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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS (ZOOLOGIA)

MORFOFISIOLOGIA DAS CASTAS E FORRAGEAMENTO DO CUPIM DE CERRADO *Velocitermes heteropterus* (ISOPTERA: TERMITIDAE)

IVES HAIFIG

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.

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Orientadora: Profa. Dra. Ana Maria Costa Leonardo

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Rio Claro, 15 de abril de 2013

Aos meus pais,
Antonio e Luzia,
e a minha esposa,
Sadala,
decido este trabalho

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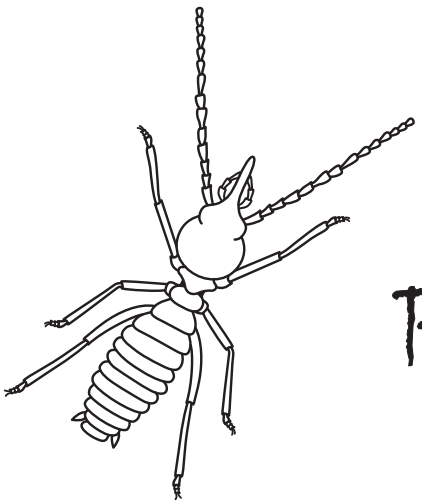
"On n'est jamais content là où l'on est"

Antoine de Saint Exupéry

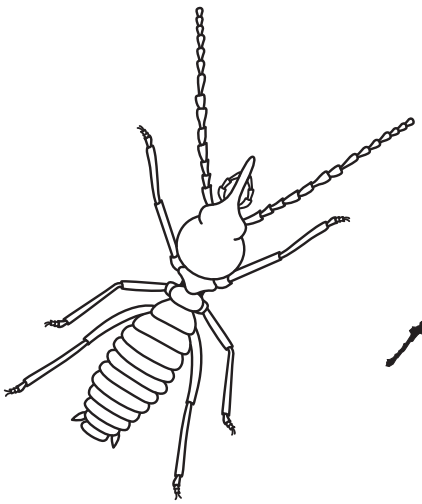


Resumo

RESUMO

Os cupins são insetos eussociais que apresentam divisão em castas entre os componentes das colônias. As castas apresentam adaptações morfofisiológicas para o melhor desempenho de determinadas funções e atividades comportamentais. A presente pesquisa objetivou analisar, do ponto morfológico, fisiológico e comportamental, as castas ápteras do cupim *Velocitermes heteropterus* que participam ativamente do forrageamento. No capítulo 1 deste volume é apresentado o estudo do polimorfismo da casta operária de *V. heteropterus* com duas subcastas, operários pequenos e grandes, baseado em caracteres morfométricos. Além disso, no capítulo 1 a rede de túneis escavadas por estes insetos foi caracterizada e as redes formadas por operários pequenos comparadas àquelas formadas por operários grandes e por grupos mistos dos dois tipos de operários. No capítulo 2 são apresentados os dados morfométricos dos outros componentes da linhagem áptera, ou seja, larvas e soldados, e também constam os dados de expressão gênica das celulases, os quais foram quantificados para ambos operários (pequenos e grandes) e soldados. Adicionalmente, no capítulo 2 é apresentado o sistema de desenvolvimento pós-embrionário da linhagem áptera de *V. heteropterus*. No capítulo 3 são apresentados dados sobre a dinâmica das trilhas de forrageamento de *V. heteropterus*. O fluxo de cupins saindo do ninho e retornando do alimento, a velocidade média individual e o número de colisões, bem como a distribuição espacial dos indivíduos nas trilhas, foram analisados durante as três primeiras horas desde o início do processo de forrageamento. A linhagem áptera de *V. heteropterus* apresenta um polimorfismo que está intimamente vinculado ao sexo e aos ínstares dos indivíduos. O polimorfismo dos componentes desta linhagem está relacionado a uma contribuição diferencial das subcastas nos processos de tunelamento e forrageamento, mas não parece influenciar a digestão de celulose, que é realizada principalmente pela casta operária.

Palavras-chave: cupim, forrageamento, Nasutitermitinae, polimorfismo, *Velocitermes*, celulases.



