam o fechamento dorsal e o desenvolvimento completo do embrião, respectivamente.

### 2.5. Anatomia das estruturas reprodutivas

#### 2.5.1. Espermateca

Cinco fêmeas virgens e rainhas de diferentes idades provenientes das colônias de *C. gestroi* (de 2 a 150-d) foram anestesiadas no freezer durante cinco minutos, e as respectivas espermatecas dissecadas em solução de Hayes (9.0 g/L NaCl, 0.2 g/L CaCl2, 0.2 g/L KCl, 0.1 g/L $NaHCO_3$, pH 8,7) sob estereomicroscópio Zeiss Discovery V8. As diferentes espermatecas foram fotografadas com o mesmo aumento, a fim de fornecer uma descrição morfológica mais detalhada, além de submetê-las a análises morfométricas (Seção 2.10.2). A espermateca de fêmeas virgens de *C. brevis* foi isolada seguindo os mesmos protocolos descritos acima e fotografadas em diferentes aumentos, uma vez que análises morfométricas não foram conduzidas utilizando essas amostras.

#### 2.5.2. Glândulas coletéricas

Fêmeas virgens de *C. gestroi* e *C. brevis* foram anestesiadas no freezer durante cinco minutos, sendo as glândulas dissecadas seguindo os mesmos procedimentos utilizados para a espermateca. As glândulas anterior e posterior foram, então, coradas com eosina aquosa por cinco minutos, lavadas, posicionadas em uma lâmina histológica e fotografadas em diferentes aumentos sob estereomicroscópio Zeiss Discovery V8.

### 2.6. Histologia da espermateca e glândulas coletéricas

O abdômen de rainhas virgens e não-virgens de *C. gestroi* e *C. brevis* foram fixados em FAA ou em Paraformoldeído por aproximadamente 24 horas. Posteriormente, os abdomens foram transferidos para álcool 70% e desidratados em uma bateria de etanol (70, 80, 90 e 95%) tendo, cada banho, duração de 60 minutos. Em seguida, as amostras foram transferidas para uma solução de resina (Leica) de infiltração e mantidas por 7 dias em geladeira. Após essa etapa, os abdomens foram incluídos em moldes especiais, preenchidos com historesina (JB-4 Polaron Instruments/ BIO RAD) mais catalisador, para polimerização em estufa a 37º C. Depois de polimerizados, os blocos foram seccionados com 3 µm de espessura utilizando navalhas de tungstênio, em micrótomo (Leica RM 2145), e as secções montadas em lâminas de vidro. Essas foram mantidas em estufa a 37º C e, posteriormente, coradas com Hematoxilina-eosina para uma melhor caracterização histológica da espermateca e glândulas coletéricas (JUNQUEIRA; JUNQUEIRA, 1983). A documentação e interpretação das lâminas foram realizadas com o auxílio de um microscópio Leica ICC50, utilizando o software Leica IM50. Secções

histológicas também foram utilizadas para avaliar a variação na altura do epitélio das glândulas coletéricas nas fêmeas de diferentes idades das duas espécies (Seção 10.3).

Para os procedimentos histológicos, pelo menos três indivíduos de fêmeas virgens e rainhas de quatro anos de *C. gestroi* foram utilizadas. Para *C. brevis*, pelo menos três indivíduos de fêmeas virgens, rainhas de seis meses, rainhas de um ano e rainhas neotênicas ninfoides foram utilizadas. Devido ao número limitado, somente uma rainha de mobiliário de *C. brevis* foi incluída nas análises.

### 2.7. Histoquímica da espermateca e glândulas coletéricas

#### 2.7.1. Método de xylidine-Ponceau para detecção de proteínas totais (ácidas e básicas)

As secções histológicas foram colocadas em solução de xylidine-Ponceau por 30 minutos em temperatura ambiente. Posteriormente, as secções foram transferidas para tampão acetato de sódio (pH = 2,6) por 1 minuto, lavadas em água destilada e mantidas a temperatura ambiente para secagem. As lâminas foram montadas em bálsamo do Canadá (sintético). Por este método, as proteínas coram-se em vermelho alaranjado. Para os procedimentos de histoquímica, utilizou-se os mesmos indivíduos descritos para análises histológicas. Protocolo seguido de acordo com Mello & Vidal (1980).

#### 2.7.2. Método de PAS (Ácido Periódico de Schiff) para detecção de polissacarídeos neutros

As secções histológicas foram colocadas em ácido periódico 0,4% por 10 minutos e, em seguida, lavadas em água destilada por cerca de um minuto e colocadas no reagente de Schiff por 60 minutos em local escuro. Após esse procedimento, as secções foram submetidas a três banhos de água sulfurosa, totalizando três minutos, lavadas e mantidas a temperatura ambiente para secagem. Depois de secas, as lâminas foram montadas em bálsamo do Canadá (sintético). Por este método, os polissacarídeos neutros e glicoproteínas coram-se intensamente de vermelho-púrpura. Protocolo seguido de acordo com Mello & Vidal (1980).

Para os procedimentos histoquímicos, foram utilizadas as mesmas fêmeas (quantidade e idade) detalhadas na seção 5.

### 2.8. Ultramorfologia da espermateca em fêmeas de *C. gestroi*

As espermatecas de fêmeas virgens de *C. gestroi* foram isoladas e fixadas em solução de Karnovsky por 24 horas. A seguir, foram desidratadas em banhos crescentes de acetona (50,

70, 90 e 95%) seguidos de dois banhos de acetona 100%, por 5 minutos cada. Esses materiais foram então levados ao ponto crítico para completa secagem (Balzers CPD/030) e, posteriormente, fixados em suporte de alumínio com auxílio de fita dupla face. As amostras foram metalizadas com ouro (Balzers CPD/050) e analisadas sob microscópio eletrônico de varredura Hitachi TM3500, visando uma descrição anatômica do órgão. Para esse procedimento, utilizou-se no mínimo três fêmeas virgens de *C. gestroi*.

### 2.9. Ultraestrutura da espermateca e glândulas coletéricas em fêmeas de *C. gestroi* e *C. brevis*

As espermatecas de fêmeas virgens e rainhas de dois anos de *C. gestroi*, assim como as de fêmeas virgens de *C. brevis*, foram dissecadas e fixadas em glutaraldeído 2,5% em tampão cacodilato de sódio 0,1 M (pH = 7,2). O mesmo procedimento foi realizado para as glândulas coletéricas de fêmeas virgens e rainhas de quatro anos de *C. gestroi* e fêmeas virgens de *C. brevis*. Para cada amostra, foram feitas duas lavagens em tampão cacodilato de sódio, de 15 minutos cada, pós-fixação em tetróxido de ósmio 1% em cacodilato de sódio 0,1 M por duas horas, seguido de dois banhos 15 minutos em tampão fosfato-salino (PBS). Posteriormente, as amostras foram desidratadas em acetona (nas concentrações de 50, 75, 90, 95 e 100%), com 10 minutos de duração cada banho e embebidas em resina Epon-araldite diluída em acetona 1:1. As espermatecas e glândulas coletéricas foram incluídas em resina Epon pura com catalisador, e mantidas em estufa a 70 ºC por 48 horas. Os cortes foram realizados em ultra-micrótomo Leica Reichert Supernova, e as secções fotografadas em microscópio eletrônico de transmissão Jeol JEM 1400. Para esta metodologia, pelo menos três indivíduos de cada grupo foram selecionados.

### 2.10. Análises morfométricas

#### 2.10.1. Morfometria das larvas e pseudergates de colônias incipientes de *C. brevis*

A prole amostrada das colônias incipientes nos diferentes meses (Seção 2.3.2) foram fotografadas em estereomicroscópio Zeiss Discovery V8 utilizando o mesmo aumento. As imagens e referidas escalas foram transferidas para o software Zeiss ZEN, onde as seguintes medidas foram quantificadas a fim de determinar os ínstares dos estágios larvais e pseuderagtes: largura máxima da cabeça, largura do pronoto, comprimento do pronoto e comprimento da tíbia anterior esquerda. Adicionalmente, o número de artículos antenais foi contabilizado. Os diferentes estágios larvais foram separados e analisados por meio de uma Análise de

Componentes Principais (PCA), considerando os diferentes parâmetros morfométricos utilizados. Os scores dos principais componentes (PC1 e PC2) foram submetidos a uma análise de variância (ANOVA) seguida pelo teste de Tukey para múltiplas comparações. As análises estatísticas foram executadas no software R (R Core Team, 2020) e os gráficos gerados pelo software GraphPad Prism 9.3.1.

### 2.10.2. Morfometria da espermateca em fêmeas de *C. gestroi*

Fêmeas virgens e rainhas de diferentes idades foram dissecadas em solução de Hayes sob estereomicroscópio Zeiss Discovery V8, conforme descrito na seção 2.5.1. As espermatecas foram então isoladas e fotografadas com aumento de 80x. A área superficial da espermateca e do lúmen foi estimada com auxílio do software ImageJ (disponível em https://imagej.nih.gov/ij/), utilizando o recurso "freehand tool". Os dados foram submetidos ao teste de normalidade de Shapiro-Wilk e as variações morfométricas entre as fêmeas foram comparadas por meio de uma análise de variância (ANOVA) seguida pelo teste de Tukey para múltiplas comparações. Todas as análises foram executadas no software RStudio (R Core Team, 2020).

### 2.10.3. Morfometria das glândulas coletéricas em *C. gestroi* e *C. brevis*

A morfometria comparada das glândulas coletéricas foi realizada a partir da altura do epitélio glandular em secções transversais (adaptado de BEDNARSKA et al., 2016). Para tal, cortes transversais dos diferentes túbulos das glândulas anterior e posterior em fêmeas virgens e não-virgens das duas espécies foram fotografados com aumento de 400x. As imagens, com as referidas escalas, foram transferidas para o software ImageJ, sendo a altura do epitélio glandular medida utilizando o recurso "straight line tool". Quatro secções transversais de cada glândula foram selecionadas para cada um dos três indivíduos dos seguintes grupos: fêmeas virgens e rainhas de quatro anos em *C. gestroi*; fêmeas virgens, rainhas de seis meses, rainhas de um ano e rainhas neotênicas ninfoides em *C. brevis*. Para cada secção, três medidas foram extraídas para estabelecer uma média de altura do epitélio (BEDNARSKA et al., 2016). Os dados obtidos foram submetidos a teste de normalidade de Shapiro-Wilk e comparados utilizando um teste *t* para *C. gestroi* e uma análise de variância (ANOVA) seguida pelo teste de Tukey para múltiplas comparações para *C. brevis*. Os dados foram analisados utilizando o software R (R Core Team, 2020) e os gráficos gerados pelo software GraphPad Prism 9.3.1.

# *Capítulo 1*

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# On the reproductive strategies post-colony foundation: major termite pest species with distinct ecological habits differ in their oviposition dynamics

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## Abstract

Termite colony foundation precedes the incipient stage, when the first oviposition cycle takes place, followed by months of reproductive inactivity. The royal couple is supposed to cease oviposition during this period, investing energy to care for the first brood. When a suitable number of alloparents differentiate, egg-laying resumes. Here we followed oviposition dynamics, embryo development and queen/king body changes in laboratory colonies of the major pest species *Coptotermes gestroi* (Rhinotermitidae) and *Cryptotermes brevis* (Kalotermitidae) during 9 months. We show that they differ in these oviposition dynamics, as *C. gestroi* queens displayed an uninterrupted oviposition whereas *C. brevis* laid a cohort of eggs and ceased oviposition during a 3-month period (*lag phase*). *C. gestroi* oviposition dynamic was remarkable and suggests that occurrence of progeny was not a limiting factor, thus queens and kings were able to concomitantly invest energy in reproduction and parental care. These findings contrast those reported for rhinotermitids from temperate areas, and we discuss the likely reasons for such a condition, including endogenous rhythms, avoidance of a high mortality rate of the first progeny and adaptation to the weather conditions of the Neotropical region. Oviposition dynamic in *C. brevis* resembled those of several termite species, in which the royal couple cease reproduction to care for the first brood. Rearing conditions did not influence oviposition dynamics (egg-laying cycle followed by a *lag phase*), thus our results on the oviposition of *C. gestroi* and *C. brevis* correspond to different reproductive strategies post-foundation adopted by these pest species.

## Introduction

Termite colony foundation is a critical event and takes place after the swarming flights, when alates disperse from the natal nest and form monogamous pairs to establish a nest (Nutting, 1969; Nalepa and Jones, 1991). Colony foundation success depends on several intrinsic and extrinsic factors (Chouvenc *et al.*, 2014; Kusaka and Matsuura, 2018; Chouvenc, 2019), and reproductives may spend energy in activities such as nest building, offspring production and initial parental care (Nutting, 1969). Termite colonies go through several developmental stages. The first stage, referred to as 'incipient colony', lasts around seven months and is marked by the first oviposition cycle (Chouvenc and Su, 2014), followed by the ergonomic phase, in which colonies increase the production of sterile castes (Oster and Wilson, 1978). Oviposition rate in termites might be affected by different factors. For instance, incipient colonies tend to cease egg production during some months when the primary reproductives are still responsible for brood care, as detailed below. Moreover, field samplings have shown that weather fluctuations impact egg production, which is interrupted during colder months (Weesner, 1969; Nozaki and Matsuura, 2021).

A successful oviposition and progeny differentiation during earlier stages of a termite colony is crucial for its development and subsequent spread (Chouvenc and Su, 2014). Such dynamics, therefore, deserve attention among invasive species of economic interest (e.g. families Rhinotermitidae and Kalotermitidae) (Evans, 2021). Egg-laying has been well studied within subterranean species (Rhinotermitidae) from temperate regions, in which the first oviposition cycle occurs within 30–70 days after pairing (table S1), followed by a period of reproductive inactivity, referred to as *lag phase*, that lasts from 4 to 6 months (Weesner, 1969; Raina *et al.*, 2003; Ghesini and Marini, 2009; Maekawa *et al.*, 2010; Janowiecki, 2012; Brossette *et al.*, 2017). Within drywood species (Kalotermitidae), the oviposition dynamic at earlier developmental stages has been poorly studied and restricted to few species (McMahan, 1962; Wilkinson, 1962; Lenz, 1987; Scheffrahn *et al.*, 1998). Queens of *C. brevis* reared in subtropical areas lay eggs during the first 6 months after colony foundation and go through a *lag phase* that lasts 3 months (McMahan, 1962). Within Rhinotermitidae, parental care towards the

first brood is pointed as a reason for the reproductives to cease oviposition during the first months of the colony, which is only resumed when a suitable number of workers differentiate to assist the next generation of the brood (Nutting, 1969; Nalepa and Jones, 1991; Maekawa *et al*., 2010). Equivalent reports on the oviposition dynamics for colonies of subterranean and drywood species distributed in the Neotropical region are unknown.

The major pest species *Coptotermes gestroi* (Rhinotermitidae) and *Cryptotermes brevis* (Kalotermitidae) are native from Southeast Asia and Coastal Chile and Peru, respectively. However, both species have been introduced in several countries, including Brazil (Li *et al*., 2013). In São Paulo state, for example, *C. gestroi* and *C. brevis* are the main wood pests and show a relevant economic impact (Fontes and Milano, 2002; Romagnano and Nahuz, 2006). The ecological habits differ greatly between these species, since *C. gestroi* has a well-established nest, separated from foraging sites, while *C. brevis* spends the entire life cycle of the colony in a single piece of wood, which serves as both shelter and food source (Korb and Thorne, 2017). Reproductive aspects also vary greatly, as *C. gestroi* queens show a notorious egg-laying rate (Costa-Leonardo, 2002) whereas those of *C. brevis* display extremely low oviposition rates, which therefore impact the colony population in different ways (McMahan, 1962; Costa-Leonardo, 2002).

Due to the cryptical habit of termites, sampling and studying reproductive individuals is challenging, resulting in knowledge gaps about their reproductive biology. Laboratory rearing systems is thus a suitable way to evaluate colony development and access the reproductive status of the royal couple (Costa-Leonardo and Barsotti, 1998, 2001; Laranjo *et al*., 2019; Costa-Leonardo *et al*., 2022; da Silva and Costa-Leonardo, 2022, 2023a, 2023b). Here, colonies of *C. gestroi* and *C. brevis* were reared and kept under laboratory conditions in Southeast Brazil and monitored over 9 months to evaluate their development and egg-laying dynamic of the queen. Our main goal was to evaluate if queens of these termite species based on the Neotropical region stop laying eggs following a first oviposition cycle, similar to what has been reported for termite species from temperate areas. Assuming the pest status and invasive success of both the species (Evans, 2021), a better understanding of their reproductive activity during earlier developmental stages of the colony is crucial to evaluate breeding strategies and spread patterns.

## Material and methods

### Termites

*Coptotermes gestroi* (Wasmann, 1896): Alates were collected during dispersal flights in the city of Rio Claro, SP, Brazil (22°23′S, 47°31′W). Sex of dealates was determined by the presence of the genital plate in the females, and the initial wet mass of each individual was measured. Alates were then paired in 6 cm Petri dishes containing decayed and moistened *Pinus* sp. sawdust (figs 1A–C), and kept in the dark at 25 °C.

*Cryptotermes brevis* (Walker, 1853): Alates were collected during swarming flights of laboratory colonies at the São Paulo State University, Rio Claro, SP, Brazil (22°23′S, 47°32′W). Dealates were sexed based on the stylus present in the ninth sternite of males. The initial wet mass of each individual was measured, and male–female pairs were placed in colonies made of *Pinus* sp. tongue blades (adapted from McMahan, 1962). For this purpose, tongue blades of different lengths were attached to each other using a non-toxic glue, leaving a central chamber where termites were placed. Such a chamber was then covered with a glass slide held by rubber bands (figs 1D–F). The total wood volume for each colony was 24.28 $cm^3$, indicating an abundant amount of food for the paired reproductives (Korb and Lenz, 2004). Wooden termitaries were individually placed in plastic containers and kept in the dark at 25 °C.

### Egg-laying dynamic and colony development

*Coptotermes gestroi:* Five colonies were randomly sampled at 2, 10, 20, 30, 50, 80, 100, 120, 150, 180, 200, 220, 250 and 270 days after foundation, totalizing 9 months (adapted from Raina *et al*., 2003). These colonies are hereafter termed according to their age (i.e. 10-, 20-, 30-day colonies). Total eggs and progeny (larvae, workers and soldiers), if present, were counted, fixed in FAA (absolute alcohol, glacial acetic acid, 40% formaldehyde, in the ratio 3:1:1) for 24 h and transferred to 80% alcohol. Larval instars were recognised according to Barsotti and Costa-Leonardo (2005). The final wet mass of the reproductives was measured for each sampling period.

*Cryptotermes brevis:* Given the extremely low egg-laying rate of the referred species, five colonies were randomly sampled per month during the 9-month period (adapted from McMahan, 1962). These colonies are hereafter referred according to their age (i.e. 1-, 2-, 3-month colonies). The number of eggs laid and progeny (larvae and pseudergates) were also counted, fixed in FAA for 24 h and transferred to 80% alcohol. Aiming to discriminate the larval instars and pseudergates of *C. brevis*, the following measurements were extracted from the progeny and compared among individuals: maximum head width (MHW), pronotum width (PW), pronotum length (PL) and tibia length (TL). Finally, the final wet mass of the reproductives was measured.

### Embryo development

Assuming the necessary hatching period (see Results section), the presence of eggs themselves could not be used to infer egg-laying activity. It occurs because queens could cease oviposition but there would still be remaining eggs at later developmental stages. Thus, eggs sampled from all colonies of *C. gestroi* and *C. brevis* were analysed under Zeiss Discovery V8 stereomicroscope aiming to classify their developmental stages (Matsuura and Kobayashi, 2007).

### Statistical analyses

Variation in the number of eggs, larvae, workers and soldiers of *C. gestroi* over the 9-month period was calculated using ANOVA (one-way) followed by Tukey's test or Kruskal–Wallis followed by a Student–Newman–Keuls (SNK) comparison test according to data distribution. For *C. brevis*, in turn, variation in the number of eggs, larvae and pseudergates among samplings were evaluated only performing a Kruskal–Wallis + SNK comparison test. The morphometrical data extracted from the progeny of *C. brevis* were submitted to a principal component analysis (PCA). The scores of PC1 and PC2 were analysed using ANOVA (one-way) followed by Tukey's test. Furthermore, changes in queen and king wet mass over the sampling period were compared using a paired *t*-test.

All data were analysed using the software R (version 4.1.0) and images were generated using GraphPad Prism 9.3.1.

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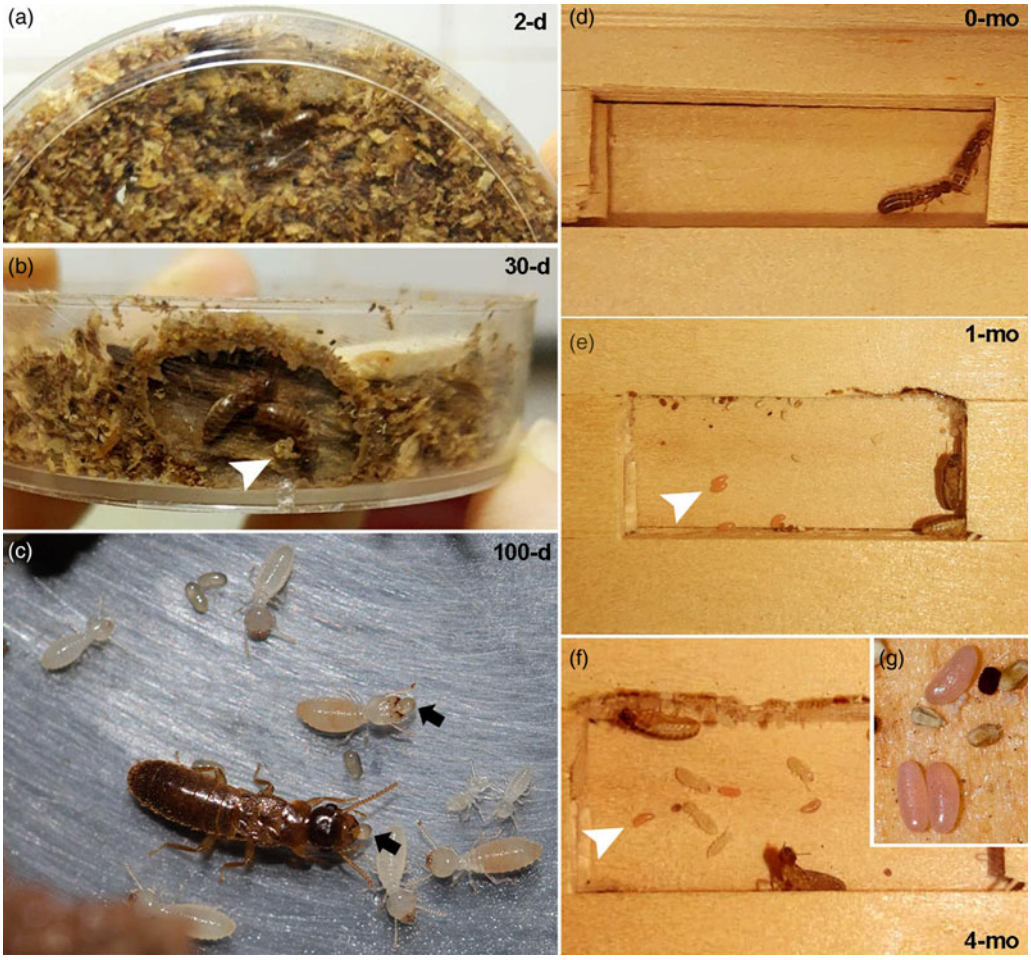


**Figure 1.** Incipient colonies of *Coptotermes gestroi* (A, B and C) and *Cryptotermes brevis* (D, E and F) reared under laboratory conditions. (A) Recently founded colony containing the royal couple. (B) 30-day colony with eggs organised into a pile (white arrowhead). (C) 100-day colony showing the queen, larvae and workers of *C. gestroi*. Black arrows indicate eggs being carried. (D) Recently founded colony. (E) 1-month colony containing eggs (white arrowhead). (F) 4-month colonies highlighting the royal couple, eggs and progeny. (G) Detail of the pinkish eggs (earlier developmental stages).

## Results

### *Coptotermes gestroi*

The royal couple performed tandem behaviour right after their introduction into the Petri dishes and quickly started to excavate a chamber. Egg-laying took place around 5 days and eggs were always stuck together into a single pile. Oviposition rate varied over the 270-day period, with all colonies showing a significantly higher number of eggs when compared to 10-day colonies ($P < 0.0001$, ANOVA), in which oviposition had just started (fig. 2A). The first peak of eggs was reported among 50-day colonies, with mean values of $17.8 \pm 1.6$ eggs per colony. After a period of egg-laying reduction from 80 to 180 days, oviposition increased again, reaching a second peak among 220-day colonies, with mean values of $15.4 \pm 0.7$ eggs per colony. It is interesting to note that eggs were reported during the whole sampling period (fig. 2A).

Eggs hatched between 50 and 60 days and larvae number remained similar (fig. 2B), but showing a slight reduction within 270-day colonies ($P = 0.0228$, ANOVA). Larvae comprised two instars, moulting into workers in the interval between 70 and 80 days, as 80-day colonies already showed both larvae and workers. Worker number increased significantly from 80- to 270-day samplings ($P < 0.0001$, ANOVA), agreeing with the slight decrease in larva number over time. At the end of the samplings (270-day colonies), workers comprised the most abundant caste (fig. 2C). Workers moulted into pre-soldiers between 90 and 100 days (not shown) and soldiers were firstly reported among 120-day colonies, but certainly differentiated from pre-soldiers before that. Such a caste was always in a low number, as expected, but increased slightly within 250-day colonies ($P = 0.0246$, Kruskal–Wallis), with mean values of $2.2 \pm 0.4$ per colony (fig. 2D).

Number of eggs at different developmental stages varied among sampling colonies and periods. The latest stage V was firstly reported among 50-day colonies, right before larval eclosion (fig. 2A and 3A), and was reported in all the following samplings, suggesting a constant progeny production (figs 3A–B). Interestingly, eggs at early development (stage I) were reported among all samplings, indicating that oviposition did not cease over the 9-month period (figs 3A–B). It therefore agrees with the occurrence of eggs in all colonies (fig. 2A).

The wet mass of reproductives fluctuated over the period. Among queens, the initial and final wet mass differed significantly

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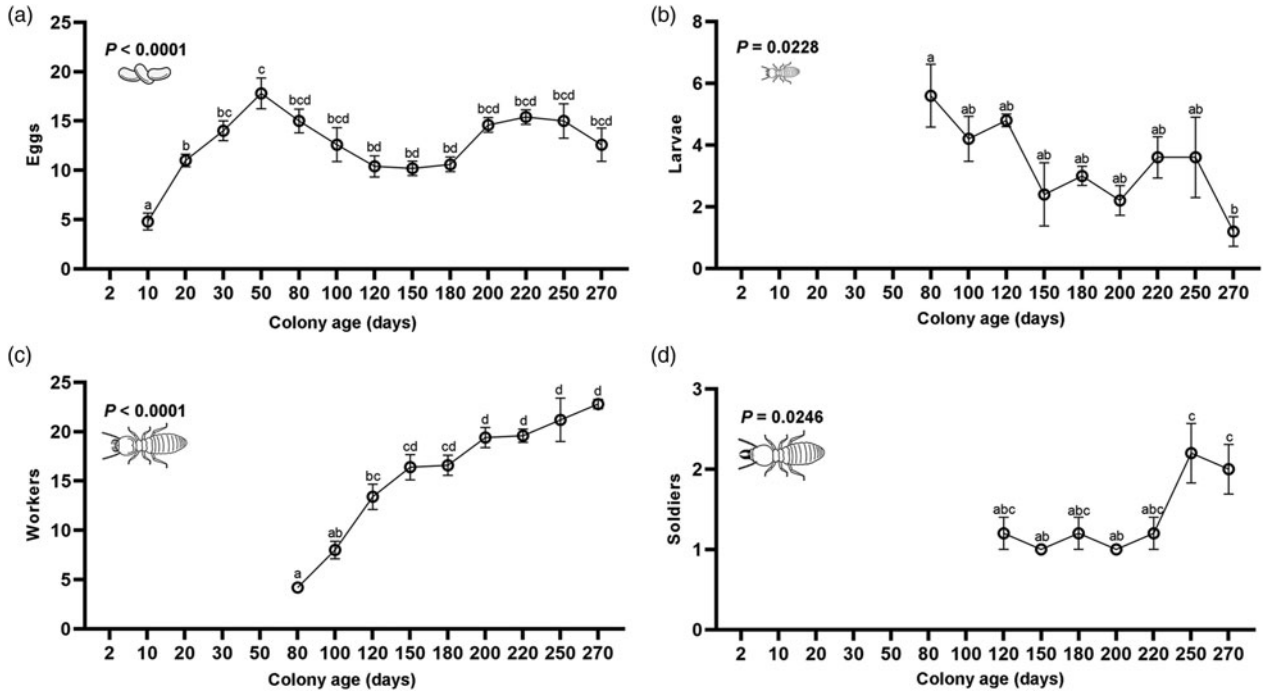


**Figure 2.** Colony development in *C. gestroi*, with mean number ± SE of (A) eggs, (B) larvae, (C) workers and (D) soldiers sampled over the 270-day sampling period. Different letters mean statistical differences (ANOVA + Tukey's HSD test).

from 2- to 80-day colonies (fig. 4A). During the following samplings, wet mass did not vary until 250-day, in which queens started to show a significant loss of the wet mass (fig. 4A). For kings, wet mass started to increase significantly among 20-day colonies, following a similar pattern observed in *C. gestroi* queens, with no wet mass change from 120- to 220-day, but with significant loss among 250- and 270-day colonies (fig. 4B).

### *Cryptotermes brevis*

After their introduction into the wooden termitaries, reproductives took about 6 days to start excavating the wood. Oviposition started about 20 days later, with a mean number of 2 ± 0.44 eggs among 1-month colonies. During the 9-month sampling period, egg-laying rate remained extremely low, with the

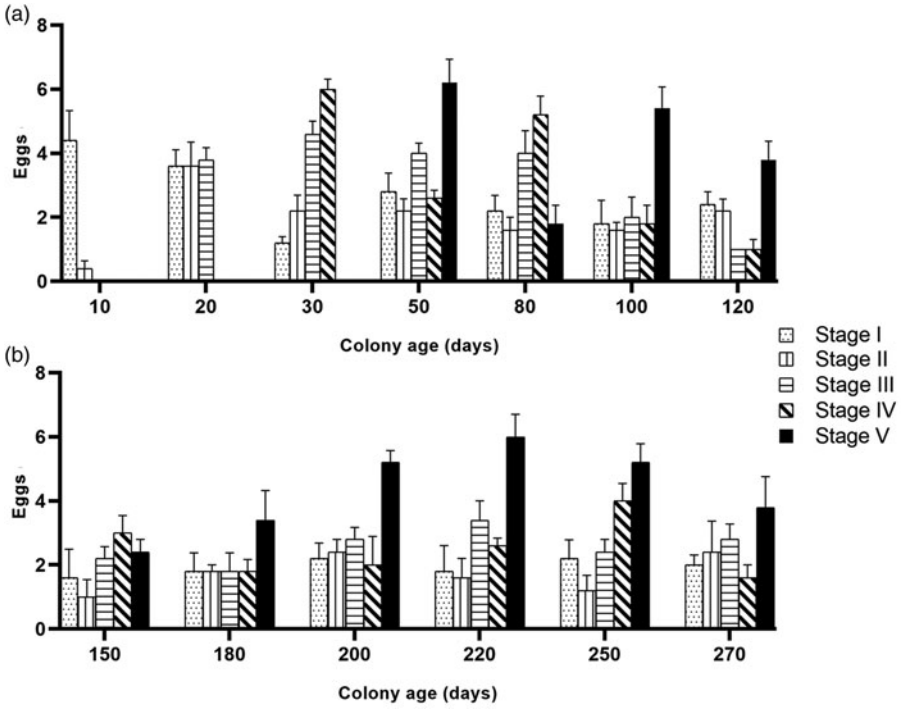


**Figure 3.** (A and B) Developmental stage of the eggs sampled from the *C. gestroi* colonies (mean ± SE). Note that stage I eggs (the earliest stage) were present over the whole 270-day sampling, suggesting that oviposition was uninterrupted.

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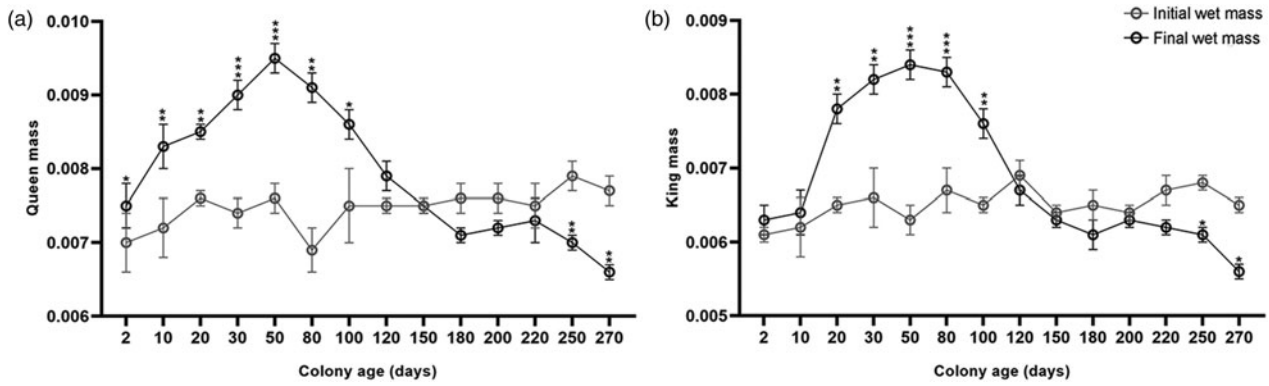


**Figure 4.** Comparison between initial and final wet mass (mean ± SE) of *C. gestroi* queens (A) and kings (B) over the 270-day period. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Samplings without asterisks did not show statistical difference (paired *t*-test).

highest value observed among 2-month colonies (3.8 ± 0.37 eggs per colony) (fig. 5A), being significantly higher when compared to all other samplings, except for 1- and 3-month colonies ($P_{2\text{-mo/1-mo}} = 0.1895$; $P_{2\text{-mo/3-mo}} = 0.5002$, Kruskal–Wallis + SNK). Notably, eggs reduced in number after the 4-month sampling and were completely absent among 7-month colonies. Oviposition resumed in the following month, without statistical difference until the end of the samplings (fig. 5A). Eggs ranged from pink to white, according to their developmental stages, and were never kept together into piles.

Eggs hatched right before the third month, with an increasing number of larvae in the subsequent samplings (fig. 5B). However, the number of larvae decreased, although not significantly, after the sixth month ($P = 0.1152$, Kruskal–Wallis), corresponding to the period in which egg-laying showed the lowest values. Early-instar pseudergates, in turn, were firstly reported among 4-month-old colonies and differentiated after three larval instars (figs 5B–C). Pseudergate number increased until the ninth month, with slight fluctuations, comprising the most abundant caste of the colonies at this time (fig. 5C). Our PCA analysis significantly separated all three larval instars, as well as two pseudergate instars ($P < 0.0001$, ANOVA; fig. 5D). The first component (PC1) explained 96.31% of the variance, followed by the second component (PC2), with 2% of the variation. ANOVA performed on the scores of PC1 and PC2 evidenced morphometric differences among all larval and pseudergate instars ($P < 0.01$, Tukey's test). Soldiers were not observed over the 9-month period.

Concerning embryo development, egg stages I and II, the earliest stages, were absent from 5- to 7-month samplings, in accordance with the decreasing number of eggs. Only stages IV and V (the last developmental stage before eclosion) were reported amongst colonies during the referred period, indicating that queens ceased oviposition during this 3-month interval (fig. 6). Such an assumption is supported by the presence of earlier developmental stage eggs in 8-month colonies, in which the oviposition resumed (figs 5A and 6).

During the whole sampling, the wet mass of the queens did not vary (fig. 7A). For kings, with exception of 4-month old males ($P = 0.02036$, paired *t*-test), the wet mass did not change either (fig. 7B).

## Discussion

Here we evaluated the egg-laying dynamics in two major pest termite species with different ecological habits. Such colonies were reared under laboratory conditions and monitored over a 9-month period. While *C. gestroi* did not cease oviposition over the whole sampling, *C. brevis* showed a 3-month interval without laying eggs. We suggest, based on the following discussion in the light of previous studies, that these oviposition dynamics reflect reproductive strategies of the sampled species after colony foundation.

### *Coptotermes gestroi*

Oviposition persisted over the 270-day period, given the presence of eggs, but especially those at earlier developmental stages. Thus, eggs were laid even in the presence of the first brood, which depend on the parental care provided by reproductives (Nutting, 1969; Chouvenc, 2022). Previous reports on Rhinotermitidae from temperate areas indicate that termite queens lay eggs during 30–70 days after pairing, followed by a period of reproductive inactivity that ranges from 4 to 6 months (table S1). Queens cease oviposition when the first larvae emerge and reproductives usually invest energy in parental care rather than reproduction (Nutting, 1969). When workers differentiate and their number reaches a threshold, oviposition resumes (Maekawa *et al.*, 2010). At this time, biparental care shifts irreversibly towards alloparental care, in which workers assist the brood (Chouvenc, 2022). The occurrence of these dependent instars, therefore, seemed not to be a limiting factor for *C. gestroi* queens to continue laying eggs.

The uninterrupted oviposition was a striking feature in our analysis, without equivalent results for other studied subterranean species (table S1). One could argue that rearing conditions could impact physiological responses of the reproductives and, therefore, the oviposition dynamic. Nevertheless, it does not seem to be the case for our analysis on *C. gestroi*, as we discuss in the light of previous studies. The first oviposition cycle in Rhinotermitidae species (*Coptotermes* and *Reticulitermes*) from temperate areas was evaluated under a variety of rearing systems (i.e. using different container sizes, substrate composition and temperature), but always showed a pattern in respect to the oviposition dynamics, comprising the first egg-laying cycle followed by several months of reproductive inactivity (Raina *et al.*, 2003; Ghesini and Marini, 2009; Maekawa *et al.*, 2010; Janowiecki, 2012; Brossette *et al.*, 2017). Thus, rearing conditions do not impact the egg-laying dynamic, although they influence the number of eggs and progeny (Ghesini and Marini, 2009; Maekawa *et al.*, 2010; Janowiecki, 2012; Brossette *et al.*, 2017). Based on this, we suggest that our *C. gestroi* reproductives do not go

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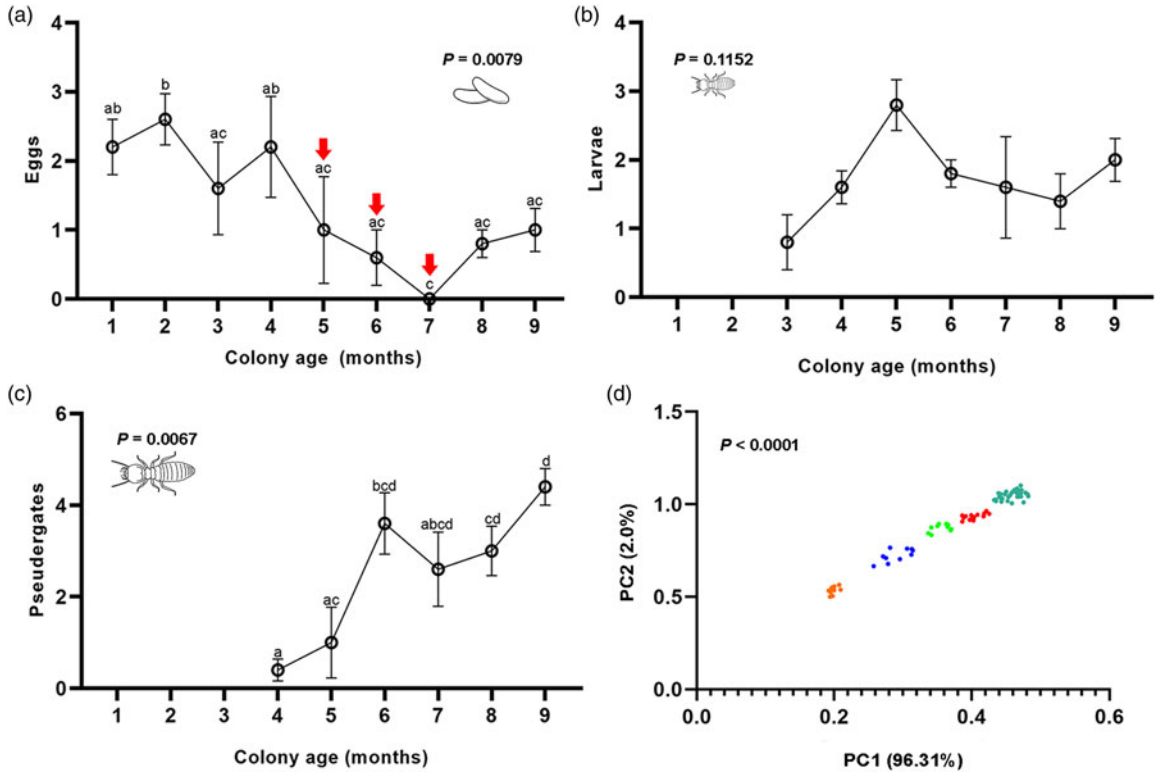


**Figure 5.** Colony development in *C. brevis*, with mean number ± SE of (A) eggs, (B) larvae and (C) pseudergates over the 9-month sampling period. Red arrows indicate the period in which egg-laying ceased. Different letters mean statistical differences (Kruskal–Wallis + SNK comparison test). Samplings without asterisks did not show statistical significance. (D) PCA analysis showing morphometrical differences among the instars (ANOVA + Tukey's HSD test). First instar larvae = orange, second instar larvae = blue, third instar larvae = green, first instar pseudergates = red, second instar pseudergates = cyan.

through a *lag phase*, but spend energy in both the activities (egg-laying and parental care), respecting their energetic reserves, as discussed below. In this way, queens may adjust the number of eggs laid while caring for the brood aiming to reach a suitable number of alloparents. Subterranean termite species show high mortality during the founding period (King and Spink, 1974; Higa, 1981; Kitade *et al.*, 2004). Even when colonies survive, death of larvae and workers are high in *Coptotermes formosanus*, indicating that the first brood has a short life expectancy (King and Spink, 1974; Higa, 1981). At the end of the 270-day sampling, the survival rate of our colonies was about 65%, all of them containing several eggs, larvae, workers and soldiers. Thus, we suggest that the strategy adopted by *C. gestroi* may reduce the probability of the colony failure during earlier developmental stages.

It is possible that the *lag phase* that usually follows the first oviposition cycle is under endogenous control rather than triggered by the presence of an offspring and its number. It was already stated that circadian rhythms play a major role in regulating oviposition cycles in ants and fruit flies (Sheeba *et al.*, 2001; Kipyatkov, 2006; Kuroki *et al.*, 2018; Shaw *et al.*, 2018). Furthermore, the physiology of termite reproductives changes due to endogenous signals rather than controlled lab conditions (Li *et al.*, 2021). Beyond the *lag phase*, in which reproductives stop egg-laying to support the first brood (Nutting, 1969; Raina *et al.*, 2003; Maekawa *et al.*, 2010), weather fluctuations are known to impact oviposition and other termite reproductive events (Nutting, 1969; Li *et al.*, 2021; Nozaki and Matsuura, 2021). We do not have sufficient evidence to infer that our colonies of *C. gestroi*, whose oviposition was uninterrupted, display such a behaviour in response, among other factors, to weather conditions offered by the Neotropical region. However, it was already pointed out that Neotropical termitids lay eggs all year round and suitable weather


**Figure 6.** Developmental stage of the eggs sampled from the *C. brevis* colonies (mean ± SE). Red arrows indicate the 3-month interval in which queens ceased oviposition. Note the absence of earlier developmental stages in these samplings.

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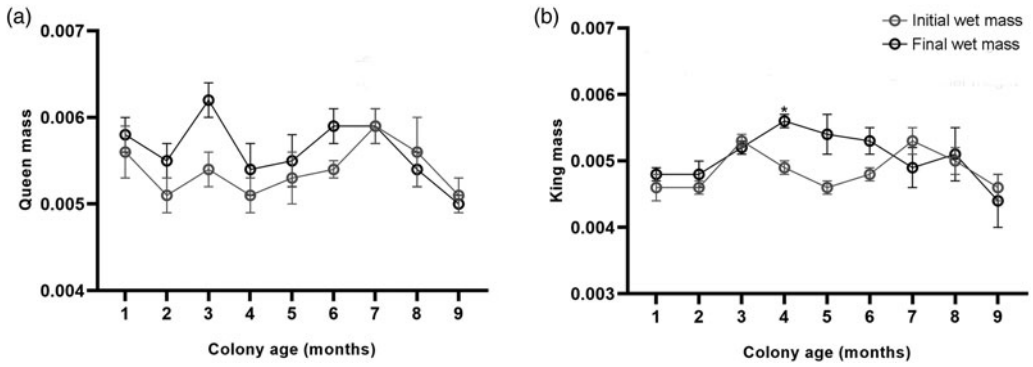


**Figure 7.** Comparison between initial and final wet mass (mean ± SE) of *C. brevis* queens (A) and kings (B) over the 9-month period. *$P < 0.05$. Samplings without asterisks did not show statistical difference (paired *t*-test).

conditions may be related to this (Fernandes *et al*., 1998; Torales and Coronel, 2004; Etcheverry *et al*., 2010; Laffont *et al*., 2012; Annoni *et al*., 2013). Future studies comparing the oviposition dynamics of *C. gestroi* between natural and introduced areas would be valuable to evaluate if there are other mechanisms underlying this strategy, especially given that *C. gestroi* was introduced in Southeast Brazil around 100 years ago and is well established in this region (Fontes and Milano, 2002).

Wet mass of the *C. gestroi* reproductives varied over time and corroborates data for the same species reared under other conditions (Chouvenc, 2022), increasing significantly until 50-day post-foundation (gaining weight phase), but decreasing from 50 to 100 days (losing weight phase), and remained equal or lower to their initial wet mass in the following samplings (stagnant weight phase). The increase in wet mass over the first 50 days coincides with the reabsorption of environmental water and inflation of the hindgut due to the replication of flagellate mutualists (Shimada *et al*., 2013; Inagaki *et al*., 2020; Velenovsky *et al*., 2021). Subsequently, wet mass loss among 50–100-day reproductives is probably due to the emergence of the offspring, which are provided with mutualistic gut loads and salivary secretions from the reproductives through proctodeal and stomodeal trophallaxis, respectively (Nalepa, 2015; Brossette *et al*., 2019; Velenovsky *et al*., 2021). Moreover, reproductives also deplete their nutritional reserves to produce eggs and assist the first brood (Zhang *et al*., 2021; Chouvenc, 2022), which therefore impact their wet mass. Although the similar values in the following samplings, probably due to differentiation and support of alloparents, both queens and kings from 250- and 270-day colonies lost significant wet mass. Queens and kings of *Reticulitermes chinensis* reared at 25 °C lose wet mass around 7.5-month post-foundation, a likely dehydration in response to colonies entering the winter season. It was suggested by the authors, as well as discussed above, that endogenous signs predominate over the controlled rearing conditions (Li *et al*., 2021). Such an assumption may be applied for *C. gestroi* colonies studied here, since the last samplings (250- and 270-day) occurred in May, when external temperature oscillates greatly.

### *Cryptotermes brevis*

Based on our results, queens of *C. brevis* sampled in southeast Brazil lay eggs until the fifth month after colony foundation and cease such an activity during a 3-month period. It therefore agrees with the 3-month *lag phase* found for the same species sampled in Hawaii (McMahan, 1962). Egg-laying took about 20 days to start, similar to that reported for other kalotermitids such as *Kalotermes flavicollis* and *Cryptotermes havilandi* (Grassé and Noirot, 1958; Wilkinson, 1962). Queens of *C. brevis*, as well as of other drywood termites, are known for laying extremely low amounts of eggs (McMahan, 1962; Wilkinson, 1962; Noirot, 1990; Scheffrahn *et al*., 1998), resulting in colonies comprising a few hundred individuals (Korb and Thorne, 2017). Even so, *C. brevis* queens sampled here ceased oviposition during a 3-month period after the eclosion of the first larvae, probably to care for the first brood, despite its low number (McMahan, 1962; Steward, 1983; Lenz, 1987).

It is suggested that *C. brevis* reproductives rarely feed and must nourish the first brood by depleting their body reserves (Lenz, 1987). Nevertheless, we observed faecal pellets on our *C. brevis* termitaries before the eggs hatched, suggesting that reproductives fed on nest wood. It is interesting to note that we offered wood termitaries of 24.28 $cm^3$, corresponding to an abundant amount of food (Korb and Lenz, 2004), against those of 2.62 $cm^3$ from the pioneer study of McMahan (1962). Even so, the egg-laying dynamics were quite similar, suggesting that the different rearing conditions did not influence the 3-month *lag phase*. As discussed above, such a dynamic within drywood species may also be controlled by endogenous signals whereas egg number and progeny may reflect rearing conditions, in this case, the avoidance of intraspecific competition and pre-existence of a nuptial chamber (McMahan, 1962; Scheffrahn *et al*., 1998).

Egg-laying resumed among 8-month colonies, 3 months after queens ceased oviposition. Our morphometrical analysis suggests the differentiation of at least two pseudergate instars, referred to as fourth+ nymphal instar by McMahan (1962), and three larval instars, in accordance with data for *Cryptotermes* spp. (Korb and Katrantzis, 2004; Cesar *et al*., 2019). The increasing number of larvae among 9-month colonies, following the resumed egg-laying, indicates that another generation of brood had developed, probably supported by nestmates. Brood care performed by alloparents of drywood species has been discussed by several studies (LaFage and Nutting, 1978; Steward, 1983; Crosland *et al*., 2004; Nalepa, 2015, 2016).

With the exception of 4-month-old kings, we did not observe a difference between the initial and final wet mass of *C. brevis* reproductives. Assuming that mass gain during the initial stages of the colony is partially attributed to environmental water resorption, it seems plausible that *C. brevis* do not gain significant wet mass, given that colonies are founded under low moisture

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levels, around 7–12% (Haigh *et al.*, 2022). Similar to other termite species, neonates of drywood termites must rely on the parents to receive protozoan loads by feeding on hindgut fluids (Nalepa, 2016). Even so, the relation between wood consumption (assumed due to the occurrence of faecal pellets) and gut protozoa duplication remains to be explored for *C. brevis*, as well as its impact on wet mass.

## Conclusions

Our scientific outcomes indicated contrasting colony demography between *C. gestroi* and *C. brevis*. Such features were not likely to be influenced by rearing conditions and seem to be controlled by endogenous signals, reflecting on different post-colony foundation reproductive strategies. The uninterrupted oviposition performed by *C. gestroi* queens was remarkable, differing from that reported for subterranean species from temperate areas, with absence of the well-known period of reproductive inactivity that follows the first oviposition cycle. The concomitant production of eggs and the parental care towards the first brood would reflect the capacity of the royal couple in investing energy in different reproductive tasks and are likely to reduce the chances of the colony failure during earlier developmental stages. The long-term impact of such a dynamic remains to be explored, given that laboratory colonies of *C. formosanus* and *Reticulitermes flavipes* kept for longer periods than ours show distinct oviposition cycles with intervening *lag phases* (Raina *et al.*, 2003; Janowiecki, 2012). *C. brevis* queens, on the other hand, ceased oviposition during a 3-month period, when the royal couple cared for the first brood until the differentiation of alloparents. With exception to *C. gestroi*, such a dynamic is in accordance with that of most termite studies sampled so far.

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**Competing interests.** None.

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Table S1. Oviposition dynamics in incipient colonies of subterranean termite species (Rhinotermitidae)

| Species | Rearing conditions (T ºC) | First oviposition cyle | Inactive period | Reference |
|---|---|---|---|---|
| *Coptotermes formosanus* | 5 cm Petri dishes with agar + moistened sawdust (28 ºC) | 60-70 days | About 4 months | (Raina et al., 2003) |
| *Reticulitermes speratus* | 20 mL glass vials with mixed sawdust (25 ºC) | About 75 days | About 5 months | (Maekawa et al., 2010) |
| *Reticulitermes lucifugus* | - | About 30 days | 4-6 months | (Nutting, 1969) |
| *Reticulitermes flavipes* | 5.4 cm plastic containers with moistened filter paper + soft and hardwood mulch (26 ºC) | 30-60 days | 4-5 months | (Janowiecki, 2012) |
| *Reticulitermes flavipes* | 5 cm Plastic boxes with cellulose discs + nutritive solution (26 ºC) | 30-60 days | At least 6 months | (Brossette et al., 2017) |
| *Reticulitermes grassei* | 5 cm Plastic boxes with cellulose discs + nutritive solution (26 ºC) | 30-60 days | 5-6 months | (Brossette et al., 2017) |
| *Reticulitermes urbis* | 9 cm Petri dishes with granite sand + block of poplar wood + filter paper (20-26 ºC) | 30 days | - | (Ghesini e Marini, 2009) |

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# Capítulo 2

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Entomologia Experimentalis et Applicata

ORIGINAL ARTICLE

# Mating mediates morphophysiological changes in the spermathecae of *Coptotermes gestroi* queens

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## Abstract

Insect spermathecae play a crucial role in sperm storage and maintenance prior to egg fertilization. Within eusocial insects, this structure is well studied in the Hymenoptera, whose queens copulate during a short period early in life and store sperm for up to decades. Within Isoptera, sperm storage and maintenance inside the spermatheca are poorly understood, especially due to the presence of a sperm-providing king. Here, we compared the morphometric and morphophysiological features of the spermathecae of virgin and mated queens of the invasive termite *Coptotermes gestroi* (Wasmann) (Isoptera: Rhinotermitidae). The spermatheca comprises a finger-shaped reservoir divided into two regions and a duct limited by a narrow transition. The superficial spermatheca area, as well as the luminal area, increase significantly after insemination, even among queens whose reproductive activity was reduced, suggesting that sperm storage continues during such conditions. The secretion of proteins and polysaccharides into the spermathecal lumen was a remarkable feature for both virgin and 4-year-old queens, although the concentration of the secreted content increased in the latter group. It suggests that spermatheca activation occurs before pairing, but its secretory activity intensifies to nourish and provide energy for the stored spermatozoa. Ultrastructure of the spermathecal epithelium showed a bicellular unit, composed of a secretory cell and associated canal cells. Secretory vesicles of various electron densities were observed next to the receiving canal of the secretory cells in both virgin and 2-year-old queens. Nevertheless, strongly electron-dense vesicles were only recorded for mated queens, which were associated with the increasing synthesis of proteins. The occurrence of rough endoplasmic reticulum and richness of mitochondria reinforces the protein synthesis and transport of contents towards the spermathecal lumen. In conclusion, the spermatheca of *C. gestroi* undergoes morphometric and physiological changes after mating, and further analysis may provide insights into the chemical nature of the spermathecal secretion prior to and after this event.

**KEYWORDS**
Blattaria, *Coptotermes gestroi*, eusocial insect, histochemistry, Isoptera, pest species, reproductive system, Rhinotermitidae, sperm storage, spermatheca, termite, ultrastructure

  

## INTRODUCTION

The reproductive system in insect females is generally composed of two ovaries connected to lateral oviducts, which join in a common canal. Additionally, associated structures such as spermathecae occur in all insect taxa (Simmons, 2013; Pascini & Martins, 2017). The spermatheca is ectodermal in origin and displays a crucial role in sperm storage and maintenance prior to fertilization of eggs (Engelmann, 1970). This organ varies in number and morphology, assuming that the occurrence of a single spermatheca is an ancestral condition. The general structure of the spermatheca comprises a reservoir, accessory glands, spermathecal duct, and associated muscles (Pascini & Martins, 2017).

The capacity of sperm storage by the spermatheca includes several benefits, such as the spatial separation between mating and egg fertilization, and the possibility of egg fertilization in the absence of males (Baer et al., 2016). For this purpose, the spermatheca provides protection, energy, and nourishment to the stored spermatozoa (Al-Lawati et al., 2009; King et al., 2011). Within eusocial Hymenoptera, whose queens copulate during a short period early in life, a suitable environment created by the spermatheca is even more crucial for long-term sperm storage (Baer et al., 2009; Malta et al., 2014; Gotoh et al., 2017; Dávila et al., 2018).

In contrast to eusocial Hymenoptera, termite colonies are generally founded and headed by a royal couple, in which the queen is regularly inseminated by the king (Nutting, 1969). After mating, termite queens also store spermatozoa inside the spermatheca, but such a storage capability is believed to be limited due to the presence of a sperm-providing king (Hartke & Baer, 2011). As a consequence, the functional morphology and mechanisms underlying sperm storage in termite queens remain largely unknown. Thus, pre- and post-copulatory assessments of the spermatheca would be useful to shed light on these aspects.