Abstract

ABSTRACT

Termites are eusocial insects that exhibit a division in castes among the members of the colonies. The current research had the aim to analyze, on the morphological, physiological and behavioral points of view, the apterous castes of the termite *Velocitermes heteropterus* that actively participate in foraging. The castes show morpho-physiological adaptations to develop certain functions and behavioral activities. In the chapter 1 of this volume it is presented the study of the polymorphism in the worker caste of *V. heteropterus* with two subcastes, major and minor workers, based on morphometric characters. In addition, in the chapter 1, the tunnel network excavated by these insects is characterized and the networks excavated by minor workers are compared to those excavated by major workers and mixed groups containing the two types of workers. In the chapter 2, the morphometric data on the other apterous individuals are presented, i. e., larvae and soldiers, and it is shown the cellulase gene expression, which were quantified for both workers (minors and majors) and soldiers. Additionally, in the chapter 2, the post-embryonic developmental system of the apterous line in *V. heteropterus* is presented. In the chapter 3, it is exhibit data on foraging trail dynamics of *V. heteropterus*. The flow of termites leaving the nest and returning from the food, the individual speed and the number of collisions, as well as the spatial distribution of the individuals in the trails, were analyzed for the first three hours from the beginning of the foraging process. The apterous line exhibit a polymorphism intimately related to the sex and instars of the individuals. The polymorphism of the members of this line is related to a differential contribution of the subcastes in tunneling and foraging processes, but does not seem to influence the cellulose digestion, which is mainly executed by the worker caste.

Key words: termite, foraging, Nasutitermitinae, polymorphism, *Velocitermes*, cellulases.

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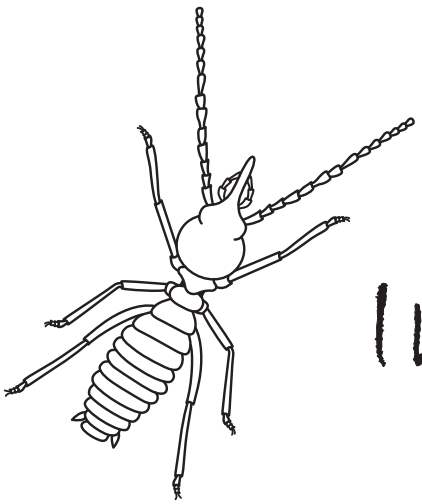


Introdução

INTRODUÇÃO

Os cupins compreendem um grupo de insetos hemimetábolos eussociais pertencente à Infraordem Isoptera (ENGEL, 2011). O nome da infraordem (do grego: “isos” = igual; “pteron” = asas) advém dos indivíduos imagos, os quais apresentam dois pares iguais de asas membranosas. Atualmente, existem 292 gêneros e 2878 espécies descritas, distribuídos em nove famílias (ENGEL et al., 2009; ENGEL, 2011; CONSTANTINO, 2013).

Estes insetos são dominantes em ambientes terrestres tropicais, em termos de biomassa e influência nas comunidades biológicas, e ocorrem desde florestas úmidas até savanas, sendo encontrados até mesmo em regiões desérticas (LEE; WOOD, 1971; PRINGLE et al., 2010). Embora sejam mais conhecidos pelo seu potencial como pragas, os cupins desempenham um papel ecológico fundamental nos ecossistemas terrestres, atuando na decomposição e reciclagem de matéria orgânica, principalmente de origem vegetal (WOOD; SANDS, 1978; NOIROT, 1982; TAYASU et al., 1997).

O sucesso evolutivo dos térmitas foi decorrente da combinação de diversos fatores, entre eles, a disponibilidade de várias fontes alimentares celulósicas, a existência de simbiose com micro-organismos no sistema digestório e a bem desenvolvida organização social, baseada no polimorfismo dos indivíduos, com castas especializadas (NOIROT, 1982; COSTA-LEONARDO, 2002).

A sociedade dos cupins é formada por castas pertencentes a duas linhagens distintas, a linhagem reprodutiva, que engloba as ninfas, os alados, o rei e a rainha, e a linhagem áptera, da qual fazem parte os operários, os soldados, e seus precursores (GRASSÉ, 1982; ROISIN, 2000).

A diferenciação de castas nos cupins ocorre quando indivíduos imaturos desviam da via hemimetabólica normal que vai do ovo ao indivíduo imago (ROISIN, 2000). Essa diferenciação ocorre por meio de modificações durante o desenvolvimento pós-embrionário, levando a produção de fenótipos alternativos, isto é, diferentes castas, e este processo é denominado polifenismo (MIURA, 2005).

Todas as castas dos cupins, com exceção dos reprodutores, são tecnicamente imaturas, pois elas retêm as glândulas protorácicas. Os operários e os soldados mantêm essas glândulas de muda mesmo quando atingem ínstares terminais (NOIROT, 1969b, 1989; NOIROT; BORDEREAU, 1991). Contudo, convencionou-se denominar de "larvas" somente os indivíduos imaturos brancos que não apresentam tecas alares ou morfologia de soldados, e que correspondem aos primeiros ínstares do desenvolvimento pós-embrionário dos cupins (ROISIN, 2000).

Em uma colônia de cupins, a casta mais numerosa é a operária. Os operários são indivíduos estéreis, ápteros e podem ser machos ou fêmeas, com ou sem simetria sexual (NOIROT, 1969a, 1982; GRASSÉ, 1982). Estes indivíduos realizam um repertório de atividades na colônia, como forrageamento, construção e reparo do ninho, alimentação das outras castas e imaturos, e cuidado com os ovos e a cria (NOIROT, 1969a, 1982). Os operários também são ativos na defesa da colônia e, em algumas espécies, possuem especializações voltadas para essa função (COSTA-LEONARDO, 2004; ŠOBOTNÍK et al., 2012). Em algumas espécies de cupins, os indivíduos que realizam as funções dos operários são conhecidos como "pseudergates", pseudo-operários ou ainda falsos operários, porque desempenham o papel de auxiliares ("helpers") nas colônias, mas podem se diferenciar em qualquer outra casta, ou seja, alados, reprodutores neotênicos ou soldados (GRASSÉ; NOIROT, 1947; THORNE, 1996; ROISIN, 2000; KORB; HARTFELDER, 2008; ROISIN; KORB, 2011).

Os soldados constituem um ínstar terminal derivado de larvas ou operários, sempre por meio de um estágio intermediário e não esclerotizado, o pré-soldado (GRASSÉ, 1982; ROISIN; KORB, 2011). Os soldados atuam primordialmente na defesa das colônias, sendo em algumas espécies equipados com poderosas mandíbulas, enquanto que em outras exibem uma cabeça bastante expandida, denominada fragmótica, que é posicionada nos orifícios do ninho para ocluir as galerias. Existem ainda espécies com soldados especializados que ejetam, através de um tubo, o conteúdo da glândula frontal, que pode ser tóxico para seus inimigos naturais (ROISIN, 2000; NOIROT; DARLINGTON, 2000; COSTA-LEONARDO, 2002; COSTA-LEONARDO; HAIFIG, 2010; ŠOBOTNÍK et al., 2010).

Reprodutores funcionais são, geralmente, o rei e a rainha, denominados reprodutores primários, advindos de indivíduos alados que perderam suas asas após o vôo nupcial. Além desses indivíduos, em uma colônia de cupins podem existir reprodutores secundários, denominados reprodutores suplementares quando os reprodutores primários ainda estão presentes, ou de substituição, se estão ausentes (GRASSÉ, 1982; MYLES, 1999; ROISIN, 2000). Os reprodutores secundários podem ser adultóides, se derivados de indivíduos alados que permaneceram no ninho natal, neotênicos ninfóides, se derivados de ninfas, ou neotênicos ergatóides, se derivados de operários (NOIROT, 1990; MYLES, 1999; ROISIN, 2000).

Polimorfismo das castas

O polimorfismo em insetos sociais já foi considerado essencialmente fenotípico, como uma expressão diferencial de um pool gênico compartilhado por todos os membros da colônia (WILSON, 1971; WEST-EBERHARD, 1987). De fato, todos os indivíduos da colônia compartilham a mesma constituição genética, exceto pelos genes que determinam o sexo (NOIROT; BORDEREAU, 1991). Neste sentido, o dimorfismo sexual dos operários, observado em muitas espécies de cupins, é um tipo de polimorfismo determinado geneticamente (NOIROT, 1969a, 1989).

Em Isoptera, as diferenças morfológicas pós-embrionárias entre os indivíduos estão geralmente relacionadas com a idade e o sexo (ROISIN, 1996). A casta operária é frequentemente polimórfica, tanto pelo dimorfismo sexual como pelo número de ínstares sucessivos pelos quais os operários passam durante o seu desenvolvimento, ou por ambos os processos (NOIROT, 1989). Já os soldados também são frequentemente polimórficos, mas diferentemente dos operários, formam um ínstar terminal e não podem mudar, embora ainda retenham a glândula protorácica (NOIROT, 1969b).