The subterranean termite *Coptotermes gestroi* (Wasmann) (Isoptera: Rhinotermitidae) is an important pest worldwide (Chouvenc et al., 2016). Reproductive studies regarding this species are quite difficult due to its cryptic habit and the occurrence of satellite nests (Lelis, 1999). Thus, rearing laboratory colonies is a suitable way to conduct comparative analyses on different-aged reproductives (Laranjo et al., 2019). Aiming to contribute to the

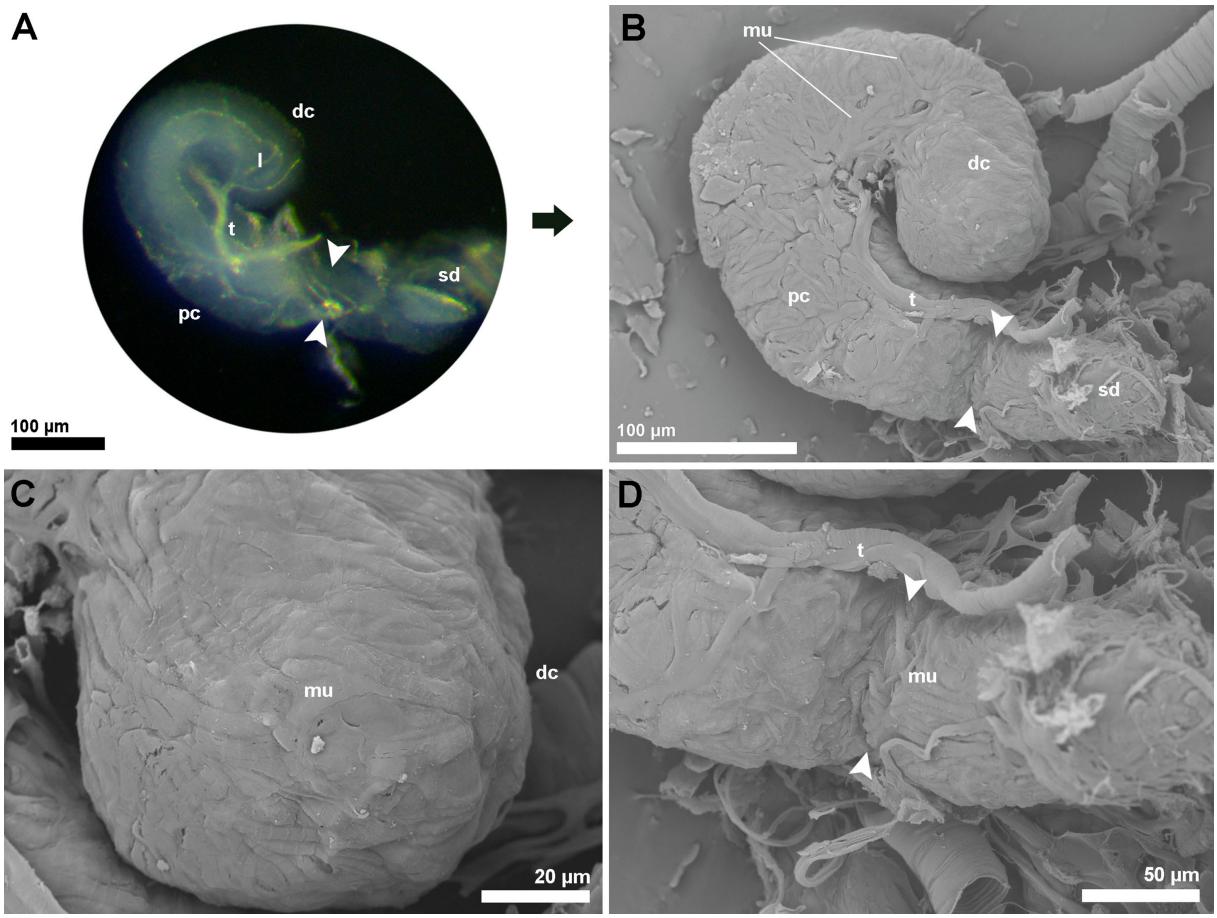


**FIGURE 1** Anatomy of the spermatheca in *Coptotermes gestroi*. (A) Wholemount of a spermatheca isolated from an alate female and photographed under stereomicroscope on a black background. Note the finger-like shape of the reservoir and short spermathecal duct (sd). (B) Scanning micrograph of the spermatheca, detailing the distal (dc) and proximal (pc) chambers of the reservoir connected to the duct via a narrow-like region, interpreted as a spermathecal pump (arrowheads). (C) Detail of the distal chamber surrounded by muscles (mu). (D) Detail of the spermathecal pump involved by several muscular layers and a trachea.

knowledge about termite reproduction and sperm storage dynamics, we provide a comparative analysis on the morphometry and morphophysiology of the spermathecae of virgin and inseminated queens of *C. gestroi*.

## MATERIALS AND METHODS

### Termites

Virgin females (alates) and mated queens (2 and 4 years old) of *C. gestroi* were used in the present study. For morphometric assessment, virgin females and mated queens of various ages (10, 20, 30, 50, 80, 100, 120, and 150 days old) were selected. The ultramorphology of the spermatheca was accessed using virgin females, whereas histological and histochemical techniques were applied in virgin and 4-year-old individuals. Additionally, virgin and 2-year-old females were selected for ultrastructural analyses. Alate females of *C. gestroi* were collected during swarming flights in the city of Rio Claro, SP, Brazil (22°23′S, 47°31′W). Mated queens were sampled from colonies maintained under laboratory conditions at the São Paulo State University (Unesp), Rio Claro, SP, Brazil. All sampled colonies contained eggs, indicating that queens had been inseminated.

### Spermatheca morphometry

A total of five individuals of virgin females and five for each group of mated queens (from 10 to 150 days old) were dissected in Hayes saline solution (9 g NaCl, 0.2 g $CaCl_2$, 0.2 g KCl, and 0.1 g $NaHCO_3$ in 1000 ml $H_2O$) under a Discovery V8 stereomicroscope (Zeiss, Oberkochen, Germany). The translucent spermatheca was carefully removed to prevent any damage to the spermathecal reservoir and duct. After that, each spermatheca was separately placed on a histological slide in a drop of Hayes solution and photographed at a magnification of 80× on a black background (Figure 1A). The images, with correspondent scale bars, were loaded in the software ImageJ (available in http://imagej.nih.gov/ij/) to extract the superficial areas of the spermatheca and spermathecal lumen. For this purpose, the measurements were calibrated and the spermatheca was outlined. The software automatically provided the enclosed area (adapted from Bednarska et al., 2016; Costa-Leonardo et al., 2021). Data obtained for virgin and mated queens were compared with a one-way ANOVA followed by Tukey's honestly significant difference (HSD) test for multiple comparisons using the software RStudio v.1.3.1093 (R Studio, Boston, MA, USA).

### Scanning electron microscopy (SEM)

Spermathecae from at least three virgin females were isolated in Hayes saline solution and fixed in Karnovsky (2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4) for 2 h at 4 °C. The samples were then dehydrated in an ethanol series (70–100%), subjected to critical point drying (Balzers CPD 030; Leica Microsystems, Wetzlar, Germany). Then, the material was placed on aluminum stubs and sputtered with gold (Balzers SCD 050). Each spermatheca was examined and photographed using a TM3000 SEM (Hitachi, Tokyo, Japan), operated at 15 kV.

### Histology and histochemistry

The abdomen of virgin females and mated queens (4 years old) were sectioned and fixed in FAA (absolute alcohol, glacial acetic acid, and 40% formaldehyde, in a ratio of 3:1:1) or alcoholic Bouin fixative for 24 h. The samples were transferred to ethanol 70% and dehydrated in a subsequent series until ethanol 95%. Then, they were transferred to Leica historesin solution and kept in a refrigerator for 7 days. The abdomens were placed in molds filled with Leica historesin plus catalyzer for polymerization, and then sectioned to a 3-μm thickness using a Leica RM 2245 microtome. Abdominal sections were stained with hematoxylin–eosin for a general description of the spermatheca. Moreover, sections were submitted to histochemical tests of xylidine-Ponceau (xP) for detection of total proteins, and periodic acid-Schiff (PAS) for detection of polysaccharides.

### Transmission electron microscopy (TEM)

The spermatheca was isolated from virgin females and 2-year-old queens of *C. gestroi*, fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 h at 4 °C, and post-fixed in 1% $OsO_4$ in the same buffer. The spermathecae were embedded in Epon-Araldite resin, included in pure epon resin, and then polymerized at 60 °C for 24 h. Ultrathin sections were obtained with the aim of a Leica Reichert Supernova ultramicrotome and stained with 4% uranyl acetate and lead citrate. The material was then examined and photographed using a JEM 1400 (Jeol, Tokyo, Japan) TEM.

**TABLE 1** Mean (± SD) superficial area of the spermatheca and spermathecal lumen in different-aged females of *Coptotermes gestroi*

| Age (days) | Spermatheca (μm²) | Spermathecal lumen (μm²) |
|---|---|---|
| Virgin | 624.15 ± 15.58 | 415.18 ± 0.76 |
| 10 | 825.44 ± 13.55 | 544.30 ± 7.61 |
| 20 | 843.16 ± 14.00 | 516.72 ± 6.45 |
| 30 | 816.98 ± 2.38 | 540.25 ± 1.12 |
| 50 | 810.66 ± 8.74 | 546.38 ± 9.34 |
| 80 | 827.18 ± 2.42 | 543.68 ± 12.36 |
| 100 | 837.20 ± 2.42 | 583.05 ± 11.23 |
| 120 | 865.67 ± 14.32 | 573.42 ± 11.06 |
| 150 | 794.57 ± 10.12 | 527.92 ± 3.86 |

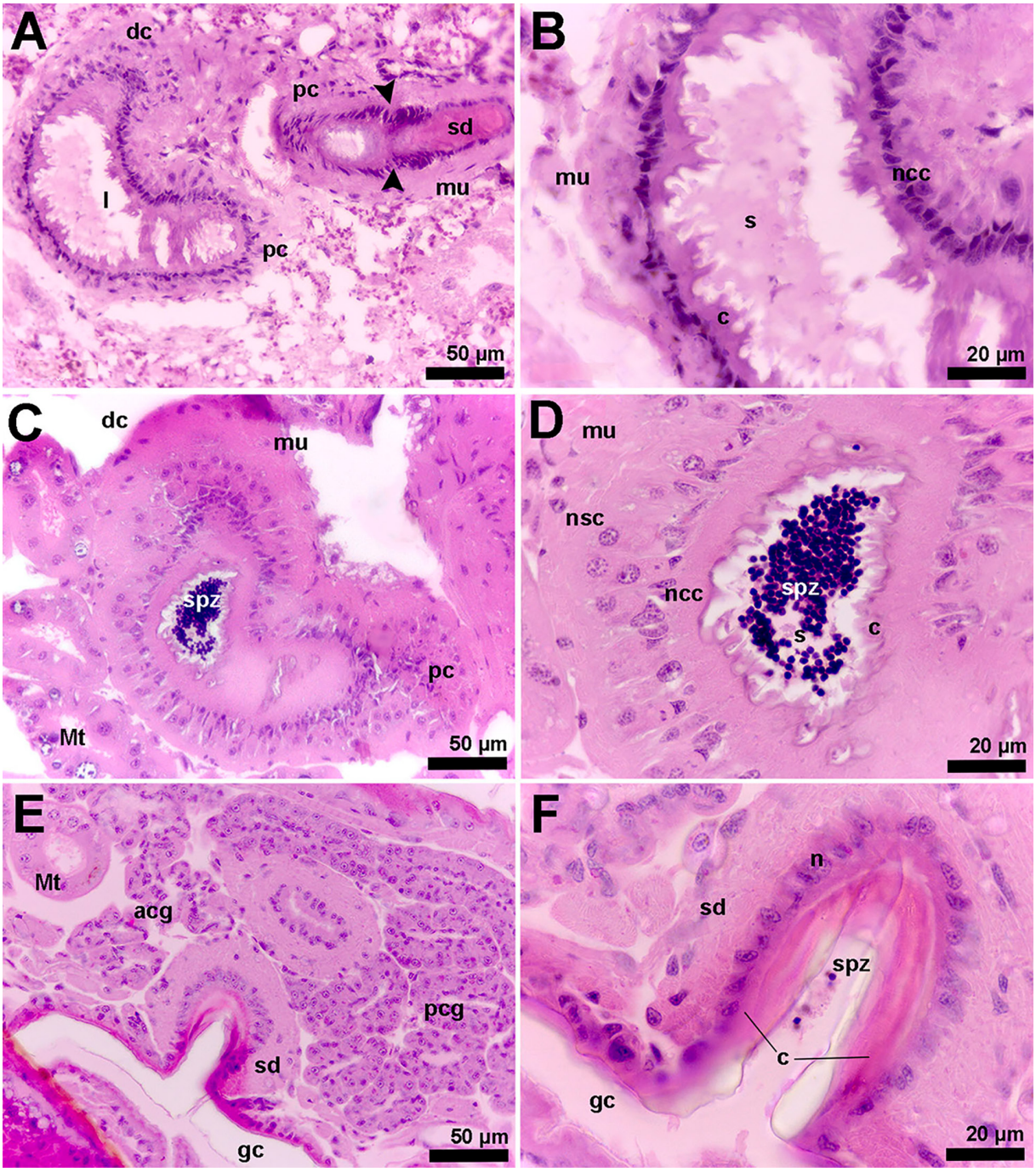


**FIGURE 2** Histological sections of the spermatheca in *Coptotermes gestroi* females. (A) Spermatheca of a virgin female with absence of spermatozoa in the lumen (l) of both distal (dc) and proximal (pc) chamber of the reservoir. Note the thickness of the cuticle surrounding the spermathecal duct (sd) and surrounding muscles (mu). Arrowheads indicate the spermathecal pump. (B) Detail of the lumen, highlighting the secretory activity of the spermatheca even in the absence of spermatozoa. (C-D) Spermatheca of a 4-year-old queen containing numerous non-flagellate spherical spermatozoa (spz) in the lumen. (E) Region of the spermathecal duct, in which the spermatheca opens into the genital chamber (gc). (F) Detail of the spermathecal duct, highlighting the thick cuticle (c) coating the lumen. Staining: Hematoxylin–eosin. acg, anterior colleterial glands; Mt, Malpighian tubules; ncc, nucleus of the canal cells; nsc, nucleus of the secretory cells; pcg, posterior colleterial glands; s, secretion.

## RESULTS

### Spermatheca anatomy and morphometry

The spermatheca of *C. gestroi* showed a finger-shaped morphology, divided into two regions (distal and proximal chambers) comprising the spermathecal reservoir, and a short spermathecal duct that connects this organ to the genital chamber (Figure 1A). Between the reservoir and spermathecal duct, there was a constricted area. Under SEM, it was possible to observe several overlapped longitudinal and circular muscular fibers covering the distal and

proximal chamber. Muscular sheets also coated the neck-like transition between the spermathecal reservoir and duct (Figures 1B-D). The spermatheca was richly supplied with trachea networks, enveloping and penetrating its surfaces in some areas (Figures 1B-D). Our morphometric assessment showed that the superficial area of the spermatheca increased significantly when comparing alate females and mated queens (ANOVA: $F_{8,36} = 9.93$, $P < 0.001$). Similarly, the area of spermathecal lumen also differed between these groups ($F_{8,36} = 17.65$, $P < 0.001$). Among mated queens, the spermathecal area did not differ, but a slight difference was observed in the luminal area of 20- vs. 100-day-old, as well as 20- vs. 120-day-old individuals (Table 1; statistical details in Tables S1 and S2).

## Histology and histochemistry

Histological sections highlighted the two regions of the finger-shaped spermatheca in both virgin and 4-year-old females (Figures 2A-D). The spermathecal epithelium contained nuclei located at different heights, always coated by muscular sheets (Figures 2A-C). The spermathecal lumen was limited by a thick cuticle and secretion was observed deposited in this region (Figures 2B-D). For 4-year-old queens, numerous rounded spermatozoa were observed in the lumen, imbedded into the secretion (Figure 2D). The spermathecal duct showed a conspicuous thick cuticle (Figure 2A), and was connected to the genital chamber by a narrow spermathecal furrow, next to the colleterial glands (Figures 2E-F).

Histochemical tests revealed a flocculate secretion store inside the spermatheca of virgin females, which was stained weakly for the xylidine-Ponceau test (Figure 3A), indicating a low concentration of proteins. Otherwise, the secretion secreted by the spermatheca of 4-year-old queens stained moderately for the same test, suggesting an increasing concentration of proteins (Figures 3B-C). A similar pattern was observed for PAS, in which secretion stored in the spermatheca of virgin females stained weakly for this test (Figure 3D), whereas that present in 4-year-old queens stained strongly, indicating high concentration of polysaccharides (Figures 3E-F). Thus, the spermathecal secretion of both groups showed a positive reaction for the presence of both proteins and polysaccharides, but at different concentrations according to their reproductive status. Moreover, the numerous spherical and non-flagellate spermatozoa observed in the spermatheca of 4-year-old females were imbedded into the referred secretion.

## Ultrastructure

The epithelium of the spermatheca was composed of bicellular units, consisting of a columnar secretory cell and associated canal cells (Figure 4). The secretory cells are always associated with a cuticular canal that is, in turn,

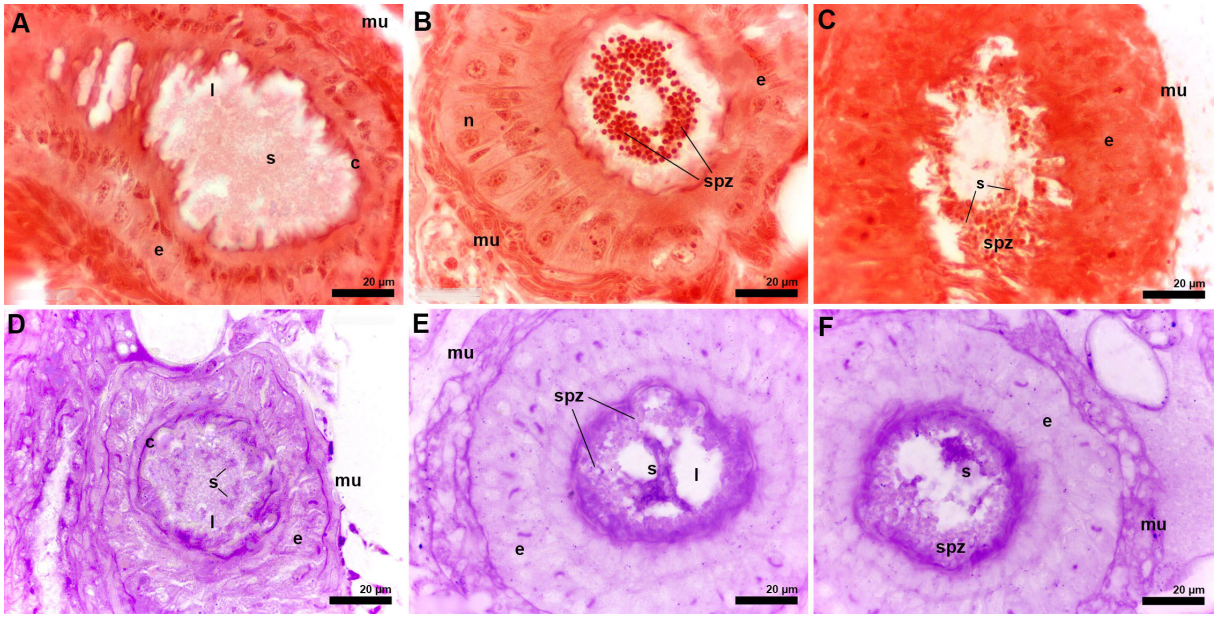


**FIGURE 3** Histochemical analysis of the spermathecal secretion in females of *Coptotermes gestroi*. (A-C) Detection of total proteins using the xylidine-Ponceau test (xP). (A) Spermatheca of a virgin female. Note the secretion (s) present in the lumen (l), which stained weakly to the occurrence of proteins. (B-C) Spermatheca of 4-year-old queens. Note the spherical spermatozoa (spz) imbedded into the secretion, which showed a stronger reaction to the xP test. (D-F) Detection of polysaccharides using the periodic acid-Schiff test (PAS). (D) Spermatheca of a virgin female. Note the weak reaction of the secretion to the PAS test. (E-F) Spermatheca of 4-year-old queens, highlighting the strongly positive reaction of the secretion to the presence of polysaccharides. c, cuticle; e, epithelium; mu, muscles; n, nucleus.

involved by the canal cell that secreted it. Such a cuticular canal has two parts: (1) the receiving canal or 'end apparatus', which is located in the extracellular space of the secretory cell and composed internally of an interrupted cuticle. Moreover, several microvilli surround the canal, where the secretion is stored. And (2) the conducting canal, whose cuticular layer is uninterrupted and is responsible for transporting the secretion towards the lumen (Figures 5–7). The receiving canal of the secretory cells in *C. gestroi* was surrounded by numerous secretory vesicles with different sizes and electron densities according to the sampled female (Figures 5–7). Many invaginations of the basal plasma membrane of the secretory cells were observed next to the layers of muscular fibers that covered the spermatheca. Moreover, the overlapped muscular sheets showed different thickness throughout this organ.

In virgin females, large secretory vesicles surrounded the receiving canal of the secretory cells, most of them weakly electron-dense (Figures 5A-D). Moreover, rough endoplasmic reticulum (RER) profiles were observed next to the secretory vesicles and receiving canal (Figures 5C-D). Few electron-lucent vesicles were also synthesized by the secretory cells of virgin females, and septate junctions separating these cells were often reported (Figure 6A). The bicellular unit, especially the apical region of canal cells, showed dense concentrations of mitochondria next to the conducting canals, suggesting an intense energy-requiring process (Figures 6A-B). Remarkably, the nuclei of the secretory cells was always rounded, whereas canal cells showed an irregular-shaped nuclei (Figures 5A-B). The lumen of the spermatheca was surrounded by a thick cuticle and stored flocculate secretion (Figure 6B). As with virgin females, the spermathecae of 2-year-old queens exhibited secretory cells containing a round-shaped nuclei when compared to those of the canal cells (Figures 7A-D). Muscular sheets were also evident, coating the whole epithelium and being frequently penetrated by tracheal networks (Figures 7B-C). Secretory vesicles of different electron densities were observed in the secretory cells, most of them surrounding the receiving canal (Figures 8A-D). Nevertheless, strong electron-dense vesicles were exclusive for the secretory cells of mated queens (Figures 8 and 9), next to the receiving canal and associated microvilli, mitochondria, and RER profiles (Figures 9A-C). Finally, numerous rounded and non-flagellate spermatozoa were stored in the spermathecal lumen of 2-year-old queens, imbedded into a high amount of flocculate and electron-dense secretion (Figure 9D).

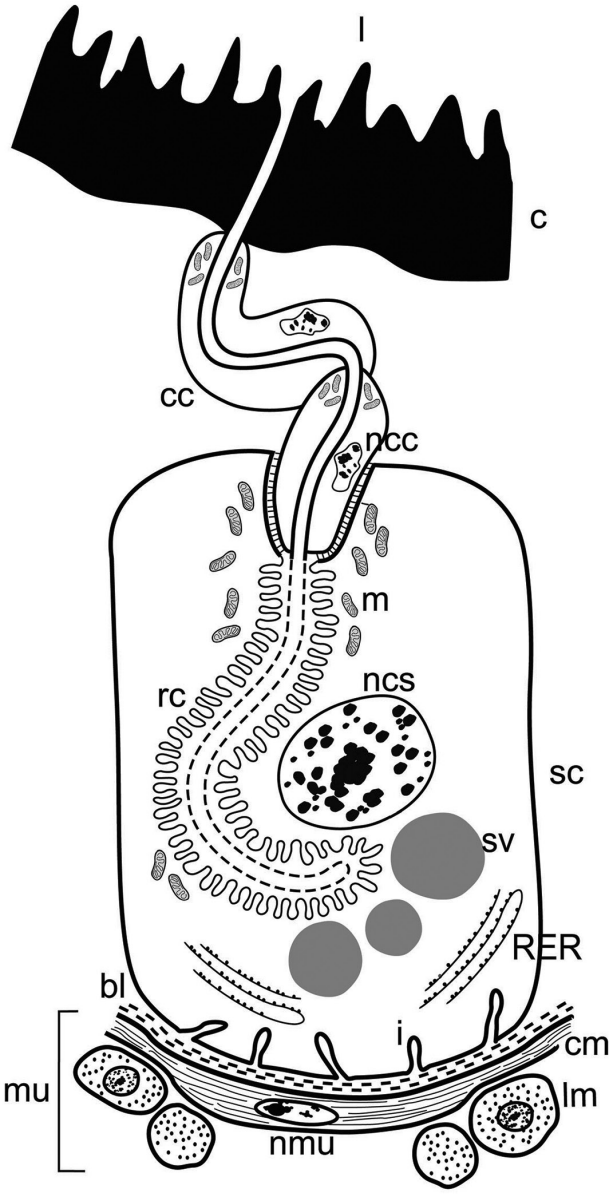


**FIGURE 4** Schematic drawing of a class III secretory cell that composes the spermathecal epithelium. Note the secretory columnar cell (sc) and associated canal cells (cc). The secretory vesicles (sv) are transported towards the lumen (l) via canals. Such canals display two distinct regions, the receiving canal (rc), which penetrates the secretory cells and is composed of an interrupted cuticle surrounded by microvilli (mv); and a conducting canal, composed of a continuous cuticle. bl, basal lamina; cm, circular muscular sheet; i, invaginations of the basal plasma membrane; lm, longitudinal muscular sheet; m, mitochondria; mu, muscles; ncc, nucleus of the canal cell; nmu, nucleus of the muscular cell; nsc, nucleus of the secretory cell; rer, rough endoplasmic reticulum.

## DISCUSSION

As far as we know, our findings comprise the first comparative analysis of the structure and mating-dependent morphophysiological changes of the spermatheca in termites. The spermatheca of *C. gestroi* queens comprises a single structure, which resembles a finger-shaped sac. Such a feature was already described for other Rhinotermitidae species, as well as for Termitidae and Serritermitidae (Costa-Leonardo & Patrício, 2005; Raina et al., 2007; Silva et al., 2019). Nevertheless, *C. gestroi* spermatheca morphology differs from that reported

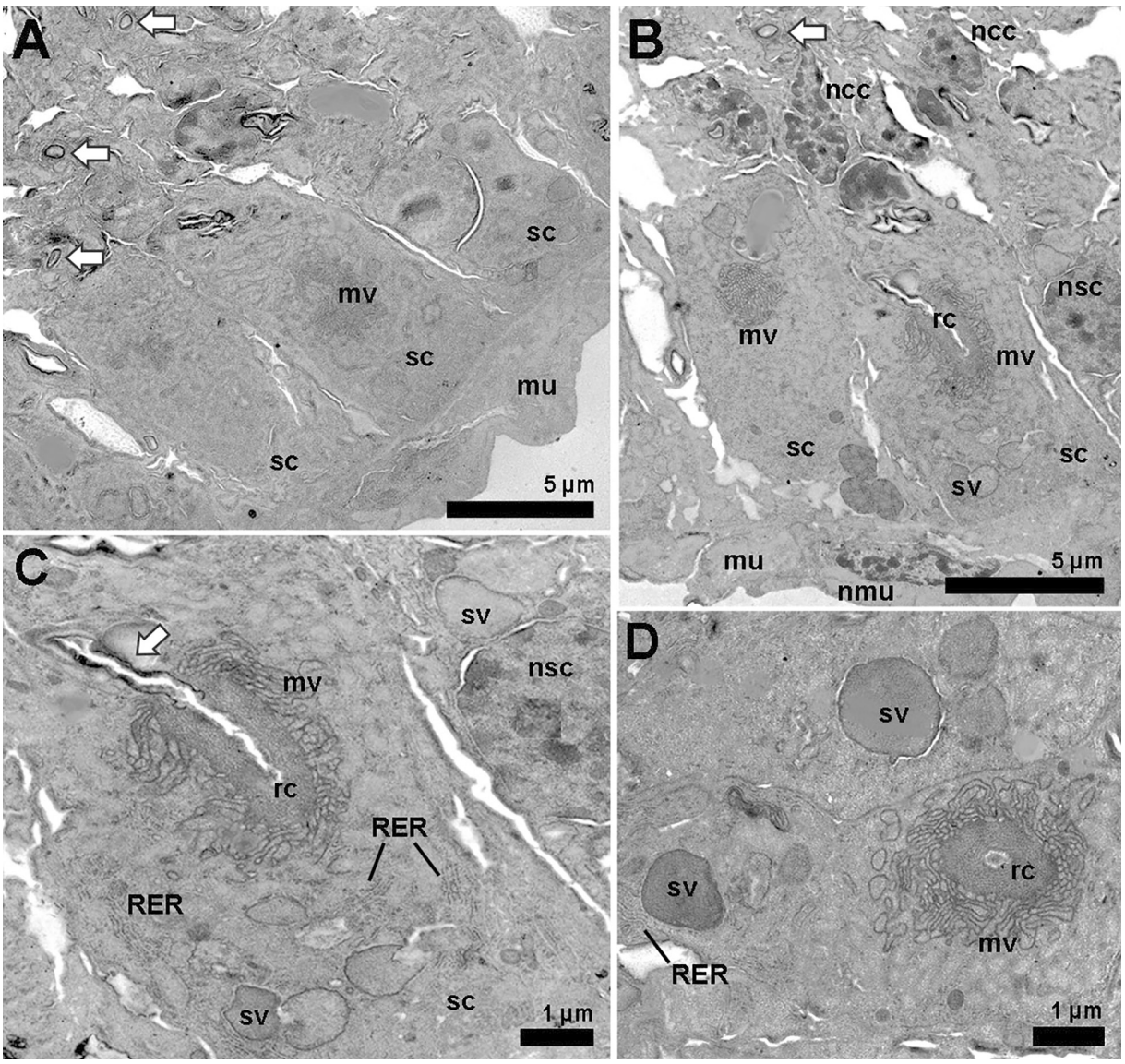


**FIGURE 5** Ultrastructure of the spermathecal epithelium in virgin females of *Coptotermes gestroi*. (A-B) Secretory cells (sc) surrounded by muscles (mu). (C) Detail of the receiving canal (rc) bounded by many microvilli (mv) and rough endoplasmic reticulum (RER). Note the transition between the receiving and conducting canals, the latter indicated by an arrow. (D) Detail of the secretory vesicles (sv) of different electron densities next to the receiving canal: ncc, nucleus of the canal cell; nmu, nucleus of the muscular cell; nsc, nucleus of the secretory cell.

for *Zootermopsis nevadensis* Hagen (Archotermopsidae) and *Hodotermes mossambicus* (Hagen) (Hodotermitidae) (Nutting, 1969; Costa-Leonardo & Patrício, 2005). Although the spermatheca has not been evaluated in any Stylotermitidae representative, the finger-like morphology of the spermatheca is probably a synapomorphy for Neoisoptera.

Our morphometrical assessment showed that the superficial area of the spermatheca, as well as the lumen, differs significantly between virgin and functional queens. It was already stated that the dimensions of the spermatheca rely on the age and reproductive status of bee queens (Martins & Serrão, 2004; Souza et al., 2008). Given the absence of spermatozoa in the spermatheca of virgin females and the initial secretory activity, the reduced size of the spermatheca and its lumen in these individuals are justified. It is interesting to note that, among mated queens, 150-day-old individuals showed lower values for the superficial area of the spermatheca and lumen. According to previous analyses, 150-day-old queens of *C. gestroi* significantly decrease their reproductive activity at this age, characterized by a reduced number of eggs and absence of oocytes at late vitellogenesis (Silva & Costa-Leonardo, 2022). Thus, these individuals may have gone through a period of reproductive inactivity which follows the first oviposition cycle (Raina et al., 2003). According to these authors, queens of *Coptotermes formonasus* Shiraki do not copulate during this period but may store up to a thousand spermatozoa from previous inseminations, thus the dimensions of the spermatheca do not seem to suffer

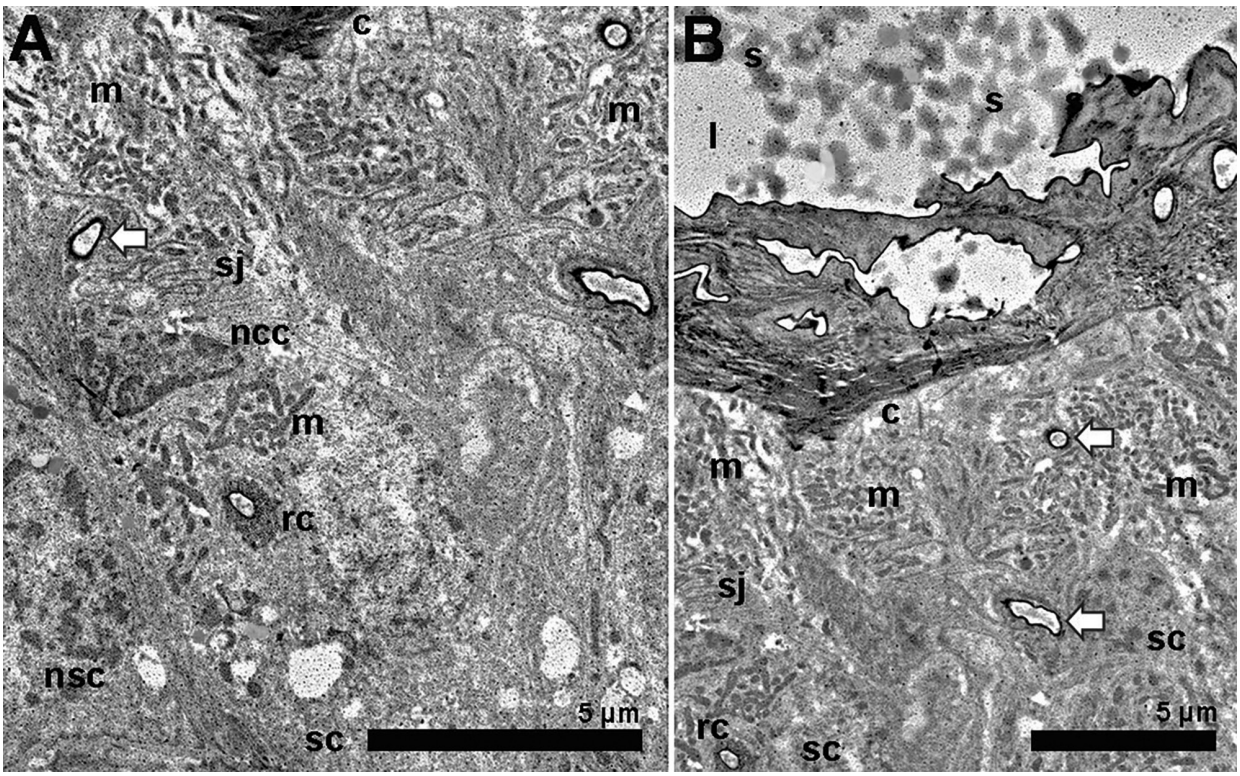


**FIGURE 6** Ultrastructure of the apical spermathecal epithelium in virgin females of *Coptotermes gestroi*. (A) Note the richness of mitochondria (m), especially next to the receiving canal (rc) and cuticle (c), suggesting intense transport of contents. (B) Detail of the epithelium and lumen (l), the latter limited by the cuticle. Flocculate secretions (s) with different electron densities are deposited in the lumen. Ncc, nucleus of the canal cell; nsc, nucleus of the secretory cell; sc, secretory cell; sj, septate junction. Arrows indicate the conducting canals.

significant variation when comparing to those of other different-aged queens (Raina et al., 2007). It would explain the similar dimensions of the spermatheca among mated queens of the present study.

Under SEM, different layers of musculature were observed, covering all the regions of the spermatheca (reservoir and spermathecal duct). The location of spermathecal muscular layers is not universal among insect taxa (Pascini & Martins, 2017). For instance, a muscular sheath may cover the whole spermathecal reservoir (Bailey & Nuhardiyati, 2005), only the spermathecal duct, and/or the transition between reservoir and duct, forming a neck-like area referred to as spermathecal pump (Dallai, 1975; Martins & Serrão, 2002). Interestingly, our study is the first to provide a SEM micrograph of the spermatheca in termites, and a similar constricted region was evident between the reservoir and the spermathecal duct of *C. gestroi*, covered by several muscular fibers. Due to this, it is plausible to assume the existence of a mechanism similar to a spermathecal pump in termites. We did not observe any spermathecal accessory gland in *C. gestroi*. Such an absence, together with the disposition of the spermatheca-associated muscles, are in agreement with previous reports on Dictyoptera (Lawson & Thomas, 1970; Raina et al., 2007; Winnick et al., 2009).

Based on our histochemical observations, proteins and polysaccharides were secreted into the spermathecal lumen of *C. gestroi* queens prior to copulation, but increased in inseminated queens. According to Greenberg & Stuart (1979) and Costa-Leonardo et al. (2022), the swarming flight might be a reproductive trigger for alate females, as their ovaries activate and develop as they leave the natal nest, dealate, and pair. Similarly, termite spermatheca might be activated during the swarming flight given that unflown females do not secrete polysaccharides into the spermathecal lumen (Ye et al., 2009), contrasting our results for alate females. The occurrence of proteins in the lumen of spermatheca has been reported for several insect groups (Souza et al., 2008, 2016; Baer et al., 2009; Malta et al., 2014). Within eusocial Hymenoptera, these proteins are likely to prevent oxidative damage and infections, as well as to be involved in energy and amino acid metabolism (Baer et al., 2009; Malta et al., 2014). Beyond their composition, proteinaceous secretion varies in its concentration according to the reproductive activity of the queens (Souza et al., 2008), in agreement with our data for *C. gestroi*.

As with proteins, polysaccharides were secreted into the spermathecal lumen of *C. gestroi* prior to copulation, even though their concentration increased in 4-year-old queens. Spermathecae of virgin females of *Aedes aegypti* (L.) contained polysaccharides in the glandular cells, ducts, and reservoir lumen, indicating that such a secretory activity takes place before sperm transfer (Pascini et al., 2012). Similar observations were also performed on the spermathecae of beetles and bees (Bhatnagar & Musgrave, 1970; Schoeters & Billen, 2000; Souza et al., 2008). Sugar-based secretion is likely to provide energy and also nourish spermatozoa during their storage (Pascini et al., 2012). Thus, this

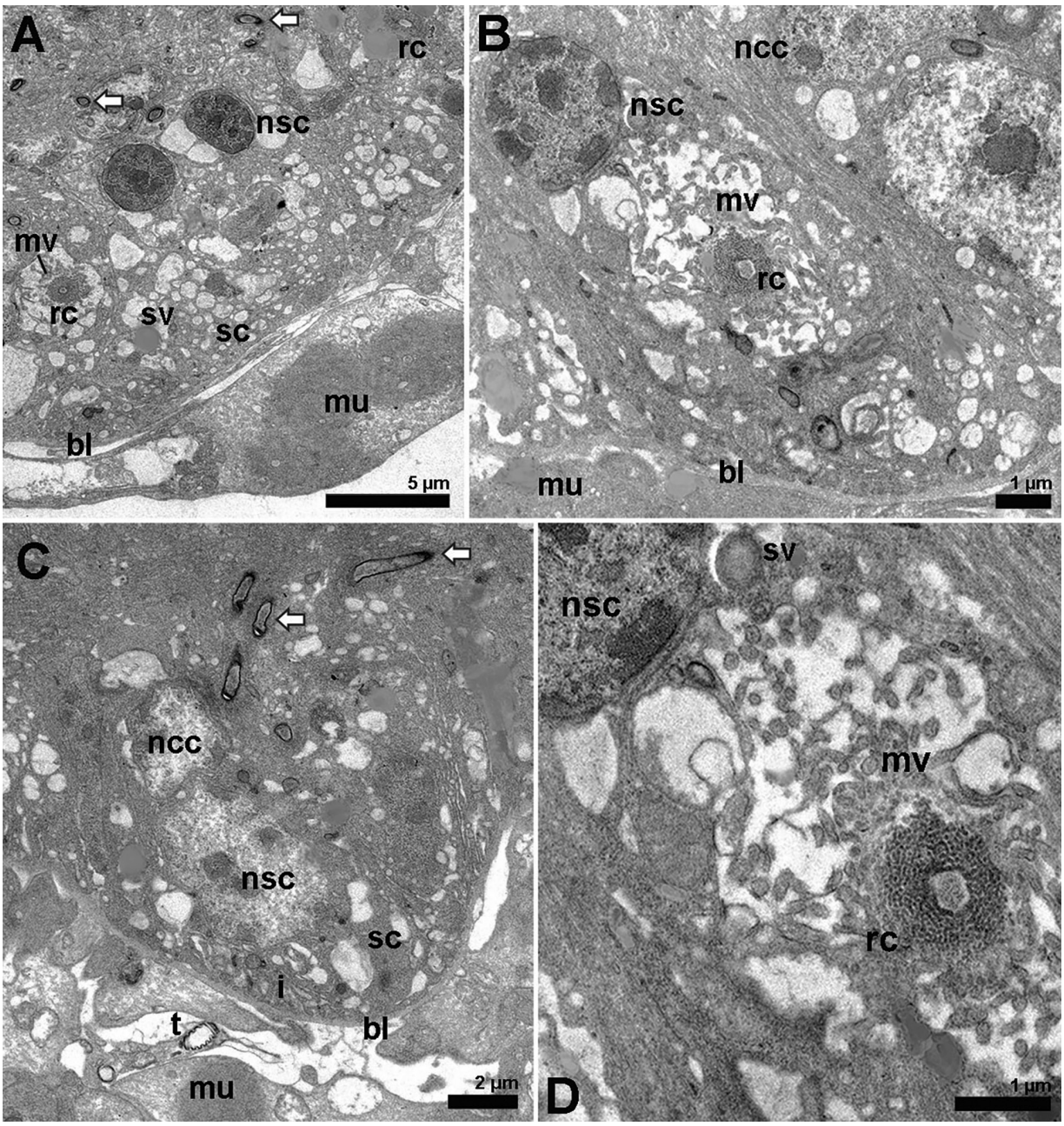


**FIGURE 7** Ultrastructure of the spermatheca in 2-year-old inseminated queens of *Coptotermes gestroi*. (A) Secretory cells (sc) coated by a thick muscular layer (mu). (B) Detail of a secretory cell, highlighting its round-shaped nucleus (nsc), receiving canal (rc) and associated microvilli (mv), and secretory vesicles (sv) of different electron densities. (C) Detail of the conducting canals (arrows). (D) Detail of the receiving canal. i, invaginations of the basal plasma membrane; bl, basal lamina; ncc, nucleus of the canal cell; nsc, nucleus of the secretory cell.

agrees with our data, in which polysaccharide concentration increased in the spermatheca of 4-year-old *C. gestroi* queens, certainly due to the storage of several spermatozoa.

Previous analyses on the seminal vesicles of different termite species, including *C. gestroi*, revealed that these structures secrete a content that reacts positively to xP and PAS tests (Laranjo et al., 2019, 2020). Then, similar to the spermathecae, the seminal fluid transferred to the female apparatus during copulation is likely to contain proteins and polysaccharides. A detailed chemical analysis of the termite spermathecal and seminal fluids has not been provided so far. Therefore, it is not possible to infer whether the content stored in the spermathecae results only from the secretory activity of the spermathecal epithelium itself or also from the seminal fluid. According to Baer et al. (2009), the proteins contained in the spermatheca of *Apis mellifera* L. differ substantially from those present in the seminal fluid of males. Thus, whereas the spermathecal fluid evolved to enhance sperm viability, seminal fluid evolved to increase paternity success over rivals (Baer et al., 2009). Assuming the general monogamy of termites (Hartke & Baer, 2011), comparative analyses of the

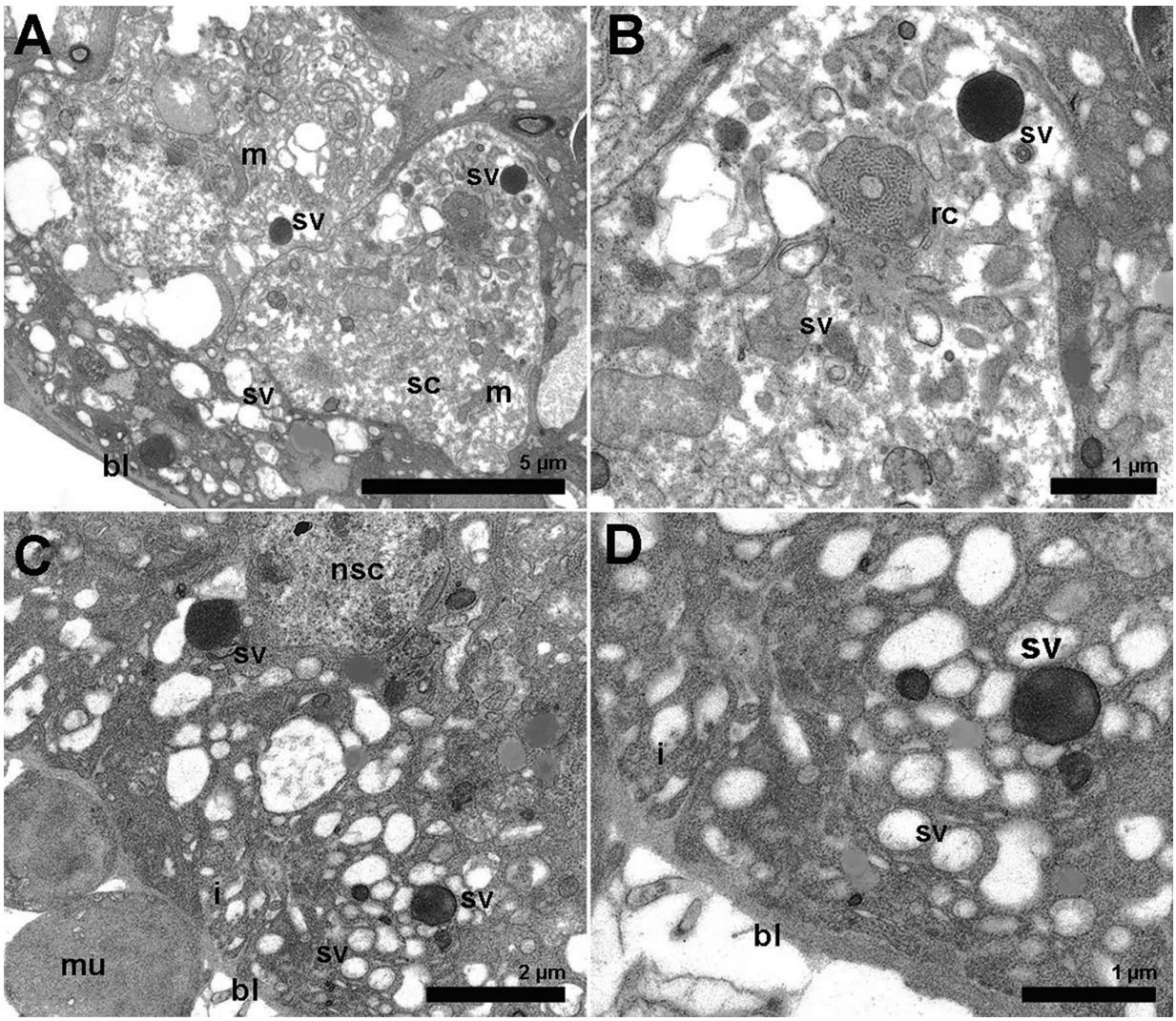


**FIGURE 8** Secretory activity of the spermatheca in 2-year-old queens of *Coptotermes gestroi*. (A-B) Secretory cells (sc) containing several secretory vesicles (sv), some of them strong electron-dense, and mitochondria (m) next to the receiving canal (rc). (C-D) Detail of the secretory vesicles of different electron densities in the cytoplasm of the secretory cells. i, invaginations of the basal plasma membrane; bl, basal lamina; mu, muscles; nsc, nucleus of the secretory cell.

contents present in the spermathecal and seminal fluids would be valuable to infer their role in sperm storage and maintenance.

The ultrastructure of the spermatheca revealed an epithelium composed of columnar secretory cells and associated canal cells, which are classified into class III secretory units (Noirot & Quennedey, 1974). Class III secretory cells may be associated with insect spermathecae in at least two different ways: they may constitute the spermathecal epithelium (La et al., 1999; Raina et al., 2007; Souza et al., 2016) or the spermathecal accessory gland (Martins & Serrão, 2002; Souza et al., 2008). In the presence of accessory glands, the epithelium of the spermathecal reservoir assumes only a transporting function (Schoeters & Billen, 2000). In the absence of these structures, the epithelium itself is responsible for synthesis and secretion of contents into the lumen via conducting canals. This is observed in the spermathecae of *C. gestroi* and other Dictyoptera representatives (Gupta & Smith, 1969; Lawson & Thomas, 1970; Winnick et al., 2009). A similar function was already postulated for other insect taxa (Brundo et al., 2011; Dallai et al., 2012; Yang & Hua, 2021).

The occurrence of secretory vesicles with different electron densities, as well as mitochondria, support the secretory activity of the epithelium (Dallai et al., 2012; Yang & Hua, 2021). Many mitochondria were observed around the receiving canal of the secretory cells and especially at the apical limit of the canal cells, suggesting that such regions are involved in energy-requiring processes: uptake and secretion of content into the lumen (Ahmed & Gillott, 1981; Lay et al., 1999; Pascini et al., 2012). Moreover, the occurrence of RER and strongly electron-dense vesicles in the secretory cells of mated queens, indicates the biosynthesis and transport of proteinaceous content into the lumen (Pascini et al., 2013; Farder-Gomes et al., 2019). Such features are in agreement with the

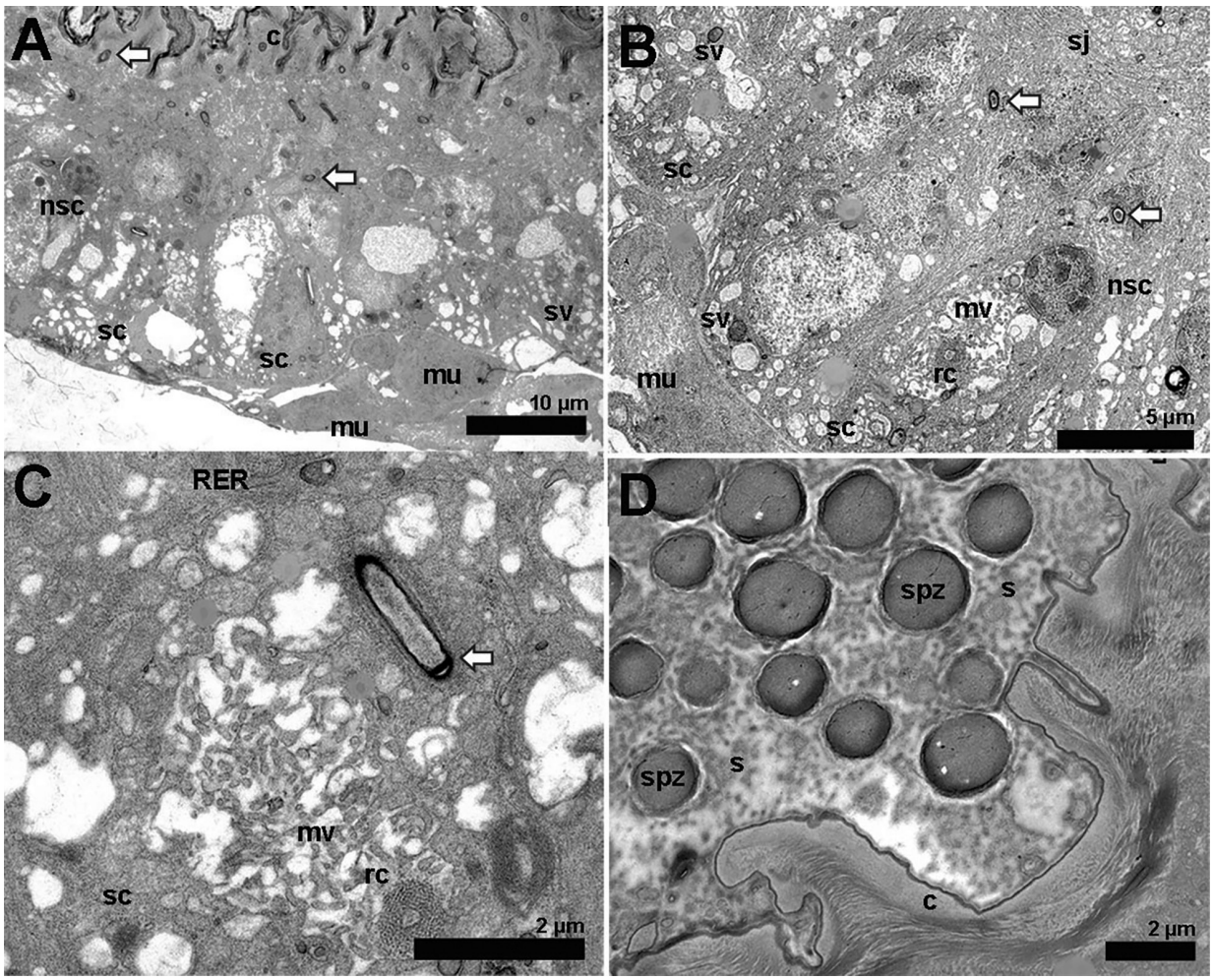


**FIGURE 9** (A-B) Ultrastructure of the secretory cells of the spermatheca in 2-year-old queens of *Coptotermes gestroi*. Note the richness of conducting canals (arrows) and secretory vesicles (sv) of different electron densities. (C) Detail of the receiving canal (rc) surrounded by microvilli (mv), conducting canal, and rough endoplasmic reticulum (RER). (D) Spermathecal lumen (l) limited by a thick cuticle (c). Note the spherical non-flagellate spermatozoa (spz) imbedded into the flocculate secretion (s). mu, muscles; nsc, nucleus of the secretory cell; sj, septate junction.

moderate positive reaction of the spermathecal secretion to the xP histochemical test.

As stated before, overlapped circular and longitudinal muscular layers surrounded the spermathecal epithelium. Muscular contractions of the spermatheca in *Periplaneta americana* L. are responsible for sperm transport and secretion release (Gupta & Smith, 1969), probably by the pressure applied to the secretory epithelium (Lawson & Thomas, 1970). The muscular sheath covering the spermatheca of *C. gestroi* is probably responsible for sperm transfer, as already suggested for other termite species (Weesner, 1956; Raina et al., 2007). Such an inference is reinforced by the immotile nature of its spermatozoa, a synapomorphy for most termite lineages (Laranjo et al., 2020).

In conclusion, morphometrical and morphophysiological changes take place in the spermatheca of *C. gestroi* as virgin females mature into functional queens. The changes in spermatheca dimensions are probably a consequence of sperm storage and increasing secretion of contents. The secretion of proteins and polysaccharides at higher concentrations in the spermathecae of mated queens corroborates its crucial role in nourishing and maintaining viable spermatozoa prior to egg fertilization. The spermathecal epithelium is composed of class III secretory units, highlighting the occurrence of secretory vesicles of different electron densities and mitochondria, as well as RER. These features are related to energy-requiring processes and increasing secretion of proteinaceous content into the lumen, which therefore agrees with our xP test on mated queens. Moreover, the *C. gestroi* spermatheca lacks accessory glands, thus the epithelium itself is responsible for both the synthesis and transport of secretion. It also agrees with previous analyses on phylogenetically related groups. Further analyses are encouraged to provide more details about the chemical profile of the content stored in the termite spermatheca prior and after copulation, and how it contributes to sperm storage.

## AUTHOR CONTRIBUTIONS

**Iago Bueno da Silva:** Conceptualization (equal); data curation (lead); formal analysis (equal); investigation (equal); methodology (equal); software (lead); writing – original

draft (lead); writing – review and editing (equal). **Ana Maria Costa-Leonardo:** Conceptualization (equal); funding acquisition (lead); investigation (equal); methodology (equal); project administration (lead); resources (lead); supervision (lead); validation (equal); writing – original draft (supporting); writing – review and editing (equal).

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## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**Table S1** Multiple comparisons of the superficial area of the spermatheca in female *Coptotermes gestroi* of different ages (days) (Tukey HSD test)

**Table S2** Multiple comparisons of the superficial area of the spermathecal lumen in female *Coptotermes gestroi* of different ages (days) (Tukey HSD test)

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**Table S1** Multiple comparisons of the superficial area of the spermatheca in female *Coptotermes gestroi* of different ages (days) (Tukey HSD test)

| | Alate | 10 | 20 | 30 | 50 | 80 | 100 | 120 | 150 |
|---|---|---|---|---|---|---|---|---|---|
| Alate | - | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| 10 | <0.001 | - | 0.998 | 0.997 | 0.998 | 0.997 | 1.0 | 0.981 | 0.950 |
| 20 | <0.001 | 0.998 | - | 0.997 | 0.981 | 0.998 | 0.997 | 0.998 | 0.838 |
| 30 | <0.001 | 0.997 | 0.997 | - | 0.998 | 0.998 | 0.997 | 0.873 | 0.996 |
| 50 | <0.001 | 0.998 | 0.981 | 0.998 | - | 0.998 | 0.995 | 0.729 | 0.998 |
| 80 | <0.001 | 0.997 | 0.998 | 0.998 | 0.998 | - | 0.999 | 0.950 | 0.981 |
| 100 | <0.001 | 1.0 | 0.997 | 0.997 | 0.995 | 0.999 | - | 0.992 | 0.913 |
| 120 | <0.001 | 0.981 | 0.998 | 0.873 | 0.729 | 0.950 | 0.992 | - | 0.411 |
| 150 | <0.001 | 0.950 | 0.838 | 0.996 | 0.998 | 0.981 | 0.913 | 0.411 | - |

**Table S2** Multiple comparisons of the superficial area of the spermathecal lumen in female *Coptotermes gestroi* of different ages (days) (Tukey HSD test)

| | Alate | 10 | 20 | 30 | 50 | 80 | 100 | 120 | 150 |
|---|---|---|---|---|---|---|---|---|---|
| Alate | - | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| 10 | <0.001 | - | 0.597 | 0.998 | 1.0 | 0.998 | 0.455 | 0.816 | 0.940 |
| 20 | <0.001 | 0.597 | - | 0.941 | 0.656 | 0.756 | 0.035 | 0.042 | 0.991 |
| 30 | <0.001 | 0.998 | 0.941 | - | 0.990 | 0.995 | 0.138 | 0.395 | 0.990 |
| 50 | <0.001 | 1.0 | 0.656 | 0.990 | - | 0.993 | 0.400 | 0.395 | 0.961 |
| 80 | <0.001 | 0.998 | 0.756 | 0.995 | 0.993 | - | 0.309 | 0.669 | 0.984 |
| 100 | <0.001 | 0.455 | 0.035 | 0.138 | 0.400 | 0.309 | - | 0.991 | 0.066 |
| 120 | <0.001 | 0.816 | 0.042 | 0.395 | 0.395 | 0.669 | 0.991 | - | 0.148 |
| 150 | <0.001 | 0.940 | 0.991 | 0.990 | 0.961 | 0.984 | 0.066 | 0.148 | - |

# Capítulo 3

*Microscopy and Microanalysis*, 2023, **00**, 1–12
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**Micrographia Article**

Microscopy AND Microanalysis

# Functional Morphology and Development of the Colleterial Glands in Non- and Egg-Laying Females of the Pest Termite *Coptotermes gestroi* (Blattaria, Isoptera, Rhinotermitidae)

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## Abstract

Colleterial glands of female insects are accessory glands responsible for producing secretions associated with egg-laying. Within Dictyoptera, they synthesize compounds of the ootheca. However, their morphology and role in termites are poorly understood. Here, we compared the morphology, development, and secretory activity of the colleterial glands between non- and egg-laying females of the pest termite *Coptotermes gestroi* under light and transmission electron microscopy. We also provide the first description of these glands for Rhinotermitidae. The glands are paired, divided into anterior and posterior units, which join in a common duct via basal trunks. They are highly developed within egg-laying females, especially the posterior gland, secreting glycoproteins to lubricate the genital chamber and/or stick the eggs together. Ultrastructure revealed glandular epithelia composed of bicellular units of Class 3, whose secretory activity varied between groups and units. Posterior gland of egg-laying females showed richness of mitochondria, rough endoplasmic reticulum, and secretory vesicles, including electron-dense secretory granules, indicating synthesis and transport of contents, especially proteins. The basal trunks were enfolded by muscles, supporting their role in conducting secretion. Morphophysiological modifications occur in the colleterial glands as females mature and lay eggs, and the mechanisms underlying the secretory cycle of the glands are discussed.