O dimorfismo sexual parece excepcional nos soldados de cupins. Das 71 espécies de térmitas para as quais o sistema de castas é conhecido atualmente, 67 espécies estudadas por diversos autores e reunidas na revisão de Bourguignon et al. (2012a), mais dados referentes a *Silvestritermes holmgreni*, analisados por Barbosa et al. (2012), *Velocitermes heteropterus* por Haifig et al.

(2012), *Microcerotermes championi* por Rasib e Akhtar (2012) e *Psammotermes hybostoma* por Bourguignon et al. (2012b), somente cinco espécies de Termitidae apresentam soldados de ambos os sexos e em uma única espécie, *Subulitermes denisae*, ocorre dimorfismo sexual (ROISIN, 1996). Estudos recentes têm avaliado e discutido possíveis hipóteses para explicar o sexo dos soldados de cupins (MATSUURA, 2006; MULLER; KORB, 2008; BOURGUIGNON et al., 2012a).

Neste sentido, atualmente ainda são aceitas as ideias aventadas por Noirot (1969a, 1985a) de que o polimorfismo dos soldados está, principalmente, relacionado a sua origem a partir de indivíduos provenientes de diversos estágios de desenvolvimento. O conceito de vários ínstares serem a base das múltiplas formas ou subcastas, ou ainda castas físicas, encontradas em uma determinada casta é denominado polimorfismo temporal (NOIROT; BORDEREAU, 1991).

No curso da evolução, a produção de soldados foi restringida a ínstares mais definidos e menos numerosos (NOIROT; BORDEREAU, 1991). Quando existe somente um ínstar produtor de soldados, estes soldados são monomórficos e esta condição é frequentemente observada nos cupins da Família Termitidae. Até mesmo quando dois ou mais ínstares podem originar soldados, um monomorfismo é obtido se estes ínstares forem de tamanhos similares (NOIROT, 1989), como é o caso de *Coptotermes lacteus* (ROISIN; LENZ, 1999), *Hodotermopsis japonica* (MIURA et al., 2000) e *Termitogeton planus* (PARMENTIER; ROISIN, 2003). O denominado polimorfismo temporal, no qual soldados de dois ou mais tamanhos são provenientes de diferentes ínstares, parece ser a regra nas espécies de Isoptera com soldados polimórficos (NOIROT, 1955, 1969a, OKOT-KOTBER, 1981; NOIROT, 1985a, 1985b, 1989; ROISIN, 1996; NEOH; LEE, 2009; HAIFIG et al., 2012).

O polimorfismo na linhagem áptera dos cupins pode estar associado à realização de diferentes tarefas durante algumas atividades desenvolvidas na colônia, como o forrageamento. Roisin (1992) levantou a hipótese de que a ocorrência de uma grande variabilidade dentro de uma casta, levando ao polimorfismo contínuo, deve representar uma adaptação ao forrageamento em ambientes heterogêneos, caso das formações vegetais que possuem solos cobertos por serapilheira.

Forrageamento de cupins

Em cupins, o forrageamento é geralmente uma atividade coletiva composta por ações individuais integradas (TRANIELLO; LEUTHOLD, 2000). O forrageamento dos cupins apresenta uma organização comportamental que muitas vezes está associada a uma divisão de trabalho entre os indivíduos que compõem a colônia (CROSLAND *et al.*, 1997, 1998). Na maioria das espécies, esta organização é sinalizada por meio de semioquímicos, principalmente pelo feromônio de trilha que é composto por diferentes substâncias, entre elas, decadienol, dodecanal, dodecenol, dodecadienol, dodecatrienol e neocembrene, que podem variar em número e quantidade nas diferentes espécies (COSTA-LEONARDO; HAIFIG, 2010; BORDEREAU; PASTEELS, 2011).

Durante o forrageamento, os cupins mostram respostas de orientação e recrutamento a substâncias produzidas por suas glândulas esternais (HALL; TRANIELLO, 1985; GESSNER; LEUTHOLD, 2001). Todas as ações relacionadas ao forrageamento dos térmitas se iniciam com a deposição do feromônio de trilha, que é secretado pela glândula esternal (STUART, 1961; COSTA-LEONARDO, 2006, 2008). Pesquisas envolvendo a comunicação por meio de feromônios de trilha em várias espécies de cupins indicam que os feromônios de recrutamento são efêmeros e os de orientação persistentes e responsáveis por diferentes fases na organização do forrageamento (TRANIELLO; LEUTHOLD, 2000). Nos Nasutitermitinae, o componente efêmero parece estar envolvido na regulação da comunicação em massa e o reforço deste sinal está diretamente conectado ao forrageamento de soldados (TRANIELLO; LEUTHOLD, 2000).