**Key words:** exocrine glands, female accessory glands, invasive species, oviposition, termites

## Introduction

The colleterial glands, also referred to as female accessory glands, are organs associated with the reproductive apparatus of female insects. These glands are not omnipresent among insect lineages (Matsuda, 1976) and comprise ectodermal- or mesodermal-derived structures, corresponding to an invagination of the oviduct and/or imaginal discs (Kaulenas, 1992; Gillott, 2002). Moreover, these glands are usually paired and open into the genital chamber right after the spermathecal duct (Gillott, 2002). The function of the colleterial glands traditionally relies on the synthesis and secretion of mucous compounds which cover insect eggs or fixate them on the substrate (Gillott, 2002). Nevertheless, other exceptional functions are known such as the transport of sperm into the spermatheca (Lococo & Huebner, 1980) and the synthesis of nutritional and/or poisonous contents in some Hymenoptera (Gnatzy et al., 2015).

Dictyoptera is a monophyletic clade that includes mantises, cockroaches, and termites (Lo et al., 2000; Legendre et al., 2015). Within this complex, the morphology and function of the colleterial glands are sufficiently understood in mantises and cockroaches (Courrent et al., 2008; Winnick et al., 2009), in which they are laterally disposed and synthesize compounds that are mixed and ultimately result in a hardened casing, the ootheca (Stay & Roth, 1962; Du et al., 2022). This casing, in turn, covers the clustered eggs during the oviposition, aiming to avoid desiccation, predation, and parasitism (Pryor, 1962; Fatouros et al., 2020; Du et al., 2022).

The colleterial glands of termites are divided into anterior and posterior units (Courrent et al., 2008). Moreover, they open via a common duct or side-by-side into the genital chamber (Grassé, 1982; Courrent et al., 2008). The basal region of each gland is involved by muscular sheets, whereas the apical portion is ramified to form convoluted tubules (Weesner, 1969; Grassé, 1982). The anterior and posterior glands show asymmetrical development and have been suggested to be analogous to the left and right posterior glands of cockroaches, respectively (Courrent et al., 2008).

The morphology of the termite colleterial glands has only been investigated in about eight species so far (Weesner, 1969; Grassé, 1982; Soltani-Mazouni & Bordereau, 1987; Courrent et al., 2008, Costa-Leonardo et al., 2023). Moreover, a comprehensive analysis of the colleterial glands in females with different reproductive status has not yet been conducted. Similarly, the chemical nature of the secretion synthesized by the colleterial glands and its likely function are also unknown, given that most termite species lay eggs individually, without any covering (Courrent et al., 2008).

The Asian termite *Coptotermes gestroi* is a subterranean species belonging to the family Rhinotermitidae. Similar to many other rhinotermitids, *C. gestroi* has a relevant economic importance, especially due to its invasive ability (Rust & Su, 2012; Evans, 2021). Colonies of this species are headed by a single pair of reproductives after the swarming flight, and its reproductive dynamics is based on the high egg-laying capacity of the queens (Costa-Leonardo, 2002). The primary objective of this study was to conduct a comparative analysis of

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the colleterial glands between non- and egg-laying females of *C. gestroi* and how the oviposition dynamic impacts the glandular development and functionality. In addition, these structures have not been hitherto described for Rhinotermitidae, so we provide the first morphological description of the colleterial glands for the referred family.

## Material and Methods

### Termites

Two different groups of *C. gestroi* (Wassman, 1896) females were used in this study: non-egg-laying (alate females) and egg-laying (4-yr-old queens). Individuals of the former group were collected during swarming flights in the city of Rio Claro, SP, Brazil (22°24′43.9″ S; 47°36′34.0″ W). They were sexed based on the presence of the genital plate in the females, which were then isolated from male alates to prevent pair formation. Egg-laying queens were sampled from 4-yr-old colonies maintained under laboratory conditions. All sampled colonies contained the royal pair, eggs, and sterile castes (workers and soldiers).

### Anatomy of the Colleterial Glands

Five non-egg-laying females of *C. gestroi* were frozen at −20°C for 10 min and dissected in Hayes solution (9.0 g/L NaCl, 0.2 g/L $CaCl_2$, 0.2 g/L KCl, 0.1 g/L $NaHCO_3$, pH 8.7) under a Zeiss Discovery V8 stereomicroscope. The colleterial glands were carefully removed, placed on a histological slide, stained with aqueous Eosin, and photographed at different magnifications using a Zeiss AxioCam 105 Color coupled to the same stereomicroscope.

### Histology, Histochemistry, and Morphometry

The posterior abdomen of non- and egg-laying females (at least four individuals of each group) were fixed in FAA (absolute alcohol, glacial acetic acid, 40% formaldehyde, in the ratio 3:1:1) or alcoholic Bouin for 24 h at room temperature. Then, samples were dehydrated in an ethanol series (from 70 to 95%, 1 h each bath), transferred to a Leica® historesin solution, and kept in a refrigerator for at least seven days. After that, the material was placed in molds filled with Leica® historesin plus catalyzer for polymerization. The abdomens were sectioned with a 3 $\mu$m thickness using a Leica RM 2245 microtome. Histological sections were stained with hematoxylin-eosin for a general description of the glandular units (for details, see Junqueira & Junqueira, 1983). Aiming to identify the chemical nature of the secreted content, xylidine-Ponceau and Periodic Acid-Schiff (PAS) tests were used for the detection of total proteins and polysaccharides, respectively (for details, see Mello & Vidal, 1980).

The development of the glands was evaluated by the morphometry of their epithelium (Bednarska et al., 2016). The glands were photographed in a Leica ICC150 photomicroscope at an instrument magnification of 400× and were then analyzed in the software Image J (https://imagej.nih.gov/ij). The measurements were calibrated and the epithelium height ($\mu$m) was measured. Four cross-sections of each gland were randomly selected from three different females of each group (non- and egg-laying females), totalizing 12 sections for each gland of each group. For each cross-section, three measures were extracted from different regions of the glandular epithelium to establish an average height (Bednarska et al., 2016). Data obtained for the female groups were compared with a *t*-test using the software R (RStudio Team, 2020).

### Transmission Electron Microscopy

At least three individuals of each group (alate females and 4-yr-old queens of *C. gestroi*) were selected and frozen at −20°C for 5 min before dissection. The colleterial glands were isolated from these individuals with the aid of forceps, fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 h at 4°C, and then postfixed in 1% OsO4 in the same buffer. The material was embedded in Epon-Araldite resin, included separately in pure epon resin, and polymerized at 60°C during 24 h. Ultrathin sections of the anterior and posterior glands were obtained using a Leica Reichert Supernova ultramicrotome, and posteriorly stained with 4% uranyl acetate and lead citrate (standard recipe after Reynolds, 1963). The material was examined and photographed using a JEOL JEM 1400 transmission electron microscope operating at an accelerating voltage of 80 kV.

## Results

### Anatomy of the Colleterial Glands

The colleterial glands of *C. gestroi* are composed of the anterior and posterior units, which are ramified tubules of distinct morphology (Figs. 1a, 1b). The glands differed in their arrangement, since the anterior one is scattered and composed of long tubules (about 4 mm), whereas the posterior unit is convoluted, comprising short tubules (about 1.1 mm) involved by tracheal networks. At the proximal region, the glands join together in a common duct (Fig. 1c). Moreover, each gland has an individual proximal region right upon the common duct, referred to as basal trunk (Fig. 1c). The colleterial glands open into the genital chamber at the ninth sternite, next to the spermatheca, which opens at the eighth sternite (Fig. 1d).

### Histology, Histochemistry, and Morphometry

The colleterial glands were wrapped in non-egg-laying females, and barely restricted to their ninth sternite (Fig. 2a). Parasagittal sections showed anterior and posterior glands very alike, comprising flattened tubules (Figs. 2a, 2b). In addition, no secretion was observed in any glandular lumen. Cross-sections of the basal trunk also showed a relatively flattened structure, involved by thin longitudinal and circular muscular sheets. Similar to the apical tubules, the lumen of the basal trunk was empty, lined by the cuticle (Fig. 2c).

Egg-laying females, however, showed well-developed and unwrapped colleterial glands, whose ramifications extended deeply into the abdomen, reaching the seventh sternite (Fig. 2d). Cross-sections of the anterior and posterior tubules of 4-yr-old individuals revealed enlarged structures, which were histologically distinguishable. The anterior gland epithelium was thinner when compared to the posterior unit, and showed an enlarged lumen (Fig. 2e). Posterior gland showed a taller and well-developed epithelium, whose nuclei were located at different heights (Figs. 2e, 2f). Moreover, the lumen of the posterior unit was narrower, but the only one filled with a flocculated secretion (Figs. 2e, 2f). The basal trunks were more swollen when compared to non-egg-laying females, and were coated by several thick muscular sheets (Fig. 2f).

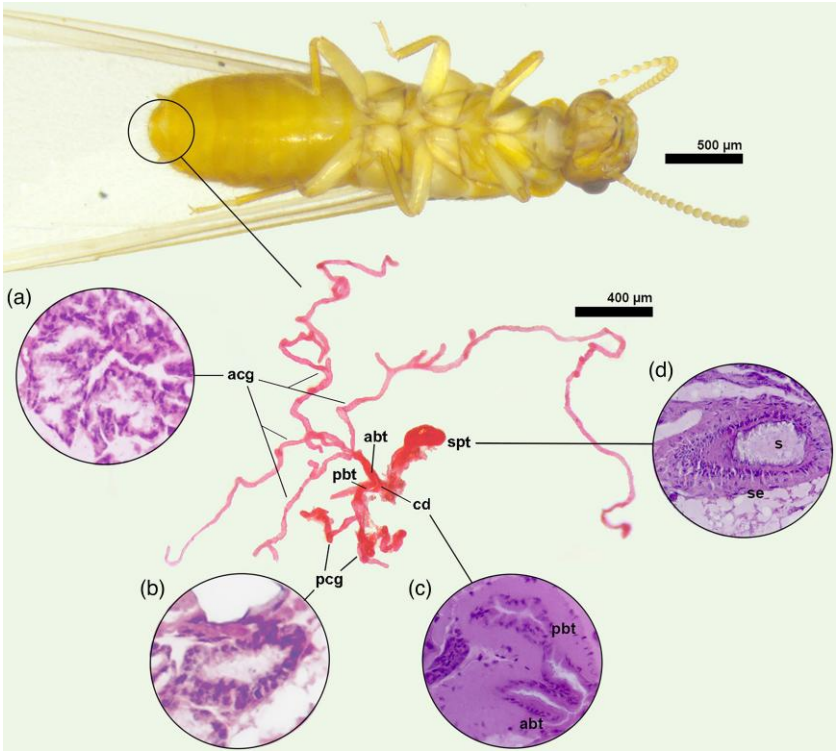


**Fig. 1.** Total mounting and histological sections of the colleterial glands in an alate female of *Coptotermes gestroi*. (**a**) Anterior gland (acg). (**b**) Posterior gland (pcg). (**c**) Anterior (abt) and posterior (pbt) basal trunks. (**d**) Spermatheca (spt). cd, common duct; s, secretion; se, spermathecal epithelium.

Histochemistry of the colleterial glands did not show positive reaction for xylidine-Ponceau test in both anterior and posterior units in alate females (Figs. 3a, 3b). The anterior gland in 4-yr-old queens also presented a negative reaction for presence of total proteins (Fig. 3c). Nevertheless, the flocculated secretion of the posterior gland stained moderately to xylidine-Ponceau test (Fig. 3d). The same pattern was observed for the detection of polysaccharides (Figs. 4a–4c), although the secretion stored in the lumen of the posterior gland in egg-laying queens stained strongly to PAS test (Fig. 4d). It suggests the occurrence of glycoproteins in the composition of the content secreted by the posterior gland of these functional queens.

Epithelium height of the anterior gland varied significantly between groups. Mean values were $7.12 \pm 1.22\ \mu$m for non- and $11.21 \pm 1.42\ \mu$m for egg-laying queens ($p < 0.0001$, *t*-test). Similarly, the epithelium height of the posterior unit also increased significantly in 4-yr-old queens, with mean values of $18.49 \pm 2.28\ \mu$m for these individuals and $10.15 \pm 1.42\ \mu$m for alate females ($p < 0.0001$, *t*-test).

## Transmission Electron Microscopy

The epithelium of both colleterial glands comprises bicellular units, each composed of a secretory cell associated to canal cells, disposed end to end along the epithelium (Figs. 5–8). The apical portion of the epithelium showed cuticular ducts secreted by the canal cells, divided into two distinct regions each: the receiving and the conducting canal. The former, also referred to as "end apparatus", is located in the extracellular space of the secretory cell and is surrounded by microvilli, while the latter is formed of an uninterrupted cuticular layer (Figs. 5–8). The secretory products are stored in the extracellular space bounded by the microvilli, which serves as a reservoir and is often dilated due to the concentration of secretion, and is delivered into the receiving canal. On the other hand, the conducting canal transports the content into the lumen by crossing the cuticle. At the basal limit, several muscular sheets coated the glandular epithelium, although the distribution of these coatings varied according to the region of the tubule (Fig. 9).

In alate females, the secretory cells of the anterior and posterior glands (Figs. 5a, 5b) showed tiny receiving canals devoid of secretion and nuclei located at the basal region (Figs. 5c–5f). Electron-lucent secretory vesicles were sparsely distributed in the cytoplasm (Figs. 5d, 5e). Moreover, few mitochondria were observed at the apical portion of the canal cells (Fig. 5e), right before a thin cuticle (about $0.027\ \mu$m) lining an empty lumen (Figs. 5c–5e). The anterior glands of 4-yr-old showed secretory cells with larger and rounded nuclei, also located at the basal region, several conducting canals secreted by the canal cells (Figs. 6a, 6c–6e), as well as septate junctions connecting the neighboring canal cells (Fig. 6c). Well-developed receiving canals were disposed in the extracellular space of the secretory cells, surrounded by microvilli and mitochondria (Fig. 6d). The receiving canal was formed of several layers of interrupted cuticle, resulting in a sponge-like appearance. Moreover, the transition between the receiving and canal cell was clearly visible (Fig. 6f). The lumen of the gland was also empty, lined by a conspicuous relatively thicker cuticle when compared to that of alate females (about $0.055\ \mu$m).

The posterior glands of 4-yr-old queens showed the most notable development (Fig. 6b) and secretory activity (Figs. 7 and 8). The epithelium was composed of different-shaped bicellular units with well-developed receiving canals surrounded by numerous microvilli and mitochondria (Figs. 7a, 7b).

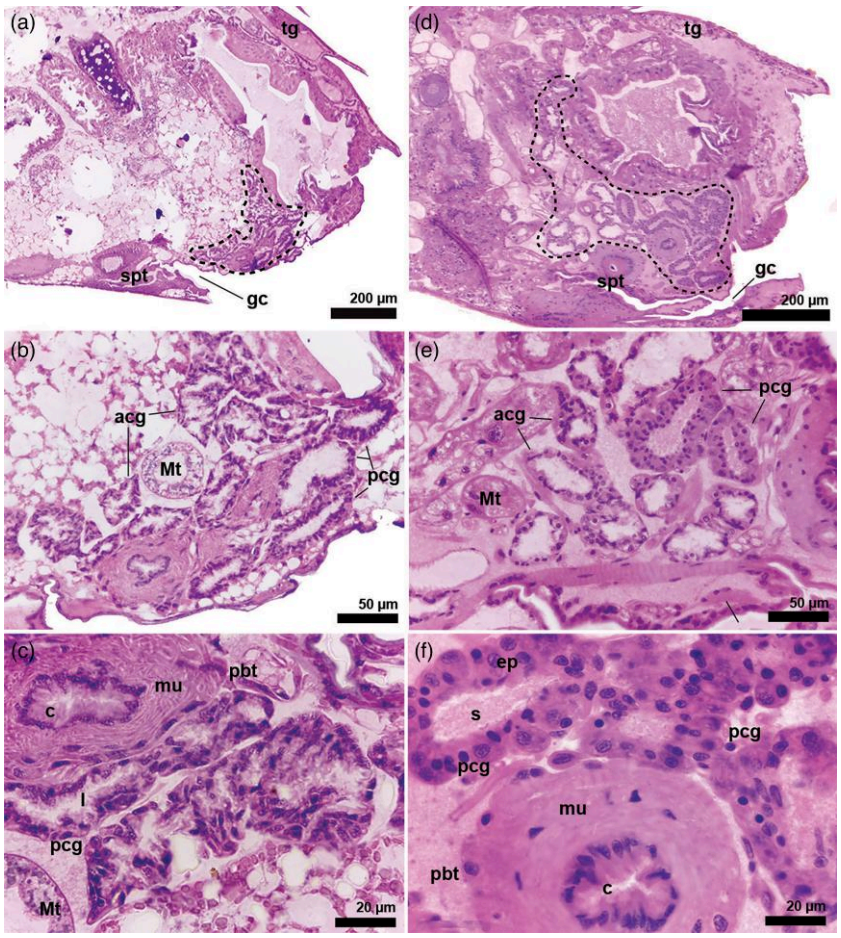


**Fig. 2.** Parasagittal sections of the abdomen of *Coptotermes gestroi* females, highlighting the cross-sectioned tubular colleterial glands with dashed lines. (**a**) The glands of alate females are restricted to the ninth sternite. (**b**) Details of the anterior (acg) and posterior (pcg) glands, which were histologically similar. (**c**) Details of the posterior gland and posterior basal trunk (pbt). Note the musculature (mu) surrounding the trunk and the shrunken lumen. (**d**) The glands in 4-yr-old queens are well developed, extending into the seventh sternite. (**e**) Details of the anterior and posterior glands. (**f**) Details of the posterior gland and its basal trunk. Note the development of the glandular epithelium (ep) and muscular sheets, and the secretion (s) in the lumen. c, cuticle; gc, genital chamber; Mt, Malpighian tubules; spt, spermatheca; tg, tergal gland. Hematoxylin-eosin staining.

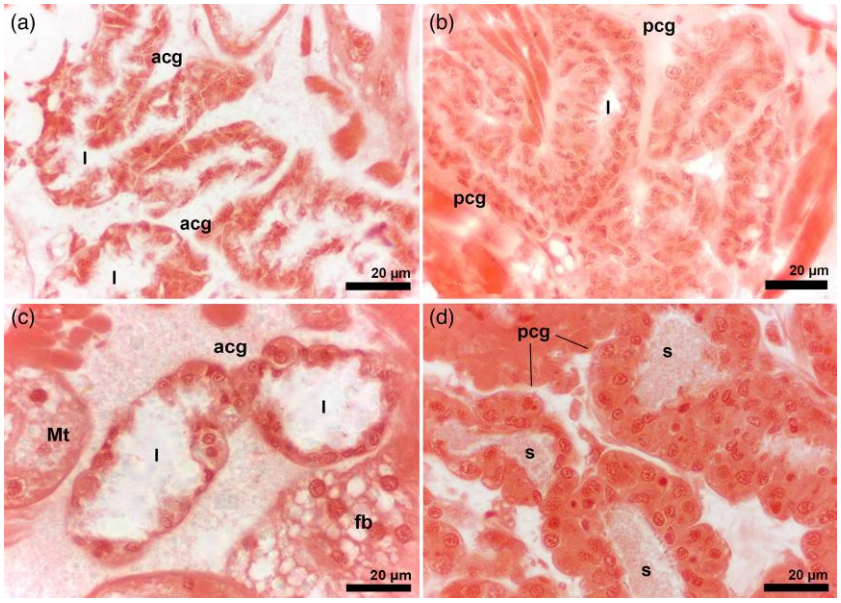


**Fig. 3.** Histochemistry of the colleterial glands of *Coptotermes gestroi* females. (**a, b**) Anterior (acg) and posterior (pcg) glands of alate females, respectively. Note the empty glandular lumen (l) and consequent negative reaction to the presence of total proteins. (**c, d**) Anterior and posterior glands of 4-yr-old queens, respectively. Note the secretion (s) of the posterior unit staining moderately to the occurrence of total proteins. fb, fat body; Mt, Malpighian tubule. Xylidine-Ponceau staining.

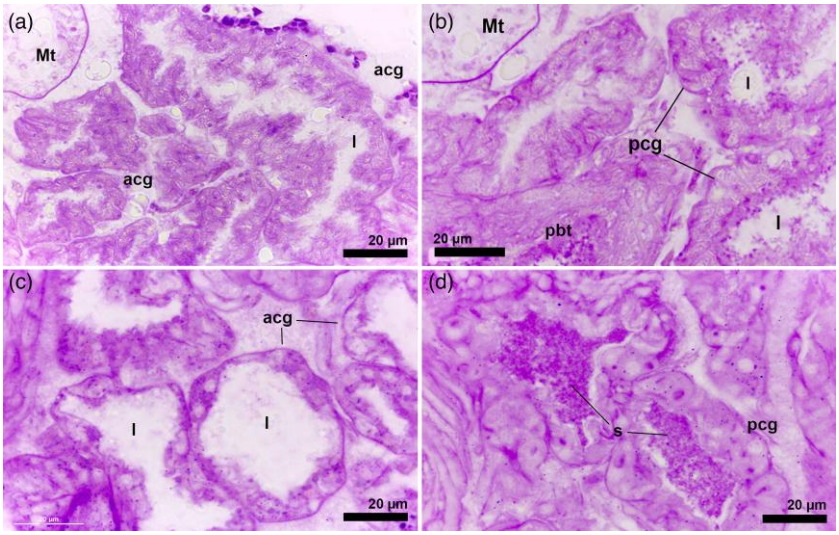


**Fig. 4.** Histochemistry of the colleterial glands of *Coptotermes gestroi* females. (**a, b**) Anterior (acg) and posterior (pcg) glands of alate females, respectively. The glandular lumen (l) showed no content, resulting in a negative reaction in the occurrence of polysaccharides. (**c, d**) Anterior and posterior units of 4-yr-old queens, respectively. While no secretion was observed in the glandular lumen (l) of the anterior gland, a strong PAS-positive secretion (s) was stored in the lumen of the posterior tubules, indicating a high concentration of polysaccharides. Mt, Malpighian tubule. PAS staining.

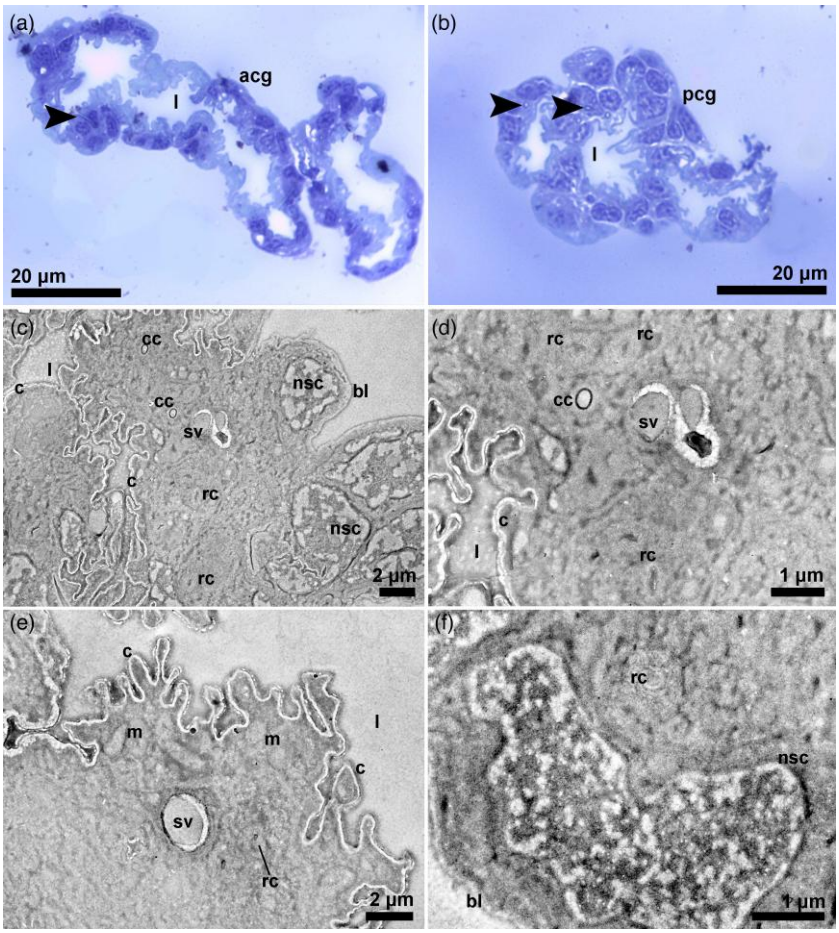


**Fig. 5.** (**a, b**) Semithin sections of the anterior (acg) and posterior gland (pcg) of an alate female, respectively. Arrowheads indicate the conducting canals. (**c–f**) Ultrastructure of the anterior gland in alate females of *Coptotermes gestroi*. (**c**) Glandular epithelium showing the rounded nuclei of the secretory cells (nsc), as well as the receiving (rc) and conducting canals (cc). (**d**) Details of the canals surrounded by small secretory vesicles (sv). (**e**) Apical portion of a canal cell, highlighting the mitochondria (m) and the lumen (l) lined by a thin cuticle (c). (**f**) Details of the basal portion of a secretory cell. bl, basal lamina.

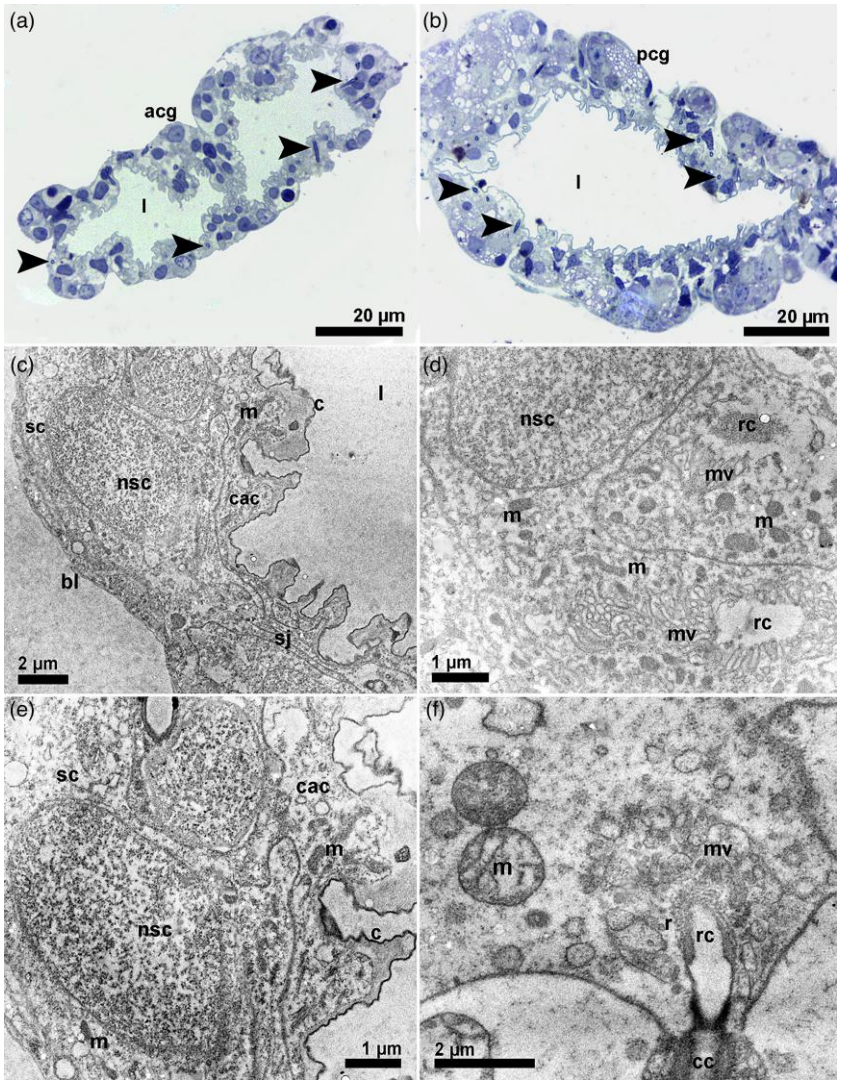


**Fig. 6.** (**a, b**) Semithin sections of the anterior (acg) and posterior gland (pcg) of a 4-yr-old queen, respectively. Arrowheads indicate the conducting canals. (**c–f**) Ultrastructure of the anterior gland in 4-yr-old queens of *Coptotermes gestroi*. (**c**) Secretory (sc) and canal cells (cac). Note the septate junctions (sj) connecting the cells. (**d**) Details of a secretory cell with receiving canals (rc) bounded by microvilli (mv) and mitochondria (m). (**e**) Details of the rounded nuclei of secretory cells (nsc). (**f**) Transition between the receiving and conducting portions of the canal. bl, basal lamina; c, cuticle, l, lumen; r, reservoir.

Externally, the glandular epithelium was coated by connective tissue penetrated by tracheae (Figs. 7a, 7e). Rough endoplasmic reticulum (RER) was very abundant, disposed in two forms: packed lamellar RER, showing a narrow lumen and disposed as long cisternae; and vesicular RER comprising vesicles with wide lumen. These arrangements were surrounded by mitochondria and myelin figures (Figs. 7c, 7d). The conducting canal was remarkably wide and showed electron-dense secretion reaching the lumen through invaginations of the cuticle. Near the end apparatus, the transition between the cuticle-lined conducting canal and the sponge-like receiving canal was evident (Figs. 7e, 7f). The conducting canal was laterally bounded by at least one canal cell, showing irregularly shaped nucleus and several septate junctions connecting it to at least one epithelial cell next to the luminal cuticle (Fig. 7f), whose thickness reached about 0.202 $\mu$m. The secretory products observed in the cytoplasm of the secretory cells varied in electron density and size (Fig. 8). Secretory vesicles of different electron densities were abundant around receiving canals, together with Golgi apparatus and mitochondria (Fig. 8a). Secretory granules of strong electron density were always associated with the receiving canal and very often concentrated in the space between the canal and the bounding microvilli, referred to as reservoir (Figs. 8a, 8b). Few electron-lucent vesicles were reported next to the receiving canals, as these larger vesicles were predominant in the basal region of the secretory cell, surrounding the nucleus (Figs. 8c, 8d).

The basal trunk of both glands showed a thinner epithelium coated by a thick musculature (Fig. 9). Similarly, the glandular lumen was narrowed at this region, especially in alate females (Figs. 9a, 9b), whose cuticular layer lining the lumen was somehow shrunken and surrounded by warped nuclei of the cells (Fig. 9a). The cuticle coating the basal trunk lumen of 4-yr-old queens was thicker and equally surrounded by irregularly shaped nuclei (Fig. 9c). The cytoplasm of the cells contained some vesicles of different electron densities (Fig. 9a), although neither receiving canals nor conducting canals were observed at the basal trunk epithelium (Figs. 9a–9d). On the other hand, well-developed longitudinal and circular muscular sheets were observed, always rich in mitochondria and

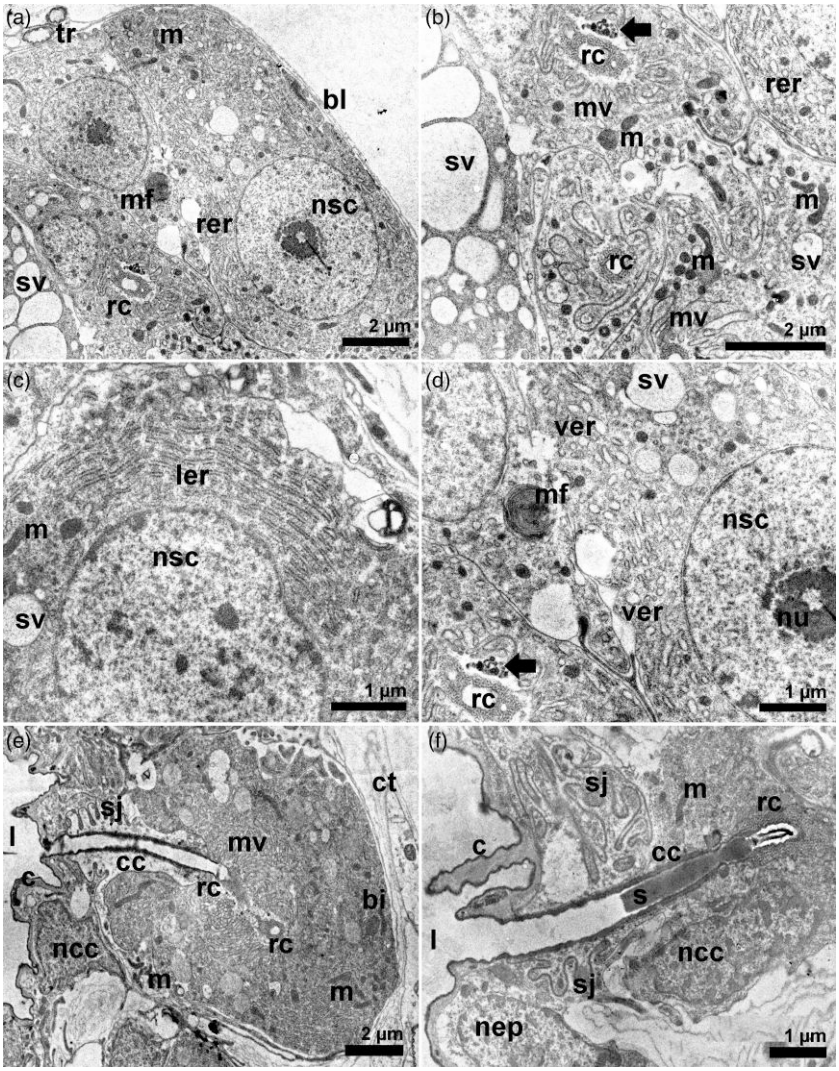


**Fig. 7.** Ultrastructure of the posterior gland in 4-yr-old queens of *Coptotermes gestroi*. (**a**) Secretory cells with rounded nuclei (nsc) and tracheal networks (tr) penetrating the connective tissue (ct) next to the basal lamina (bl). (**b**) Details of the receiving canals (rc), surrounded by microvilli (mv), mitochondria (m), and rough endoplasmic reticulum (rer). Arrows indicate the electron-dense secretory granules in the space between the receiving canal and microvilli (reservoir). (**c**) Details of the nucleus of the secretory cell and lamellar endoplasmic reticulum (ler). (**d**) Details of the vesicular endoplasmic reticulum (ver) and secretory vesicles of different electron densities (sv). (**e, f**) General view and details of the pathway made by the canal, respectively. Note the bounding canal cells and the transport of electron-dense secretion (s) from the secretory cells to the lumen (l). Before the lumen, an epithelial cell and its respective nucleus (nep) are observed. bi, basal invaginations; c, cuticle; cc, conducting canal; ncc, nucleus of the canal cell; sj, septate junctions.

often penetrated by tracheal branches (Figs. 9b, 9c). A diagrammatic representation of the bicellular glandular unit is shown in Figure 10.

## Discussion

Here, we provide a comparative analysis of the colleterial glands in different-aged females of *C. gestroi* and also the first description of these glands for Rhinotermitidae. The general morphology of the colleterial glands resembles those reported for other termites, being constituted of a set of asymmetrical tubules (Weesner, 1969; Courrent et al., 2008). Moreover, the glandular epithelia are composed of bicellular units, corresponding to class 3 secretory cells (Noirot & Quennedey, 1974). Such an organization has been reported for the colleterial glands of Termitidae and Kalotermitidae (Soltani-Mazouni & Bordereau, 1987; Courrent et al., 2008), whereas in the basal termite species *Mastotermes darwiniensis* and *Zootermopsis nevadensis*, the glandular epithelia are composed of modified class 1 cells (Courrent et al., 2008). Thus, it is likely that a shift from class 1 to class 3 cells occurred in the colleterial glands of a common ancestor to Kalotermitidae + Neoisoptera.

Each colleterial gland of *C. gestroi* possesses a basal trunk, which is ramified into convoluted tubules. Thus, both glands are paired at some point, since they bifurcate right after the basal trunk. Such an organization agrees with the description made on *Kalotermes flavicollis* (Kalotermitidae) (Soltani-Mazouni & Bordereau, 1987). For other termite species such as *M. darwiniensis* (Mastotermitidae) and *Pseudacanthotermes spiniger* (Termitidae), the anterior gland has been described as paired, while the posterior one comprises a single unit (Courrent et al., 2008). The way the secretion reaches the genital chamber in *C. gestroi* (via a common duct) is similar to *K. flavicollis*, *Cubitermes fungifaber*, *Velocitermes heteropterus*, and *Tenuirostritermes tenuirostris* (Weesner, 1969; Grassé, 1982; Soltani-Mazouni & Bordereau, 1987; Costa-Leonardo et al.,

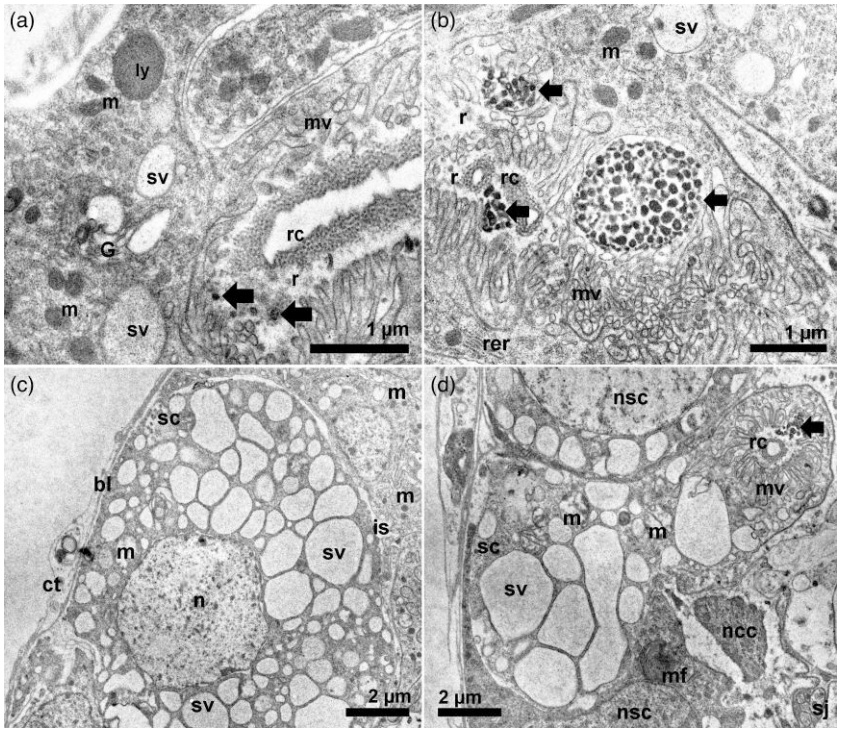


**Fig. 8.** Secretory products of the posterior gland in 4-yr-old queens of *Coptotermes gestroi*. (**a, b**) Secretory vesicles (sv) of different electron densities next to a receiving canal (rc) and Golgi complex (G). Note the abundance of strong electron-dense secretory granules (arrows) in the reservoir (r) between the receiving canal and microvilli (mv). (**c, d**) Details of the basal portion of a secretory cell (sc) with electron-lucent vesicles of different sizes. Note that some of the electron-lucent vesicles are disposed toward the receiving canal. bl, basal lamina; ct, connective tissue; is, intercellular space; ly, lysosome; m, mitochondria; mf, myelin figures; ncc, nucleus of the canal cell; nsc, nucleus of the secretory cell; sj, septate junctions.

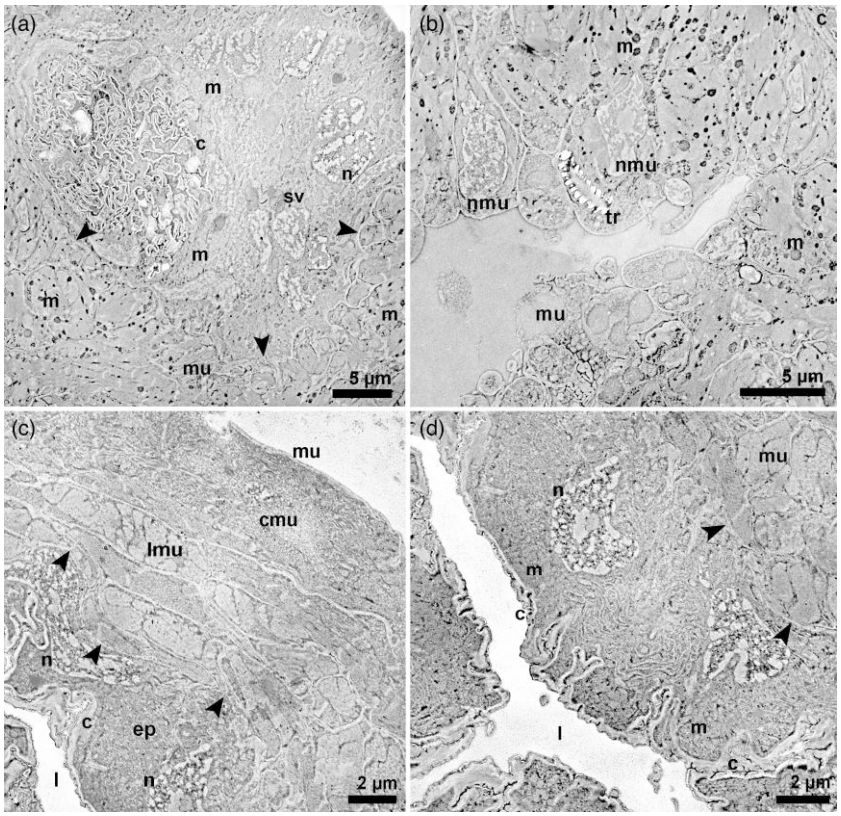


**Fig. 9.** Basal trunks of the colleterial glands of *Coptotermes gestroi* females. (**a, b**) Anterior basal trunk of an alate female. The musculature (mu) is well developed, containing several mitochondria (m), and being penetrated by tracheae (tr) at some points. (**c, d**) Posterior basal trunk of a 4-yr-old queen. Note the longitudinal (lmu) and circular (cmu) muscular sheets surrounding the epithelium (ep). Arrowheads indicate the limit between epithelium and musculature. c, cuticle; l, lumen; n, nucleus; nmu, nucleus of the muscular cell; sv, secretory vesicle.

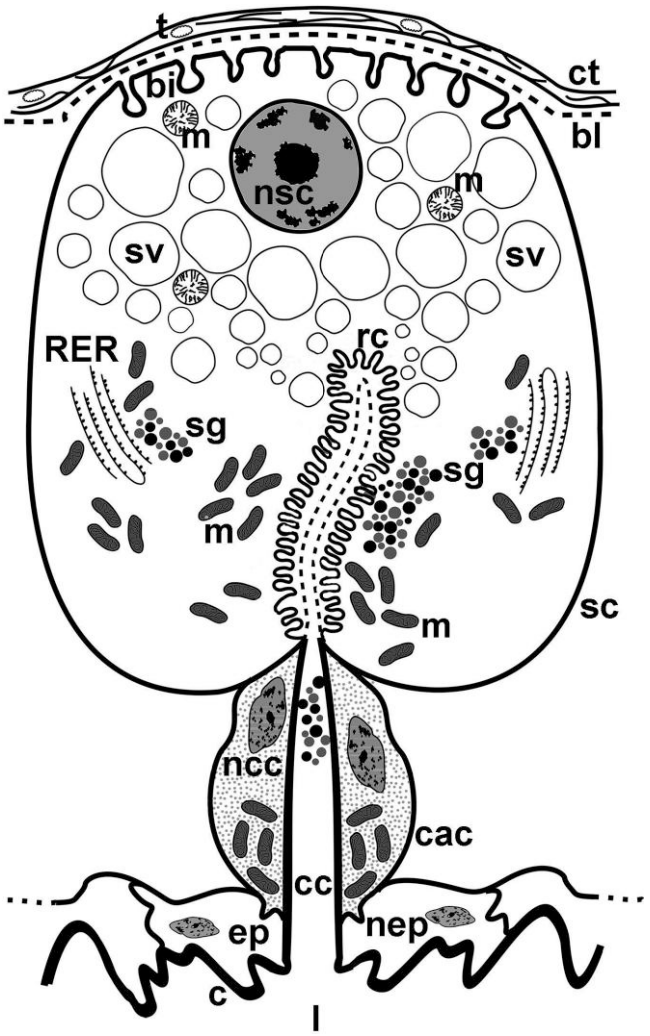


**Fig. 10.** Schematic representation of a bicellular unit of Class 3, component of the colleterial gland epithelium. bi, basal invaginations; bl, basal lamina; c, cuticle; cac, canal cell; cc, conducting canal; ct, connective tissue; ep, epithelial cell; l, lumen; m, mitochondria; ncc, nucleus of the canal cell; nep, nucleus of the epithelial cell; nsc, nucleus of the secretory cell; rc, receiving canal; rer, rough endoplasmic reticulum; sc, secretory cell; sg, secretory granules; sv, secretory vesicles; t, trachea.

2023), but differs from *M. darwiniensis*, *P. spiniger*, and *Z. nevadensis*, in which the basal trunk of each gland opens individually into the genital chamber (Courrent et al., 2008). The common duct is likely to mix and conduct the products secreted by the glands (Stay & Coop, 1974; Gillott, 1988; Ma et al., 2013).

The anterior and posterior glands were histologically similar within alate females. The flattened tubules showed a lumen lined by a thin cuticular layer. Moreover, scarce secretory activity was observed in the secretory cells, whereas there was no content in the glandular lumen. All these features suggest that the development and full activity of the colleterial glands relies on reproduction (Szopa, 1982; Soltani-Mazouni & Bordereau, 1987). Analyses on the termite *K. flavicollis* showed that the cuticle differentiation and thickening, as well as secretory activity of the colleterial glands,- only take place when pseudergates differentiate into neotenic reproductives (Soltani-Mazouni & Bordereau, 1987). Furthermore, the glands are degenerated and inactive during the period in which females of *Periplaneta americana* cease reproduction to incubate the ootheca (Stay & Roth, 1962).

Our morphometric data corroborated that the development of the glandular epithelium is a consequence of the reproductive events, similar to what happens with the accessory reproductive glands of other insects (Happ et al., 1982; Happ & Happ, 1982). The full activation of other reproductive traits such as the ovaries and spermatheca takes place before copulation, when alate females leave their natal nest, pair, and establish a nest (Greenberg & Stuart, 1979; Ye et al., 2009; Costa-Leonardo et al., 2022; da Silva & Costa-Leonardo, 2022*a*). For the spermatheca, it is likely to happen because mating seems to take place within 24 h after pairing (Raina et al., 2003) and females store spermatozoa as soon as they copulate, thus the secretion of contents must start before sperm transfer (da Silva & Costa-Leonardo, 2022*a*). On the other hand, oviposition in *C. gestroi* starts about five days after colony foundation (da Silva & Costa-Leonardo, 2022*b*), thus the absence of secretion in the lumen of the colleterial glands of non-egg-laying females is plausible. It is interesting to note that there are physiological and morphological shifts in termite queens as they mature and age. Not only the spermatheca and ovaries increase in size and activity (Costa-Leonardo et al., 2022; da Silva & Costa-Leonardo, 2022*a*) but also the fat body, tracheal system, and cuticle go through important modifications in accordance to the reproductive status of the queens (Bordereau, 1982; Han & Bordereau, 1982; Costa-Leonardo et al., 2013; da Silva et al., 2019). Other structures, in turn, case of the tergal glands (Figs. 2a, d) and thoracic muscles atrophy as queens age, suggesting their temporary role during swarming flights and pair formation, and colony stablishment (Ignatti & Costa-Leonardo, 2001; Zhang et al., 2021; Chouvenc, 2022). Therefore, our scientific outcomes on the colleterial glands of *C. gestroi* bring new insights into the shifts related to the reproductive status of termite queens.

The chemical compounds secreted by insect colleterial glands vary greatly and may play different functions (Dallai et al., 1996; Gillott, 2002). Based on our histochemical tests, only the posterior glands of egg-laying females showed secretory activity composed of a glycoproteinaceous content. Glycoproteins seem to be absent in the composition of the ootheca (Kramer et al., 1973), which agrees with the oviposition nature of termites. The secretion of these compounds has been often related to adhesive and lubricate functions (Tirone & Avancini, 1997; Ma et al., 2013; Sturm, 2016). Moreover, glycoproteins secreted by the male accessory glands are suggested to serve as lubricant to facilitate spermatophore transfer in grasshoppers (Cheeseman & Gillott, 1988, 1989). Thus, the secretion of glycoproteins by the posterior gland of *C. gestroi* could lubricate the genital chamber for the passage of eggs and also stick the eggs together after oviposition.

At the ultrastructural level, tiny and large receiving canals, the latter richly bounded by microvilli and limited by sponge-like cuticular layers, were observed in non- and egg-laying females, respectively. According to Quennedey (1998), there is a great variability in the organization of Class 3 cells, especially in what concerns the development and disposition of the canal cells and receiving canals. Based on the ultrastructure, as well as histology, scarce or none secretory activity was observed in the anterior and posterior glands of alate females, as well as in the anterior gland of mated queens. Such an absence agrees with the non-egg-laying nature of the former group.

Nevertheless, an abundance of electron-dense secretory granules was concentrated next to the receiving canals in the colleterial glands of 4-yr-old females, together with different profiles of RER, whose forms are likely to represent functional differences (Han & Bordereau, 1982). These features indicate synthesis and secretion of proteins by the cells (Quennedey, 1998; Křížková et al., 2014; Costa-Leonardo et al., 2020). On the other hand, electron-lucent vesicles concentrated at the basal portion of the secretory cells are also derived from endoplasmic reticulum and are likely to contain small-sized molecules (Quennedey, 1998). Thus, it seems that the secretory pathway of the colleterial glands in *C. gestroi* involves varied products synthesized in different sites of the secretory cell. The Golgi apparatus and richness of mitochondria in the secretory and canal cells also reinforces the active transport of secretion toward the lumen via conducting canals (Lay et al., 1999; Pascini et al., 2013). Such energy-requiring processes explain the tracheal penetrations in the connective tissue external to the glandular epithelium (Ahmed & Gillott, 1981; Wigglesworth, 1983).

The basal trunk of the glandular units was coated by a thick musculature, highlighted by ultrastructural micrographs, made up of circular and longitudinal sheets, equivalent to those previously describe for the colleterial glands of other termite species (Weesner, 1969). The well-arranged muscles of the basal trunks control the secretory activity toward the genital chamber of *C. gestroi* females. The richness in tracheal branches and mitochondria corroborates the energy demand of the muscular contractions to transport secretion (Ahmed & Gillott, 1981; Wigglesworth, 1983). Additionally, the absence of muscular sheets at the distal portion of the tubules and equal absence of receiving and conducting canals in the basal trunk epithelium supports the transport role of the proximal portion of the glands. Similar features were reported for scorpionflies (Greel, 1942; Ma et al., 2013), in which glandular units are scarce or completely absent in the common duct. The muscular contractions may be responsible for closing the gland opening and preventing the content release (Sturm & Pohlhammer, 2000; Sturm, 2008) or forcing the secretion into the genital chamber (Lococo & Huebner, 1980).

Previous reports have shown that the colleterial glands of termites and other insects do not display a regular secretory activity. For instance, posterior gland with empty lumen was already reported for some termite species (Courrent et al., 2008), whereas in other representatives, the anterior and posterior units are functional, but the contents secreted are quite different (Soltani-Mazouni & Bordereau, 1987, Silva and Costa-Leonardo, personal observation). For the scorpionfly *Panorpa sexspinosa*, the secretion diversity observed in the colleterial glands implies that several regions of the tubules secrete different compounds or that these products are in different synthesis phases (Ma et al., 2013). Variations in the secretory cycles have also been reported for other termite glands (Costa-Leonardo et al., 2009, 2021). Moreover, the different secretory activity may also be related to the age of the sampled females, as well as their reproductive activity, especially assuming that our study is the first to evaluate the colleterial glands in mature egg-laying queens. As the queens age, a higher number of eggs are laid, which ultimately reflect on the development and secretory activity of the colleterial glands (Silva and Costa-Leonardo, personal observation). In any case, further studies are necessary to shed light on the mechanisms responsible for the secretory activity of each gland.

## Conclusions

The development and secretory activity of colleterial glands in *C. gestroi* vary according to the age and reproductive status of the females. Thus, as alates mature into functional queens, morphophysiological changes take place in these organs. The posterior colleterial gland of *C. gestroi* secretes high amounts of glycoproteins, probably to lubricate the genital chamber during the oviposition. Ultrastructural features corroborate the synthesis and transport of contents toward the glandular lumen. The absence of secretory activity in the anterior gland of egg-laying females and in both glands of alate females may reflect different secretory cycles and the non-egg-laying nature of the individuals, respectively. If the colleterial glands of termites secrete other compounds beyond glycoproteins, it still requires further approaches. Moreover, it is important to note that *M. darwiniensis* is the only termite species whose eggs are laid enclosed in a modified ootheca (Nalepa & Lenz, 2000), and that females of *Odontotermes obsesus* lay eggs embedded into a matrix probably secreted by the colleterial glands (Roonwal, 1975). Assuming that the chemical nature of the termite egg coverings is unknown, it would be valuable if future comparative studies included these species.

## Availability of Data and Materials

The data that support the findings of this study are available from the corresponding author, da Silva IB, upon reasonable request.

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## Conflict of Interest

The authors declare that they have no competing interest.

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# Capítulo 4

Protoplasma
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ORIGINAL ARTICLE

# Mating- and oviposition-dependent changes of the spermatheca and colleterial glands in the pest termite *Cryptotermes brevis* (Blattaria, Isoptera, Kalotermitidae)

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**Abstract**
The spermatheca and colleterial glands of female insects are organs associated with the reproductive system, responsible for sperm storage and secretion of egg coverings, respectively. Here we compared the development, secretory activity, and chemical nature of the secretion in the spermatheca and colleterial glands of different-aged females of the drywood termite *Cryptotermes brevis*. We also provide the ultrastructure of these organs in alate females. These structures have been poorly investigated in termites when compared to other eusocial insects (Hymenoptera) and termite-related dictyopterans (mantises and cockroaches). The spermatheca of *C. brevis* comprises a cone-shaped structure, connected to the genital chamber by a short duct. The colleterial glands, in turn, are divided into anterior and posterior tubules, each showing a basal trunk, and join into a common duct. Histological and histochemical analyses showed that the secretion of proteins and polysaccharides by the spermatheca takes place before pairing, but increases as females mate and store sperm. Colleterial glands of alates showed non-synchronous secretory activity, but the synthesis of products increased in egg-laying queens, together with the epithelium height. Ultrastructure of the spermatheca and colleterial glands revealed epithelia composed of class III secretory cells. Richness of mitochondria and electron-dense secretion in the spermatheca indicates synthesis and transport of content. Presence and absence of colleterial gland secretion in different individuals may reflect variable maturation stages of the females and secretory cells. Assuming that termites are iteroparous, the development and secretion of the spermatheca and colleterial glands play a crucial role for *C. brevis* queens.

**Keywords** Reproduction · Pest species · Ultrastructure · Exocrine glands · Sperm storage · Oviposition

## Introduction

The reproductive system of female insects comprises several structures, directly or indirectly involved in the production, fertilization, and deposition of eggs. These include paired ovaries, lateral and common oviducts, genital chamber, spermatheca (associated or not with spermathecal glands), accessory glands, and external genitalia. Although these organs may not occur in all insect lineages, they vary greatly in number, size, ontogeny, and location (Matsuda 1976; Heming 2003; Simmons 2013).

The spermatheca is an epidermal-derived sperm storage organ omnipresent in all insect orders (Engelmann 1970). The number of spermathecae varies across the taxa, as well as their morphological features (Pascini and Martins 2017). The function of the spermatheca is to provide a suitable environment for sperm storage and maintenance, especially based on the synthesis and secretion of contents from the associated spermathecal glands or the spermathecal epithelium itself (Baer et al. 2009; Pascini and Martins 2017; Gotoh et al. 2017; da Silva and Costa-Leonardo 2022). The spermatheca opens into the genital chamber via a spermathecal duct, right before the colleterial glands (Gillott 2002). Such glands, also referred to as female accessory glands or cement glands, comprise paired structures widespread among insects. They show non-homologous origins according to the species, developing from epidermal or mesodermal tissues (Matsuda 1976; Chapman

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1998; Gillott 2002). Their main role is to synthesize and secrete contents responsible for covering eggs or attaching them to substrate during oviposition (Gillott 2002; Simmons 2013; Du et al. 2022).