As atividades de forrageamento dependem dos hábitos alimentares das espécies de cupins, e podem ocorrer por meio de túneis subterrâneos, galerias cobertas na superfície do solo ou mesmo a céu aberto, sendo esta última ocorrência restrita a poucas espécies de cupins no mundo (MIURA; MATSUMOTO, 1998a).

As espécies de cupins subterrâneos forrageiam por meio de túneis que conectam o ninho, que encontra-se geralmente sob o solo, ao alimento (COSTA-LEONARDO, 2008). Algumas espécies, como *Macrotermes carbonarius*,

forrageiam na superfície do solo, mas a procura do alimento é inicialmente realizada por meio de túneis que se ramificam a partir do ninho (SUGIO, 1997). Os cupins que forrageiam na superfície do solo fazem longas trilhas de expedição, geralmente durante a noite, como ocorre em *Hospitalitermes* (COLLINS, 1979; MIURA; MATSUMOTO, 1998a), *Longipeditermes* (MIURA; MATSUMOTO, 1998b) e *Constrictotermes* (MOURA *et al.*, 2006). Estas trilhas podem ser gradualmente cobertas com sua contínua utilização, como ocorre em *Velocitermes* (MATHEWS, 1977) e *Nasutitermes* (TRANIELLO; BUSHER, 1985).

Traniello e Busher (1985) descreveram três fases de organização no forrageamento em *Nasutitermes costalis*. No estágio inicial, os soldados saem do ninho para explorar o ambiente e informam a outros soldados sobre os recursos alimentares encontrados. Relativamente poucos operários aparecem nas trilhas de forrageamento ou no alimento neste período (menos de uma hora da descoberta da fonte alimentar). O recrutamento de um grande número de operários caracteriza a segunda fase do forrageamento, e a razão de castas nas trilhas tende a operários. Na terceira fase, o recrutamento de soldados diminui e o recrutamento de operários pequenos e grandes aumenta. Com um aumento do fluxo bidirecional de operários, os soldados posicionam-se nas bordas ao longo da trilha. O recrutamento diferencial de soldados e operários sugere aos soldados um papel essencial na exploração (“scouts”) e comunicação da localização do alimento aos operários. Esta comunicação parece ser predominantemente química e as diferenças nas respostas comportamentais entre soldados e operários parecem estar ligadas à sensibilidade na percepção das substâncias da trilha (TRANIELLO, 1981).

Estudos com espécies de *Trinervitermes*, outro gênero de Nasutitermitinae, indicam uma organização inicial do forrageamento realizada simultaneamente por soldados e operários (TSCHINKEL; CLOSE, 1973; OLOO; LEUTHOLD, 1979). Em *Trinervitermes bettonianus*, colunas de exploração compostas por operários e soldados emergem do ninho a céu aberto, permanecendo em contato com o mesmo por meio de trilhas de feromônio. Quando o alimento é detectado, o processo de recrutamento é iniciado. Durante este processo, a informação necessária para a orientação dos demais cupins ao alimento é provida e ocorre

um aumento na frequência de saída de forrageiros potenciais do ninho (OLOO; LEUTHOLD, 1979).

Em poucas espécies, como em *Hodotermes mossambicus*, os operários tem olhos compostos funcionais e o forrageamento pode também ser orientado visualmente. A orientação química ocorre no escuro e na luz difusa, o que capacita este cupim a forragear ativamente durante o dia e a noite (LEUTHOLD et al., 1976; HEIDECKER; LEUTHOLD, 1984).

Apesar das diferentes maneiras que os cupins coletam o alimento e da variedade de materiais orgânicos que são utilizados para sua nutrição, a maioria das espécies consome alimentos derivados de celulose (LIMA; COSTA-LEONARDO, 2007). Este fator põe o grupo em destaque na natureza, visto que poucos animais são capazes de digerir celulose.

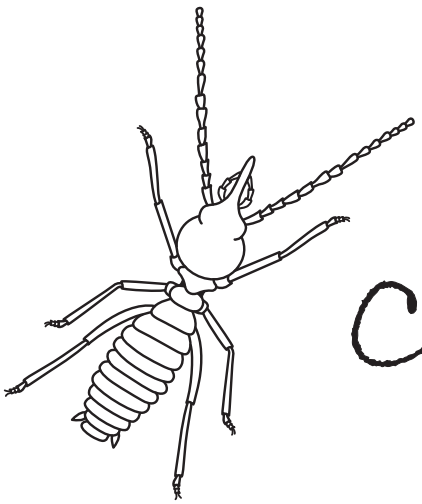
Digestão de celulose

A celulose é um polissacarídeo formado por unidades de glicose unidas por ligações β-1,4. As enzimas que quebram a celulose em glicose são conhecidas como celulases, e estão agrupadas em três principais categorias: as celobiohidrolases, as endo-β-1,4-glicanases e as β-glicosidades, sendo que somente as duas últimas ocorrem nos cupins (LO et al., 2011). As celobiohidrolases são restritas a bactérias, fungos e protistas, e atuam nas regiões terminais do polissacarídeo, sendo, por isso, denominadas exoceluloses (WATANABE; TODUKA, 2010; LO et al., 2011).

A digestão de celulose nos cupins inferiores é assistida pelos flagelados simbiontes, os quais são transferidos para outros indivíduos por meio de trofalaxia proctodeal (OHKUMA; BRUNE, 2011). Já os cupins da família Termitidae não apresentam protozoários simbiontes e a digestão de materiais celulósicos se dá por meio de atividade bacteriana e de enzimas endógenas, as quais desempenham um papel importante no metabolismo destes insetos (LO et al., 2011). As endo-β-1,4-glicanases hidrolisam a parte interior da cadeia de celulose, quebrando a ligação β-1,4 para gerar glicose (WATANABE; TOKUDA, 2010; LO et al., 2011). As β-glicosidades degradam a celobiose, que é um dissacarídeo, e outros celo-oligossacarídeos em glicose (TOKUDA et al., 2002; WATANABE; TOKUDA, 2010).

Tokuda et al. (1997) avaliaram a atividade enzimática envolvida no processo de digestão de celulose nas diferentes partes do canal alimentar do cupim *Nasutitermes takasagoensis*. Os autores encontraram uma maior atividade de β-glicosidade nas glândulas salivares do que no intestino médio, sendo o oposto observado para endo-β-1,4-glicanase, que apresentou aproximadamente 90% de atividade no intestino médio dos insetos. A digestão de celulose pode variar também de acordo com a fase de vida ou com a casta de um determinado indivíduo. Por exemplo, no início da formação da colônia, antes da emergência dos primeiros operários, o casal real apresenta níveis de expressão gênica de celulases maiores quando comparados com o período após o surgimento dos primeiros operários (SHIMADA; MAEKAWA, 2010). Os operários são responsáveis pela nutrição da colônia, sendo portanto os indivíduos com maior atividade enzimática. Uma maior expressão gênica da enzima endo-β-1,4-glicanase foi observada nos operários pequenos de *N. takasagoensis* quando comparados aos outros operários, médios e grandes, e aos soldados (FUJITA et al., 2008).

O estudo de cupins de ocorrência no Cerrado é primordial, visto que ocorrem muitas lacunas no conhecimento da biologia desses insetos, principalmente no que diz respeito à organização social do grupo. O estudo da dinâmica de forrageamento em *Velocitermes heteropterus* é importante porque nesta espécie ocorre polimorfismo da casta áptera, e a divisão de trabalho decorrente desse fenômeno é um processo pouco esclarecido em Isoptera, especialmente para as espécies neotropicais. Em vista do exposto, a presente pesquisa visou estudar aspectos da biologia do cupim *V. heteropterus* por meio da caracterização morfofisiológica das castas ápteras, e dos diferentes indivíduos componentes de uma mesma casta, que participam do forrageamento. Este trabalho objetivou compreender a divisão de trabalho entre as castas que estão envolvidas no forrageamento, bem como a dinâmica desse processo. Características morfológicas, comportamentais e genéticas expressas nos diferentes indivíduos, componentes da linhagem áptera, auxiliam no entendimento das diferentes funções desenvolvidas pelos cupins nos ambientes terrestres.