The spermatheca plays an important role in eusocial insects, especially within Hymenoptera, in which queens copulate during a brief period early in life and store sperm up to decades (Baer 2005; Boomsma et al. 2005). Thus, the different mechanisms underlying sperm storage in these insects has received great attention (Baer et al. 2009; Gonzalez et al. 2018; Rangel et al. 2021), and are suggested to rely on the age and reproductive status of the queens (Martins and Serrão 2004; Souza et al. 2008). In termites, insects in which the eusociality evolved through different pathways (Nalepa 2015), the queen and king form a royal couple, who performs regular copulations during its lifetime (Nutting 1969; Raina et al. 2003). In contrast to eusocial Hymenoptera, mechanisms underlying sperm storage in termites have been neglected so far, as comprehensive analyses are restricted to few subterranean species (Raina et al. 2007; Ye et al. 2009; da Silva and Costa-Leonardo 2022).

In a similar way, the colleterial glands have received little attention in termites when compared to other related groups. Within Dictyoptera, a monophyletic clade comprising mantises, cockroaches, and termites, the morphology, content composition, and function of the colleterial glands are well known for the former groups (Stay and Roth 1962; Winnick et al. 2009; Du et al. 2022). The glands are paired and laterally disposed in mantises and cockroaches, and secrete different compounds which mix together to form the ootheca, a hardened case in which the eggs are grouped and protected from pathogens, water loss, and predation (Stay and Roth 1962; Gillott 2002; Du et al. 2022). Termites, in turn, possess colleterial glands following an anteroposterior pattern, being composed of convoluted glandular tubules (Grassé 1982; Courrent et al. 2008; Costa-Leonardo et al. 2023). With exception of *Mastotermes darwiniensis*, termite queens lay eggs individually and without any covering (Nalepa and Lenz 2000; Courrent et al. 2008). Thus, the likely function of the content secreted by colleterial glands of termites remains speculative and has been addressed to few species (Courrent et al. 2008; da Silva and Costa-Leonardo 2023).

The maturation of the spermatheca and colleterial glands of insects are in concert with that of other reproductive traits and mating events (Stay and Roth 1962; Szopa 1982; Hosken et al. 2002; da Silva and Costa-Leonardo 2022, 2023). Therefore, comparative analyses on females of different ages and reproductive status would be valuable to understand how such aspects influence the functionality of the reproductive system. In this paper, we compared the development and functional morphology of the spermatheca and colleterial glands in different-aged females of the West Indian dry-wood termite, *Cryptotermes brevis*. This species is native from the coastal desert of Chile and Peru and known as an important structural pest in the tropics (Scheffrahn et al. 2009). Colonies of *C. brevis* show a slow development and low egg-laying rate (Costa-Leonardo 2002), and go through a reproductive cycle that lasts from five to six months after colony foundation (McMahan 1962; da Silva and Costa-Leonardo, unpublished). Therefore, we predict that both aging and reproductive events trigger morphophysiological changes in the spermatheca and colleterial glands of *C. brevis* females.

## Material and methods

### Termites

For this study, five different groups of *Cryptotermes brevis* (Walker 1853) females were used: alates, 6-mo, 1-yr-old, neotenic, and mature queens. Alate females were sampled from swarming flights from colonies kept under laboratory conditions. These females were separated from males to prevent pairing. *C. brevis* queens (6-mo and 1-yr-old) were obtained from laboratory colonies whose reproductives were initially paired after swarming flights. For this purpose, alates were sexed under Zeiss Stemi 6 stereomicroscope, paired, and placed in a wood container made of tongue blades stuck together, containing a central chamber. Neotenic queens were obtained from orphaned colonies maintained under laboratory conditions, whereas a single mature queen was sampled from infested wood furniture. All the individuals were collected in the city of Rio Claro, SP, Brazil (22°24′43.9″ S; 47°36′34.0″ W).

### Anatomy of the colleterial glands

Five alate females of *C. brevis* were frozen for 5 min at -20 °C and then dissected in Hayes saline solution (9 g NaCl, 0.2 g $CaCl_2$, 0.2 g KCl and 0.1 g $NaHCO_3$ in 1000 ml $H_2O$) under a Zeiss Discovery V8 stereomicroscope. The spermatheca and colleterial glands were carefully isolated, stained with aqueous Eosin and photographed at different magnifications.

### Histology

The posterior abdomen of at least three individuals from each group were fixed in FAA (absolute alcohol, glacial acetic acid, 40% formaldehyde, in the ratio 3:1:1) or alcoholic Bouin for 24 h. Each sample was then dehydrated in an ethanol series (70%-95%), transferred to a Leica® historesin solution, and kept for at least seven days in a refrigerator. The abdomens were placed in molds and filled with Leica®

historesin plus catalyzer for polymerization. Histological sections of the posterior abdomens were obtained using a Leica RM 2245 microtome (3 µm thickness). Finally, the sections containing the spermatheca and colleterial glands were stained with hematoxylin–eosin (HE) for a proper description of their histological features.

### Histochemistry

Aiming to understand the chemical nature of the secretion of the spermatheca and colleterial glands, histological sections of alate females, 6-mo, 1-yr-old, neotenic, and mature queens were stained with xylidine-Ponceau (XP) to detect the presence of total proteins. Additionally, sections were also stained with Periodic acid-Schiff (PAS) for estimating the occurrence of polysaccharides. At least three individuals of each group were analyzed, with exception of the mature queen, which included a single sample.

### Morphometry of the colleterial glands

The age-dependent development of the colleterial glands was evaluated by measuring the epithelium height (Bednarska et al. 2016; da Silva and Costa-Leonardo 2023). To do so, three sections of each glandular unit (anterior and posterior) from two individuals of each group were photographed in a Leica ICC150 photomicroscope at 400× and the images were uploaded in the software Image J (https://imagej.nih.gov/ij) with their respective scale bars. The measurements were calibrated and the epithelium height (µm) was measured using the "straight tool". For each section, three measures were extracted to obtain a mean height (adapted from Bednarska et al. 2016). Data were compared among groups using an analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test for multiple comparisons using the software R version 1.3.1093 (RStudio Team 2020). Due to the number of samples, queens from wood furniture were not used in this analysis.

### Transmission Electron Microscopy

The spermatheca and colleterial glands of alate females, were isolated and fixed in 2.5% glutaraldehyde in 0.1-M sodium cacodylate buffer (pH 7.2) for 2 h at 4 °C. The material was post-fixed in 1% OsO4 in the same buffer and transferred to Epon-Araldite resin. Posteriorly, the spermatheca and glands were separately included in pure Epon resin and polymerized at 60 °C during 24 h. Ultrathin sections were obtained using a Leica Reichert Supernova ultramicrotome and stained with 4% uranyl acetate and lead citrate. Sections of the spermatheca, as well as of the anterior and posterior glands, were photographed using a Jeol JEM 1400 transmission electron microscope.

## Results

### Anatomy of the spermatheca and colleterial glands

The spermatheca and colleterial glands in females of *C. brevis* are located at the posterior limit of the abdomen, at the 8$^{th}$ and 9$^{th}$ sternites, respectively (Figs. 1a-f). The spermatheca comprises a cone-shaped structure that is connected to the genital chamber by a short and cuticular spermathecal duct (Figs. 1e- f). The spermathecal reservoir, which corresponds to the lumen where the secretion was stored, followed a similar shape, whose dimensions were about 180 µm in length and 100 µm in width. The colleterial glands comprise two units, the anterior and posterior (Figs. 1a-b). Both units are made up of glandular tubules at their distal portion, which join together in a common duct at the proximal limit (Fig. 1c). This duct, in turn, opens into the genital chamber next to the spermatheca opening. Moreover, each gland has a basal trunk right before the common duct (Fig. 1d).

### Histology

The spermatheca of all females followed the same disposition, with epithelium containing nuclei at different heights. At the apical portion, a cuticle-lined lumen was observed, whereas overlapped muscular sheets were disposed at the basal portion (Figs. 2 and 3). The lumen of the spermatheca of all females showed stored secretion, including alate females (Figs. 2a-b). Nevertheless, elongated and non-flagellate spermatozoa were mixed with the secretion in 6-mo-old (Figs. 2d-e), 1-yr-old (Fig. 2g), neotenic (Fig. 3a), and mature queens (Figs. 3d-e).

The colleterial glands were disposed as convoluted tubules. The anterior unit epithelium was relatively thinner when compared to that of the posterior one in all female groups. Moreover, the glandular lumen was limited by a cuticular layer. The basal trunk of each unit was richly surrounded by muscular sheets, whereas the lumen seemed to be shrunk and limited by a thick cuticle (Figs. 2 and 3). The colleterial glands in alate females were underdeveloped and devoid of secretion (Figs. 2b-c), whereas the anterior and posterior units of the glands in 6-mo-old (Figs. 2e-f), 1-yr-old (Figs. 2h-i), neotenic (Figs. 3b-c), and mature queens (Figs. 3e-f) showed secretory activity at different intensities, according to the age and development of the females. Thus, the highest amount of stored secretion was observed in neotenic and mature queens (Figs. 3b-f). Furthermore, the secretion observed in the anterior and posterior glands differed, since it was flocculated in the former and comprising opaque vesicles in the latter (Figs. 2e-i, 3b-f).

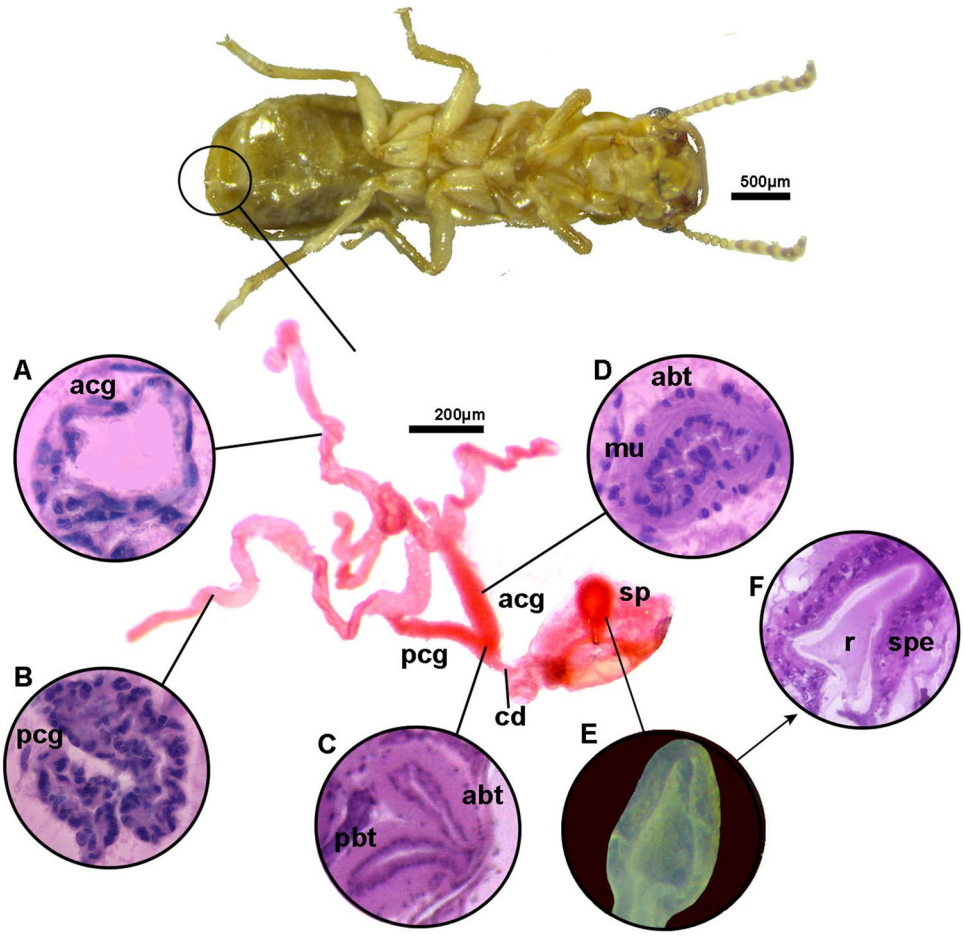


**Fig. 1** Total mounting and histological sections of the colleterial glands and spermatheca of a *C. brevis* alate female. **a** Anterior colleterial gland (**acg**). **b** Posterior colleterial gland (**pcg**). **c** Anterior (**abt**) and posterior (**pbt**) basal trunks. **d** Detail of the anterior basal trunk coated by muscular sheets (**mu**). **e** Total mounting of the spermatheca (**sp**). **f** Histological section of the spermatheca. **cd** common duct, **r** reservoir, **spe** spermathecal epithelium

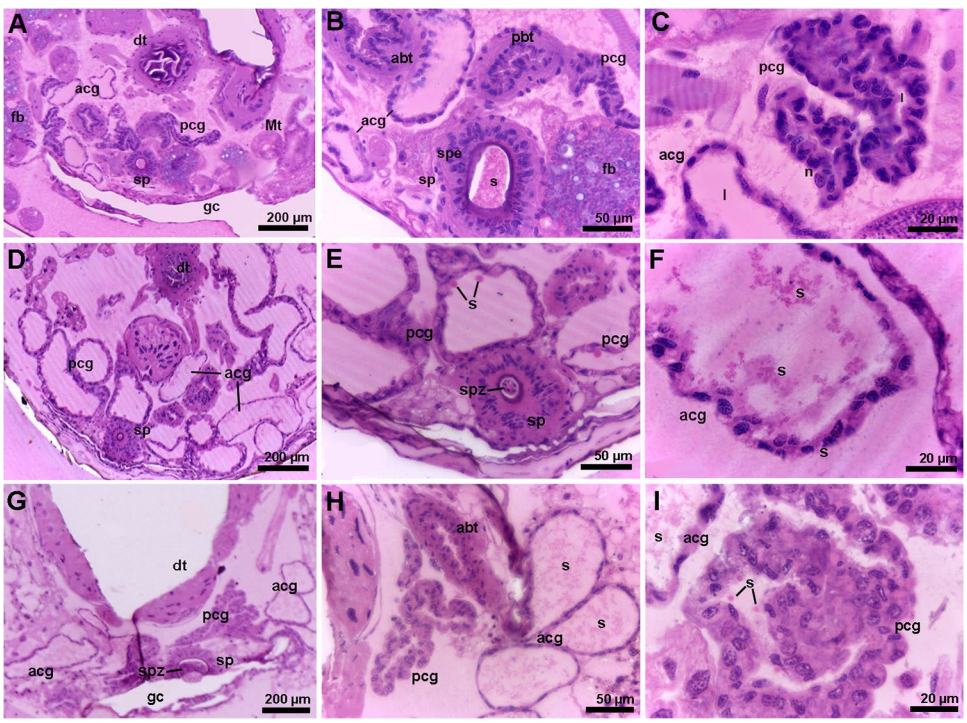


**Fig. 2** Cross sections of the abdomen of *C. brevis* females, highlighting the spermatheca and colleterial glands. **a-c** Alate females. Note the spermatheca (**sp**) filled with secretion (**s**), whereas the anterior (**acg**) and the posterior (**pcg**) glands were devoid of secretory activity. **d-f** 6-mo-old queens. The anterior gland shows a higher amount of flocculate secretion while that of the posterior unit is round-shaped, opaque, and scarce. **g-i** 1-yr-old queens. A similar pattern is observed, especially in what concerns the secretory activity of the colleterial glands. **abt** anterior basal trunk, **dt** digestive tube, **fb** fat body, **gc** genital chamber, **l** lumen, **Mt** Malpighian tubule, **pbt** posterior basal trunk, **spe** spermathecal epithelium, **spz** spermatozoa. Hematoxylin–eosin staining

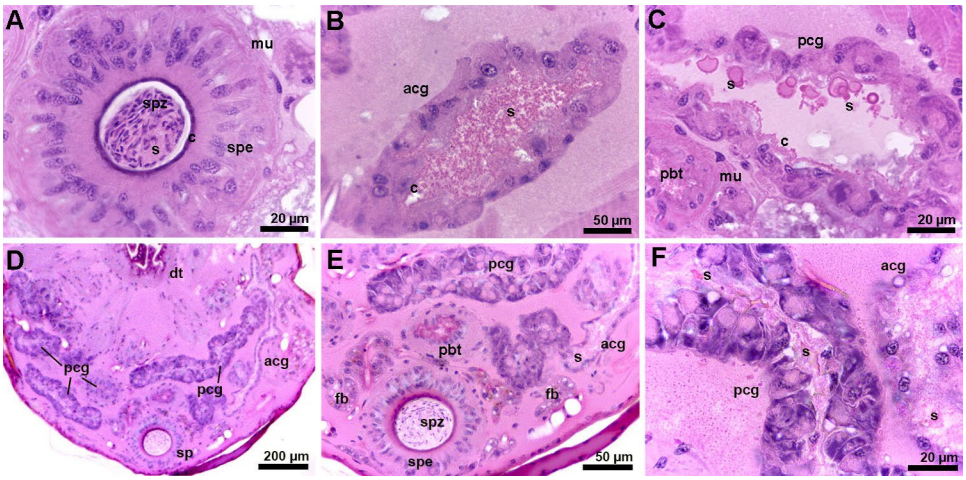


**Fig. 3** Cross sections of the abdomen of *C. brevis* females. **a-c** Neotenic queens. An increasing secretory activity of the spermatheca and the colleterial glands is observed. Note the spermathecal reservoir with numerous spermatozoa (**spz**) mixed with the secretion (**s**). **d-f** Mature queen. The spermatheca (**sp**) is also filled with spermatozoa, although a higher secretory activity is only observed in the anterior (**acg**) and posterior (**pcg**) glandular units. **c** cuticle, **dt** digestive tube, **fb** fat body, **mu** muscular sheets, **spe** spermathecal spithelium. Hematoxylin–eosin staining

## Histochemistry

The secretion stored in the spermatheca of all female groups reacted positively to the XP and PAS tests, but at different intensities (Figs. 4–6; Table S1). For alate females (Fig. 4a), there was a weak positive reaction to XP test, whereas it was moderate among 6-mo-old (Fig. 4d), 1-yr-old queens (Fig. 4g), and mature queens (Fig. 5d). Neotenic queens, in turn, showed a strong positive reaction to the presence of proteins in the stored secretion (Fig. 5a). As described above, the colleterial glands of alate females did not display secretory activity (Figs. 4b-c). For mated queens, the positive reaction of the secreted contents to XP test varied between the glands, as the flocculated secretion of the anterior unit stained moderately (Figs. 4e-f, 5b-e) while the opaque and rounded secretory vesicles of the posterior unit were stained strongly (Figs. 4f-i, 5c-f).

The secretion of alate female spermatheca (Fig. 6a) stained moderately to the PAS test, suggesting the occurrence of polysaccharides. Similarly, the content stored in the spermatheca of 6-mo-old (Fig. 6d), 1-yr-old (Fig. 6g), and neotenic queens (Fig. 6j) was sugar-based, in which several spermatozoa were embedded. Due to the absence of secretory activity, the glands of alate females did not react positively to the PAS test (Figs. 6b-c). However, a moderate reaction to the presence of polysaccharides was observed in the content secreted by the colleterial glands of 6-mo-old (Fig. 6e-f), 1-yr-old (Figs. 6h-i), whereas a strong concentration of these compounds was reported in the glandular lumina of neotenic queens (Figs. 6k-l).

## Morphometry of the colleterial glands

Epithelium height of both glands varied significantly among female groups (anterior gland: $F_{3,28} = 60.42$, $P < 0.001$; posterior gland: $F_{3,28} = 113.8$, $P < 0.001$; ANOVA + Tukey HSD). As observed in the histological sections, the colleterial glands of alate females were less developed, resulting in epithelia significantly thinner than those from any other female group (Figs. 7a-b). Measurements performed on the colleterial glands of 6-mo and 1-yr-old queens revealed no significant difference in the anterior gland epithelium ($P = 0.435$). Differences in the posterior epithelium height were also less significant between these female groups (Fig. 7a). The development of the colleterial glands was remarkable in neotenic queens, whose glandular units reached the highest values for epithelium height (Figs. 7a-b). Morphometrical data are shown in table S1.

## Ultrastructure

The spermathecal reservoir epithelium in alate females of *C. brevis* was composed of bicellular secretory units. Thus, each unit consisted of outer and columnar secretory cell and inner flattened canal cells, responsible for secreting a cuticular canal (Figs. 8a-c). The secretory cell was provided with an extracellular cavity surrounded by microvilli and crossed by the receiving canal or "end apparatus", internally coated by an interrupted cuticle. This canal continues with a conducting canal, which was internally coated by a continuous cuticle and responsible for carrying the secreted

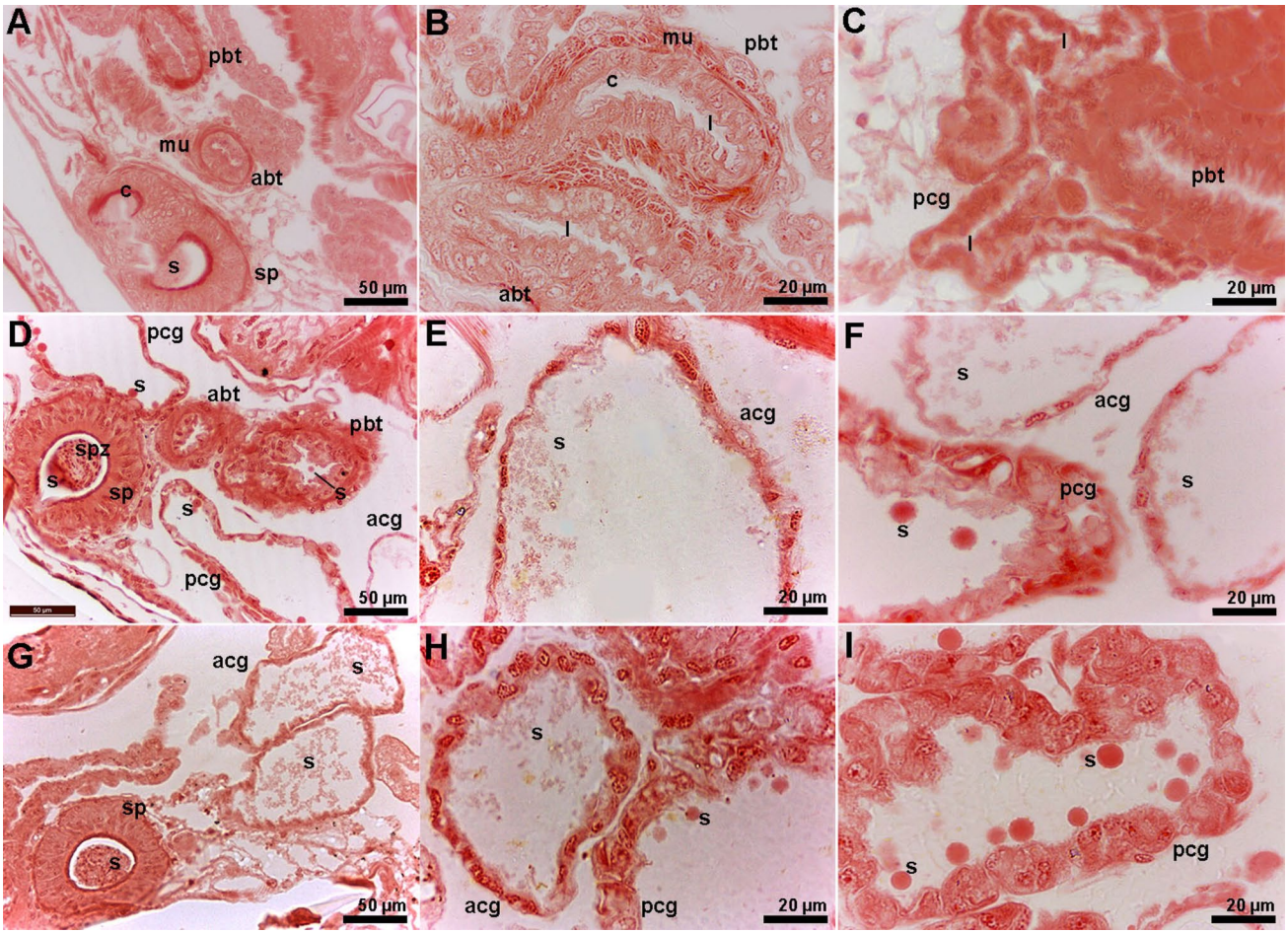


**Fig. 4** Histochemistry of the spermatheca and colleterial glands of *C. brevis* females. **a-c** Alate females. The secretion (**s**) of the spermatheca (**sp**) stained weakly to the detection of proteins, whereas there was no secretory activity in the anterior and posterior (**pcg**) colleterial glands. **d-f** 6-mo-old queens. Note the increasing concentration of proteinaceous content in the spermatheca and glandular secretion, especially in the posterior unit. **g-i** 1-yr-old queens. There was an increasing secretory activity in the posterior glands, although the concentration of proteins was similar to that observed for 6-mo-old females. **abt** anterior basal trunk, **c** cuticle, **l** lumen, **mu** muscular sheets, **pbt** posterior basal trunk, **spz** spermatozoa. Xylidine-Ponceau staining

content towards the glandular lumen. The epithelium was apically limited by a thick cuticle and externally surrounded by muscular sheets (Figs. 8a-c). The spermathecal duct epithelium, in turn, comprised cells devoid of glandular features such as receiving/conducting canals. The cuticle lining the spermathecal duct was thicker, made-up of several layers. Similar to the described above, the duct epithelium was also surrounded by muscular bundles (not shown).

The secretory cells of the spermathecal epithelium showed round-shaped nuclei, whereas those of the canal cells were irregularly shaped (Figs. 8a-c). Additionally, neighboring cells were connected by septate junctions. A rich number mitochondria was located at the apical limit of canal cell cytoplasm. The cells of the spermathecal duct always showed rounded nuclei, which occupied most part of the cytoplasm. A remarkable feature of this region was the richness of mitochondria at the apical portion of the duct cells (not shown). The conducting canals, as well as the spermathecal reservoir and duct, were filled with an opaque secretion (Fig. 8c), indicating synthesis, transport, and storage of content, probably proteins, in agreement with our histological data.

Ultrastructure of the colleterial gland epithelia also revealed bicellular secretory units at the distal portion of the tubules, with well-developed receiving and conducting canals, frequently surrounded by different mitochondrial morphotypes (Figs. 9–10). The anterior gland of alate females showed a relatively developed epithelium (Figs. 9a), with a swollen lumen lined by a convoluted cuticle and containing flocculate and opaque secretion (Figs. 9a). Cytoplasm of the secretory cells showed large round-shaped nuclei and receiving canals usually located at the basal region of the secretory cell (Figs. 9a-c), surrounding by mitochondria (Fig. 9b). Similar to the anterior gland, the

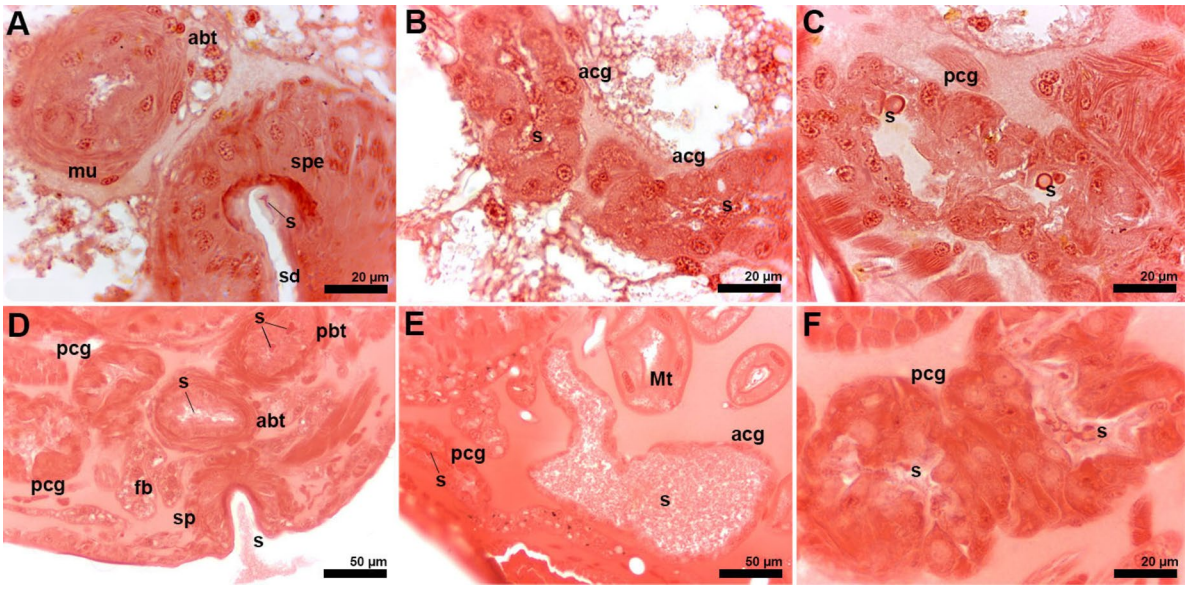


**Fig. 5** Histochemistry of the spermatheca and colleterial glands of *C. brevis* females. **a-c** Neotenic queens. A high concentration of proteins is observed in the spermathecal duct (**sd**) and colleterial glands. **d-f** Mature queen. Note the anterior colleterial gland (**acg**) filled with secretion, which stained moderately to XP test, whereas a high concentration of proteins is observed in the secretion of the posterior unit (**pcg**). **abt** anterior basal trunk, **fb** fat body, **Mt** Malpighian tubule, **mu** muscular sheets, **pbt** posterior basal trunk, **spe** spermathecal epithelium. Xylidine-Ponceau staining

posterior unit of alate females showed secretory cells with rounded nuclei and well-developed receiving canals surrounded by different mitochondrial morphotypes (Fig. 10a-b). Similarly, secretory activity varied among the analyzed females. The electron-dense secretion located in the receiving canals of both the glandular units indicate synthesis and transport of content (Figs. 9d and 10b).

## Discussion

The general anatomy of the spermatheca in females of *C. brevis* showed a cone-shaped structure with an arrowhead-shaped reservoir. Based on paraffin histological sections, Costa-Leonardo and Patrício (2005) described the spermatheca of *C. brevis* and *Rugitermes* sp. (Kalotermitidae) as a recurved finger. However, with the aid of thinner histological sections and dissection techniques, we could provide a more accurate description. Thus, the spermatheca of *C. brevis* corresponds to a unique structure, as within Neoisoptera clade, the spermatheca comprises a finger-shaped organ, whereas it resembles an umbrella in alate females of *Zootermopsis nevadensis* (Costa-Leonardo and Patrício 2005). The spermatozoon of *C. brevis* was already described as non-flagellate and elongated (Laranjo et al. 2020). Additionally, such a non-flagellate and immotile nature is a synapomorphy for the spermatozoa of most termite lineages (Baccetti et al. 1981; Grandi 1992; Costa-Leonardo and Patrício 2005; da Silva et al. 2019; Laranjo et al. 2020). The anatomy of the colleterial glands corresponds to that previously reported for other termite species, in which each gland comprises a tubular unit that bifurcates at some point (Grassé 1982; Soltani-Mazouni and Bordereau 1987; Courrent et al. 2008; da Silva and Costa-Leonardo 2023). Nevertheless, the anterior and posterior glands were very alike and fully developed, contrasting other findings that suggested an asymmetrical glandular development (Courrent et al. 2008). At the proximal portion, the glands converge into a common duct, a site probably related to the mixing of contents secreted by each gland (Stay and Coop 1974; Gillott 2002; Ma et al. 2013; da Silva and Costa-Leonardo 2023).

Based on the histological and histochemical analyses, the secretory activity of the spermatheca in *C. brevis* takes place prior to insemination. Such a condition has been reported for virgin females of different insects, in which other factors influence the secretory activity of the spermatheca rather than sperm transfer (Ye et al. 2009; Pascini et al. 2012; da Silva and Costa-Leonardo 2022). One of these factors might be the termite swarming flights, followed by pairing and colony foundation, which trigger ovary and spermatheca maturation (Greenberg and Stuart 1979; Ye et al. 2009; Costa-Leonardo et al. 2022; da Silva and Costa-Leonardo 2022). Although we reported secretory activity in the spermatheca of alate females, the concentration of proteins, given the positive reaction to xylidine-Ponceau test, increased in queens and reached the highest concentration among neotenic

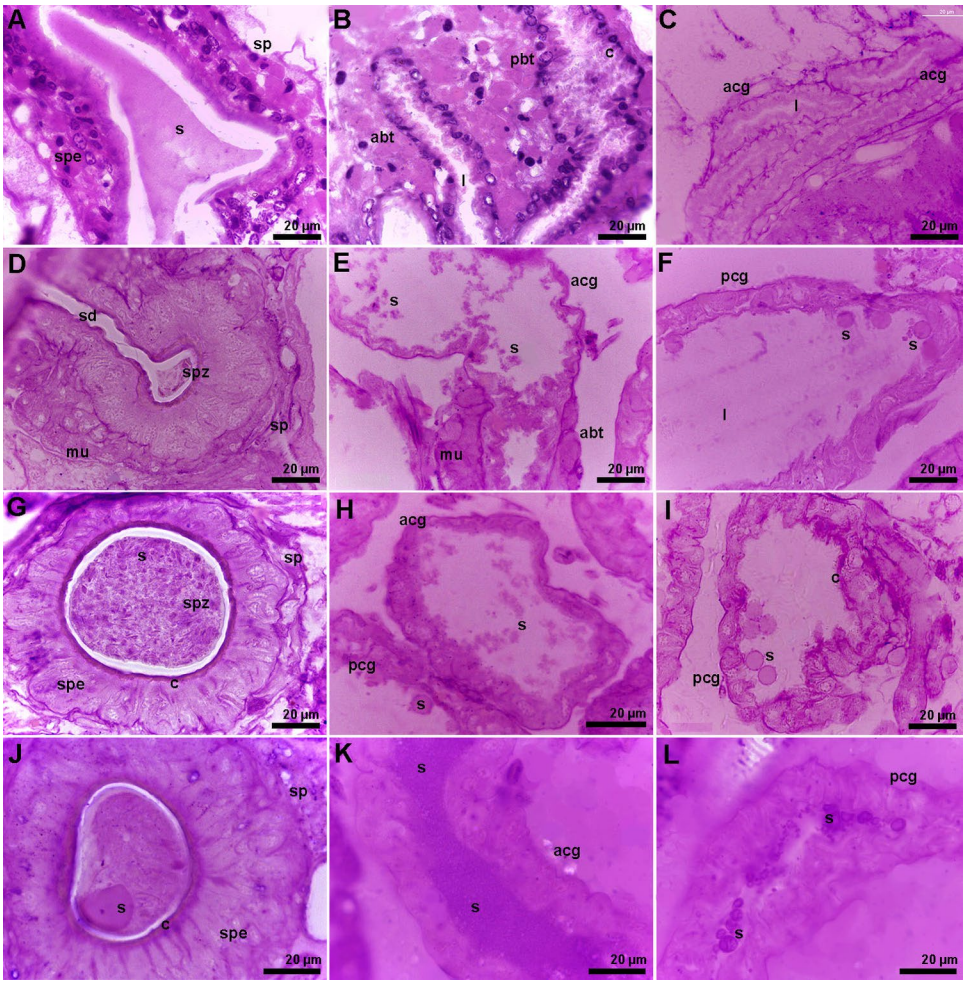


**Fig. 6** Histochemistry of the spermatheca and colleterial glands of *C. brevis* females. **a-c** Alate females. Note the sugar-based secretion (**s**) of the spermatheca, whereas both the anterior (**acg**) and posterior (**pcg**) glands are devoid of secretion. **d-f** 6-mo-old queens. There is an increasing secretion of polysaccharides in the glandular lumina. **g-i** 1-yr-old queens. A similar pattern is observed between these queens and 6-mo-old ones. Note the abundance of spermatozoa (**spz**) imbedded into the sugar-based secretion (**s**). **j-l** Neotenic queens. Both the glands show a high concentration of polysaccharides, due to the strong reaction to PAS test. A moderate staining is observed in the secretion of the spermatheca (**sp**). **abt** anterior basal trunk, **c** cuticle, **l** lumen, **mu** muscular sheets, **pbt** posterior basal trunk, **sd** spermathecal duct, **spe** spermathecal epithelium. PAS staining

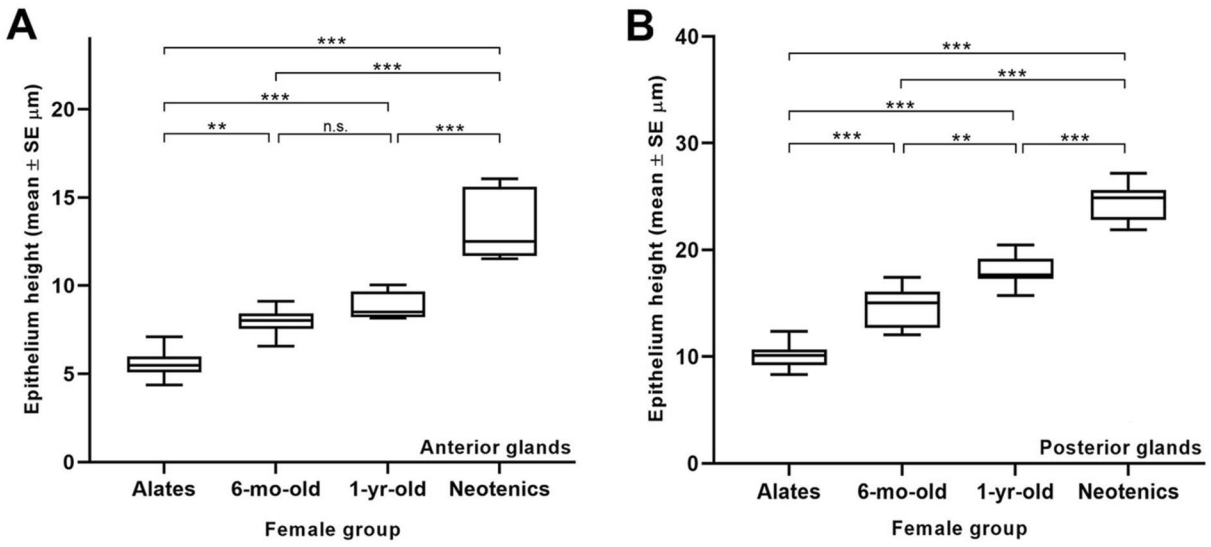


**Fig. 7** Epithelium height of the colleterial glands of different-aged females of *C. brevis*. **a** Anterior glands. **b** Posterior glands. Note the epithelium development in neotenic queens when compared to other sampled females. **ns** non-significant, ** $P < .01$, *** $P < .001$

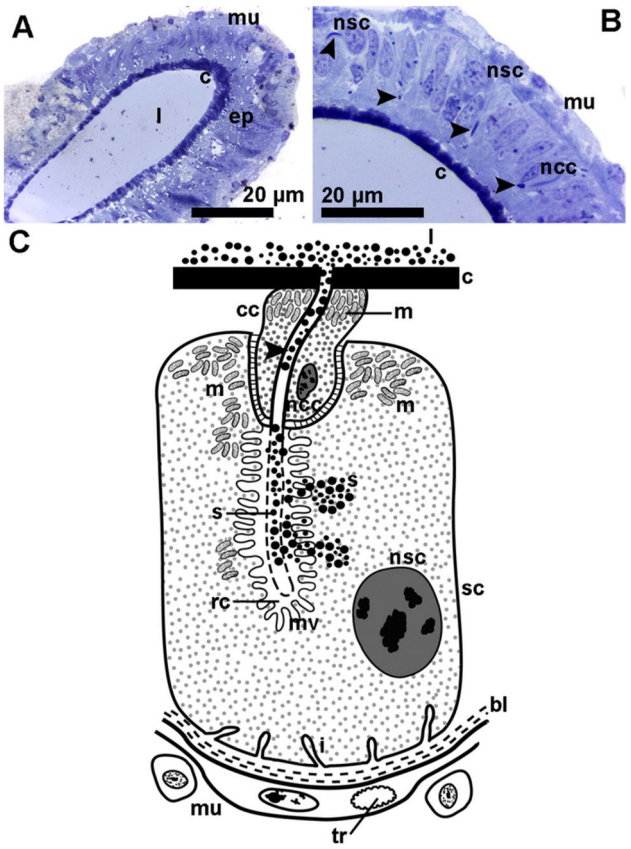


**Fig. 8** **a-b** Semithin sections showing the general view and detail of the spermatheca in female alates of *C. brevis*, respectively. Note the conducting canal (arrowheads). **c** Schematic drawing of a bicellular unit of class 3, component of the spermathecal epithelium, and its morphological features. **bl** basal lamina, **c** cuticle, **cc** canal cell, ep epithelium **i** invaginations, **l** lumen, **m** mitochondria, **mu** muscular sheets, **mv** microvilli **ncc** nucleus of the canal cell, **nsc** nucleus of the secretory cell, **rc** receiving canal, **s** secretion, **sc** secretory cell, **tr** tracheole

females. In a similar way, the secretion of polysaccharides, based on the positive reaction to PAS test, was higher in neotenic queens when compared to other individuals. Proteins and sugar-based compounds are responsible for nourishing and providing energy for the stored spermatozoa, as reported for different insect taxa (Bhatnagar and Musgrave 1970; Schoeters and Billen 2000; Souza et al. 2008; Pascini et al. 2012; da Silva and Costa-Leonardo 2022).

The colleterial glands among the different female groups varied in development and secretory activity. Thus, our histological analyses suggested that the glandular units of alate females were not fully functional in some individuals, especially based on the absence of secretion in the glandular lumen (see the discussion on the ultrastructure of the glands). It was already suggested that the complete development and activity of the termite colleterial glands rely on reproductive events (Soltani-Mazouni and Bordereau 1987; Costa-Leonardo et al. 2023). Similar results have been found for female crickets (Sturm 2016). As termite alates mature into functional queens, the glandular epithelium becomes taller and the secretory activity increases, corroborating the age-dependent morpho-physiological changes of the accessory glands observed in insects (Feng and Roelofs 1977; Happ et al. 1982; Happ and Happ 1982). The similar values for epithelium height between 6-mo and 1-yr-old females may have occurred because egg-laying does not vary significantly between these groups. *C. brevis* queens cease oviposition for three months after the first oviposition cycle, which lasts from five to six months after colony foundation (McMahan 1962; da Silva and Costa-Leonardo, unpublished). When oviposition resumes, an extremelly low number of eggs are laid in the following months (McMahan 1962; da Silva and Costa-Leonardo, unpublished) and therefore does not have a remarkable impact on the development of the colleterial glands.

The secretion of contents was notable within neotenic and mature queens, whose glandular lumen was filled with a strong-stained proteinaceous secretion. Moreover, an intense secretion of polysaccharides was recorded in neotenic queens when compared to other sampled females. The secretion of glycoproteins into the genital chamber are believed to play adhesive and lubricate functions (Cheeseman and Gillott 1988, 1989; Tirone and Avancini 1997), thus the increasing secretion of these compounds by the colleterial glands of functional queens of *C. brevis* would serve to facilitate the passage of eggs through the genital chamber and/or stick the laid eggs together (da Silva and Costa-Leonardo 2023). Moreover, glycoproteins are absent in the ootheca composition (Kramer et al. 1973), which therefore agrees with the oviposition nature of termites. According to Noirot (1990), the fecundity of neotenic queens of dry-wood species might be equal to that observed in imaginal ones, thus a notable development of the reproductive apparatus in these individuals was expected.

At the structural level, the bicellular units of the spermathecal epithelium and colleterial glands correspond to secretory cells of class 3 (Noirot and Quennedey 1974, 1991). The reproductive apparatus of *C. brevis* does not show spermathecal glands, in agreement with other termite families (Costa-Leonardo and Patrício 2005; Raina et al. 2007; da Silva and Costa-Leonardo 2022), dictyopterans (Lawson and Thomas 1970; Winnick et al. 2009; Brannoch et al. 2017), and scorpionflies (Hou and Hua 2008; Yang and Hua 2021). In this case, the spermathecal epithelium itself has a secretory function. The richness of mitochondria at the apical portion of the canal cells corroborates an epithelium associated with energy-requiring processes (i.e., secretion and transport of content) (Ahmed and Gillott 1981; Wigglesworth 1983).

The spermathecal lumen, as well as the conducting canals, filled with floculated secretion among alate females

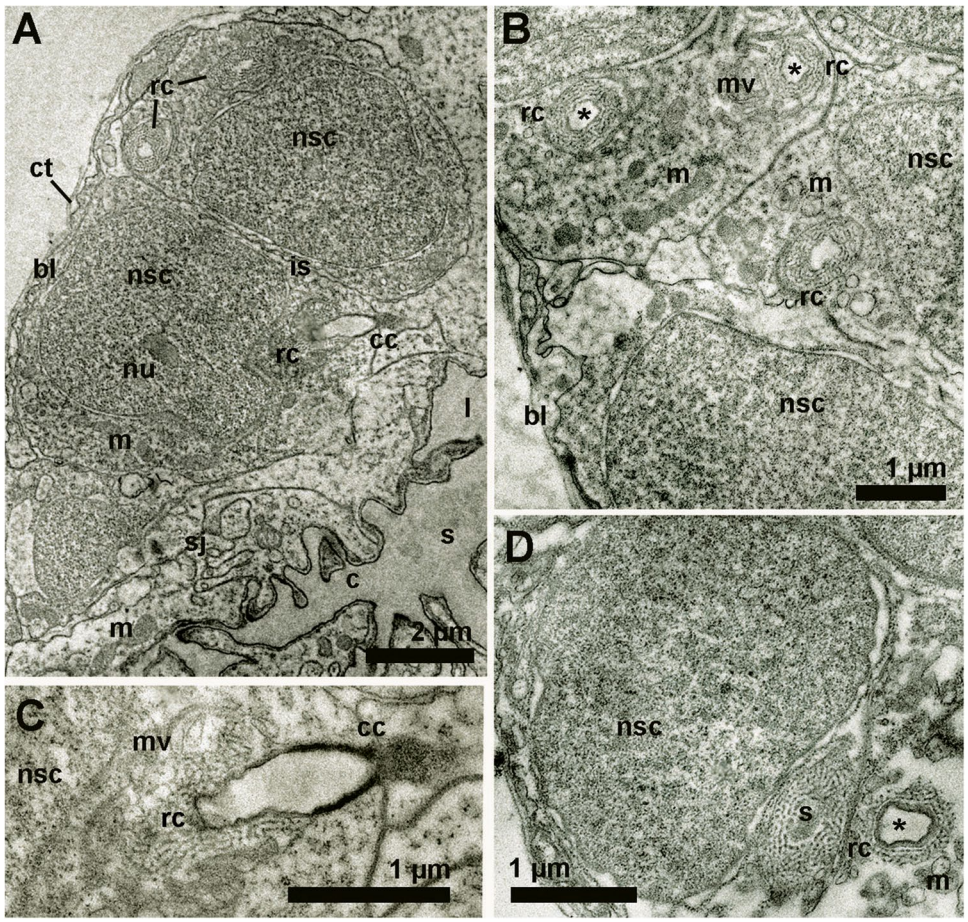


**Fig. 9** Ultrastructure of the of the anterior colleterial gland in female alates of *C. brevis*. **a** Secretory epithelium. Note the large and round nucleus of the secretory cells (**nsc**) and nucleolus (**nu**). **b** Detail of secretory cells, showing the receiving canals (**rc**) surrounded by microvilli (**mv**) and several mitochondria (**m**). **c** Transition of a receiving canal (interrupted lining cuticle) into a conducting canal (**cc**) with continuous lining cuticle. **d** Detail of the non-synchronic secretory activity into the receiving canals. Empty canals are marked with asterisk. **bl** basal lamina, **c** cuticle, **ct** connective tissue, **is** intercellular space**, l** lumen, **s** secretion, **sj** septate junction

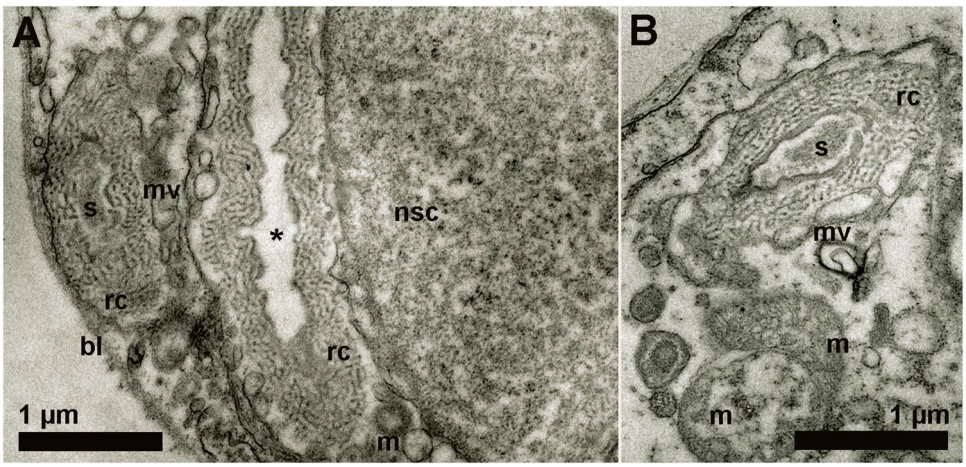


**Fig. 10** Ultrastructure of the posterior colleterial gland in female alates of *C. brevis*. **a** Secretory epithelium with well-developed receiving canals (**rc**) located at the basal epithelium, next to the basal lamina (**bl**). Different receiving canals show non-synchronous secretory activity, as some canals are filled with electron-dense secretion (**s**) whereas others are empty (*). Detail of a sponge-like receiving canal with microvilli (**mv**) and surrounded by different mitochondria (**m**). **nsc** nucleus of the secretory cell

corroborates our histological and histochemical data, indicating that sperm storage-related content is secreted prior to copulation (da Silva and Costa-Loenardo 2022). The spermathecal duct epithelium of *C. brevis*, similar to that of other termites, did not show features of secretory activity. Otherwise, the richness of mitochondria in the cells and the surrounding muscles are associated with sperm transfer through the duct (Raina et al. 2007). The thick mutilayered cuticle is likely to protect and isolate the spermatozoa (Farder-Gomes et al. 2019; Yang and Hua 2021), although such a region must be flexible enough to allow the passage of termite spermatozoa through the narrow duct lumen (Raina et al. 2007).

The ultrastructural features of colleterial glands do not follow a pattern among termites, since the basal species *M. darwiniensis* (Mastotermitidae) and *Zootermopsis nevadensis* (Archotermopsidae) show epithelium with modified class 1 secretory cells, while *Kalotermes flavicollis* and *C. brevis* (Kalotermitidae), *Coptotermes gestroi* (Rhinotermitidae), and *Pseudacanthotermes spiniger* (Termitidae) possess class 3 secretory cells (Soltani-Mazouni and Bordereau 1987; Courrent et al. 2008; da Silva and Costa-Leonardo 2023). Based on this, it is likely that there was a transition from class I to III in the colleterial glands of an common ancestor to Kalotermitidae + Neoisoptera (da Silva and Costa-Leonardo 2023).

Both the anterior and posterior units showed receiving canals surroundede by mitochondria of different morphotypes. These features, together with the electron-dense secretion inside the canals and in the lumen, indicate synthesis and transport of glandular content (Wheeler and Krutzsch 1994; Lay et al. 1999; Sturm 2002). The electron-dense secretion corresponds to a proteinaceous content, corroborating the histochemical analyses. Although alate females were not laying-eggs, we reported the secretion of contents by the colleterial glands in some individuals, whereas such an acitivity was absent in others. An asymmetrical secretory activity of the colleterial glands seems to be common in females of termites and other insects. This asymmetry relies on age, secretory phase of the glands, sites in which the secretory activity takes place, and reproductive maturity, which seems to be the case of *C. brevis* alate females (Soltani-Mazouni and Bordereau 1987; Courrent et al. 2008; Ma et al. 2013; da Silva and Costa-Leonardo 2023). Since these individuals were sampled from laboratory colonies, it is possible that not all alate females, as well as the class 3 secretory cells of their colleterial glands, were at the same stage of maturation, reflecting on receiving canals with a variable amount of content. Other reproductive traits, such as the vitellogenic oocytes, also reach distinct late-stages among termite alate females, suggesting that different mechanisms underly reproductive maturation (Costa-Leonardo et al. 2022).

The most notable function of the colleterial glands is to secrete compounds associated with egg coating. These include the ootheca, a hardened case with occurrence in several polyneopterans (Du et al. 2022), and soft jelly-like structures (Gillott 2002). Within termites, with rare exceptions, the colleterial glands do not secrete egg-coating materials. For instance, only *M. darwiniensis* queens are known to produce egg coverings (Nalepa and Lenz 2000). As termites are iteroparous, where the royal pair invests in multiple brood generations to increase colony population (Thorne 1997), regular copulations and an increasing egg-laying rate would be benefited by the secretory activity of the spermatheca and colleterial glands, whose glycoproteinaceous content nourishes the stored spermatozoa and lubricates the genital chamber/sticks laid eggs together, respectively (da Silva and Costa-Leonardo 2023). Further studies might reveal if they secrete other compounds and bring new insights into the evolution and role played by these reproductive organs in insects.

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**Data Availability** The authors confirm that the data supporting the findings of this study are available within the article and its supplementary material.

## Declarations

**Ethical approval** This article does not contain any studies with human participants performed by any of the authors.

**Conflict of interest** The authors declare that they have no conflict of interest.

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Table S1 – Histochemical and morphometrical features of the spermatheca and colleterial glands of *C. brevis* females

| Females | Reproductive organ | XP reaction | PAS reaction | Epithelium height (µm) |
|---|---|---|---|---|
| Alate females | Spermatheca | + | + + | / |
| | Anterior gland | - | - | 5.57 ±0.3 |
| | Posterior gland | - | - | 10.06 ±0.43 |
| 6-mo-old queens | Spermatheca | + + | + + | / |
| | Anterior gland | + + | + + | 7.96 ± 0.26 |
| | Posterior gland | + + + | + + | 14.67 ± 0.66 |
| 1-yr-old queens | Spermatheca | + + | + + | / |
| | Anterior gland | + + | + + | 8.84 ± 0.26 |
| | Posterior gland | + + + | + + | 18.0 ± 0.51 |
| Neotenics | Spermatheca | + + + | + + | / |
| | Anterior gland | + + + | + + + | 13.32 ± 0.68 |
| | Posterior gland | + + + | + + + | 24.48 ± 0.62 |
| Mature queen | Spermatheca | + + | / | / |
| | Anterior gland | + + | / | / |
| | Posterior gland | + + + | / | / |

- Not stained
+ weakly stained
++ moderately stained
+++ strongly stained
/ Not recorded

# *Conclusões*

## 7. CONCLUSÕES

- *Coptotermes gestroi* e *Cryptotermes brevis* apresentam diferentes dinâmicas de oviposição, uma vez que a espécie subterrânea apresenta uma oviposição contínua, com ausência de *lag phase*. Essa dinâmica reflete a capacidade dos reprodutores em investir energia concomitantemente na cópula/oviposição e no cuidado parental. Por outro lado, *C. brevis* apresenta um ciclo de oviposição que dura seis meses, seguido por uma *lag phase* de três meses, corroborando a dinâmica de oviposição observada em outras espécies de cupins.

- A espermateca de *C. gestroi* é dividida em regiões distal e proximal, sendo a última conectada à câmara genital por um duto espermatecal e separada do reservatório por uma bomba espermatecal. Ainda, notou-se uma variação na área dessa estrutura conforme rainhas são inseminadas e estocam espermatozoides. A espermateca de *C. brevis*, no entanto, possui forma de cabeça de seta, uma morfologia única entre os Isoptera, cujo reservatório também se conecta à câmara genital por meio de um duto;

- A secreção de proteínas e polissacarídeos na espermateca se inicia antes da cópula em ambas as espécies. No entanto, a transferência de espermatozoides pelo rei intensifica essa secreção, conforme constatado em rainhas funcionais e de diferentes idades de *C. gestroi* e *C. brevis*. Tal secreção está diretamente relacionada com a manutenção dos espermatozoides estocados;

- O epitélio da espermateca, em ambas as espécies, é composto de unidades secretoras de classe 3. Esse arranjo bicelular é responsável pela secreção de compostos, uma vez que as glândulas espermatecais estão ausentes. Características ultraestruturais, tais como retículo endoplasmático rugoso (RER) e mitocôndrias, reforçam essa síntese e transporte de substâncias, especialmente proteínas, dada a ocorrência de vesículas secretoras eletrondensas;

- As glândulas coletéricas de *C. gestroi* e *C. brevis* são divididas em unidades anterior e posterior, ambas apresentando regiões secretoras (apicais) e de transporte (basais). As duas unidades são compostas de células secretoras de classe 3, iguais às de representantes de Kalotermitidae e Termitidae, mas distintas das células secretoras de espécies basais (classe 1 modificada). Assim, nota-se uma transição das classes de células secretoras que compõem as glândulas coletéricas no ancestral de Kalotermitidae + Neoisoptera (clado composto por Stylotermitidae, Serritermitidae, Rhinotermitidae e Termitidae).

- As glândulas coletéricas das duas espécies são pouco desenvolvidas e desprovidas de atividade secretora em fêmeas aladas, justificada pela ausência de postura de ovos nesses indivíduos. Em rainhas inseminadas, as glândulas são desenvolvidas tanto do ponto de vista morfofisiológico quanto morfométrico, mas podem apresentar ciclos secretores

diferentes, conforme verificado em *C. gestroi,* onde somente a glândula posterior apresentava secreção de glicoproteínas. Em *C. brevis*, uma secreção notável foi observada em rainhas neotênicas ninfoides, cuja capacidade de postura de ovos pode ser igual a de rainhas imaginais. As glândulas coletéricas atuam na lubrificação da câmara genital, assim como exercendo função adesiva, auxiliando a adesão entre os ovos após a postura.

- A ultraestrutura das glândulas coletéricas apresentaram organelas características de um epitélio ativo na síntese de transporte de substâncias, como RER, mitocôndrias, complexo de Golgi, e vesículas secretoras de diferentes eletrondensidades. A região basal, no entanto, não apresentou tais características, nem mesmo canais receptores, mas abundância de camadas musculares, o que sugere sua atuação somente no transporte de secreção.

- As alterações morfofisiológicas e morfométricas da espermateca e glândulas coletéricas estão de acordo com a iteroparidade dos cupins, onde o casal real apresenta diferentes ciclos de reprodução durante os diferentes estágios de desenvolvimento da colônia.

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