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The size of excavators within a polymorphic termite species governs tunnel topology

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Caste polymorphism in termites may provide specialization for different tasks in a colony, generating a division of labour that promotes fitness. The influence of polymorphism has been widely studied for many termite activities, although general patterns are missing in the literature. The aim of our study was to verify the influence of polymorphism on the tunnelling behaviour of termites and to test how the size of workers influences the network geometry. We hypothesized that larger workers dig fewer and longer tunnels, while smaller workers dig shorter and more ramified tunnels. We tested this hypothesis by separating groups of polymorphic workers of *Velocitermes heteropterus* according to their size and analysing the topology of their networks. The workers of *V. heteropterus* were first examined to ensure their dimorphism and then sexed to confirm their sexual polymorphism. The analysis of the tunnel networks showed that major female workers dug faster but with less bifurcations compared to minor male workers. When both types of workers were placed together, the resulting network topology was closer to that of the majors, suggesting that this class may govern the activities during tunnelling. Our results show that worker size influences the network architecture and that, in the case of *V. heteropterus*, the majors dominate both digging and the bifurcation rate.

Polymorphism in termite workers may result from two phenomena, which have been well characterized by Noirot & Bordereau (1991). The first, termed temporal polymorphism, considers the successive instars of workers and the ontogenetic differences between them. This type of polymorphism is often quite inconspicuous because the intermoult growth may be small or absent. The second, known as sexual dimorphism, takes into account the sex of the individuals, with two well-defined classes of workers: males and females.

After the extensive work by Noirot (1955) on the characterization of the apterous line (comprising larvae, workers and soldiers) in Termitidae, many researchers have investigated the relationship between polymorphism and the division of labour for several behavioural activities, such as trail laying (Pasteels 1965), queen attendance (McMahan et al. 1984), food processing (Badertscher et al. 1983; Hinze et al. 2002) and foraging (McMahan et al. 1984; Miura & Matsumoto 1995, 1998; Miura et al. 1998).

Division of labour in surface gallery construction of *Nasutitermes costalis* has also been studied (Jones 1980). This particular behaviour was chosen because it is easier to replicate and measure than other behaviours. Tunnelling behaviour presents the same qualities for studying division of labour, although it remains hitherto little explored in this context. Division of labour in tunnelling has been studied for the termites *Reticulitermes fukienensis* (Crosland & Traniello 1997) and *Coptotermes formosanus* (Yang et al. 2009; Bardunias et al. 2010). In Crosland & Traniello (1997), differences in tunnelling behaviour were associated with the size of the individuals, which varied according to age. This is in agreement with the findings of Yang et al. (2009), who demonstrated that worker age affects tunnelling, with older individuals showing a higher probability than young individuals to become active excavators. In addition, Campora & Grace (2004) demonstrated that larger workers excavated fewer and longer tunnels and that the smaller workers excavated more bifurcated networks. This pattern of tunnelling suggests that the size of the workers influences the geometry of the tunnel network in termites. The number of workers may also influence the behaviour of the group and modify the final work. Bardunias & Su (2010) showed that the densities of termites modify the width of the tunnels, with higher densities excavating wider tunnels.

The Neotropical genus *Velocitermes* (Holmgren 1912) belongs to the family Termitidae and comprises nasute termite species, which

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present dimorphic workers and dimorphic or trimorphic soldiers. These species are known for grass foraging, and their pyramidal nests are spread throughout the Brazilian Cerrado (Mathews 1977). Roisin (1996) showed that worker dimorphism in *Velocitermes barrocoloradensis* corresponds to a sexual dimorphism, with major female workers and minor male workers. Owing to their polymorphic workers, we considered the genus *Velocitermes* to be a good model to study different behavioural responses within the castes.

Here we show that there is a different behavioural work product between the polymorphic workers of *Velocitermes heteropterus*. We first confirm the presence of distinct major and minor workers as previously described for other *Velocitermes* species, and clarify the relationship between sex and class. We then associate the size classes with different workloads performed during tunnelling behaviour and discuss how the size of workers affects different characteristics of the tunnel network architecture.

METHODS

Termites

Nests of *V. heteropterus* were collected in Santa Rita do Passa Quatro, SP, Brazil (21°42′S, 47°28′W), and carried to the laboratory, where they were placed in chambers containing moistened sterilized sand. Nests were maintained at 25 °C, under a 12:12 h light:dark cycle. The termites received water and were fed dried grass, which was collected from the same location as the nests, both provided ad libitum. The termite workers were extracted from the nests the same day that they were used for experiments, and the nests were kept in the laboratory no longer than 30 days. A total of eight nests were used in this study.

Polymorphism and Tunnelling Behaviour

Morphometry

Samples of 100 workers of *V. heteropterus* from three nests (totalling 300 workers) were collected at random and fixed in FAA (formaldehyde:acetic acid:ethanol, 1:1:3) for 24 h before being transferred to 70% ethanol. The workers were observed under a Zeiss Stemi SV6 stereomicroscope connected to a Motic-CAM camera. Motic Images Plus 2.0 software (Motic, Richmond, BC) was used to analyse the obtained images and measure the maximum width of the head and the length of the hind tibia for each worker. In addition, the number of antennal segments that were fully developed in each animal was recorded. We performed principal component analysis (PCA) to evaluate changes in morphological features among the workers. The sex of each physical caste was determined according to Noirot (1955).

Experimental set-up

Tunnelling behaviour was studied in the laboratory using two-dimensional arenas, which were placed horizontally. The arenas consisted of three layers: the top and the bottom layers were composed of two glass plates ($40 \times 40 \times 0.4$ cm), separated from each other by four glass laminas ($40 \times 5 \times 0.2$ cm) placed between the outer margins, and the middle 0.2 cm layer was filled with 200 ml of sifted sand (0.2 cm sieve) moistened with 34 ml of deionized water (saturation humidity of 50%). The perimeters of the experimental arenas were held together with binder clips. One access hole was drilled in the centre of the top layer, where a petri dish of 9 cm in diameter was attached through a 0.5 cm piece of plastic tube (0.5 cm in diameter). The termite workers were released in the petri dish. We tested two densities of workers, 90 and 180, which were equivalent to one and two termites per 10 cm^2, respectively. Three distinct groups of workers were used: (1) major workers; (2) minor workers; (3) mixed workers (50% major, 50% minor), totalling six experimental conditions. Each experiment was repeated 10 times. Digital photographs were taken every 24 h for 5 days. All of the experiments were conducted inside a climatic chamber set at 25 °C, in the absence of light, except when images were recorded.

Image Analysis and Modelling

The daily network topology was extracted from the images manually as a series of connected straight segments using custom-made Clicca software (programmed by Andrea Perna). Data were analysed using the R software package (version 2.12.1, The R Foundation for Statistical Computing, Vienna, Austria, 2011). The variables used to compare the network dynamics between groups were the total network length, the tunnel width, the network area (the network length multiplied by the mean tunnel width) and the branching rate (the number of bifurcations divided by the length of the network). In addition, we computed branching rates without the tunnels at the external margins of the set-up (1 cm from the borders) to avoid a possible boundary effect. A new branch was defined when the ratio of its length over its width exceeded one. Active branches (defined as tunnel ends that progressed during the previous 24 h) were counted each day to verify whether there was an effect of the experimental condition on the percentage of active branches. We used the lme function in the lme4 library to fit the linear mixed-effects model. We defined the densities and the groups of workers as fixed effects, and the time (24, 48, 72, 96 and 120 h), the replications (10 for each experimental condition), the number of days that termites were kept in the laboratory and the colonies as random effects. Moreover, we counted the primary tunnels arising from the entry point for each replication and analysed their number with a generalized linear model (GLM).

We also used survival analysis to characterize the tunnel distance between bifurcations and to compare the bifurcation patterns between the three conditions in the final networks (after 120 h). We used the survreg function in the survival library to estimate the rate (probability per unit of distance) at which workers started a new bifurcation, either for all interbifurcation distances of a group (when estimating the group's global bifurcation rate) or only for interbifurcation distances of a single replication (to compare rates between groups). Bifurcation nodes with higher orders (defined here as the number of bifurcations, including the starting one, along the shortest path from the node to the exit) were more likely to be along the arena border. Because the border may influence the branching rate, we restricted branching rate analysis to nodes with an order less than or equal to six (the highest node order for which we found no effect of node order on interbifurcation distance). To detect the effect of node order on interbifurcation distance in the final network architecture we used the mixed-effects Cox proportional hazards model in the coxme library. The effects for groups were analysed using ANOVA ($\alpha = 0.05$), and multiple comparisons were performed using the Tukey HSD test.

When necessary, we used a logarithmic transformation to achieve either homoscedasticity or normality of the data and performed a model simplification procedure to estimate the *P* values of the interaction terms (Crawley 2007).

RESULTS

Velocitermes heteropterus workers were separated into two classes: major and minor workers (Fig. 1). The two classes corresponded to the sexual dimorphism, with major workers being female and minor workers being male. The first principal component was head width, which explained 91.31% of the total variation. Mean ± SE

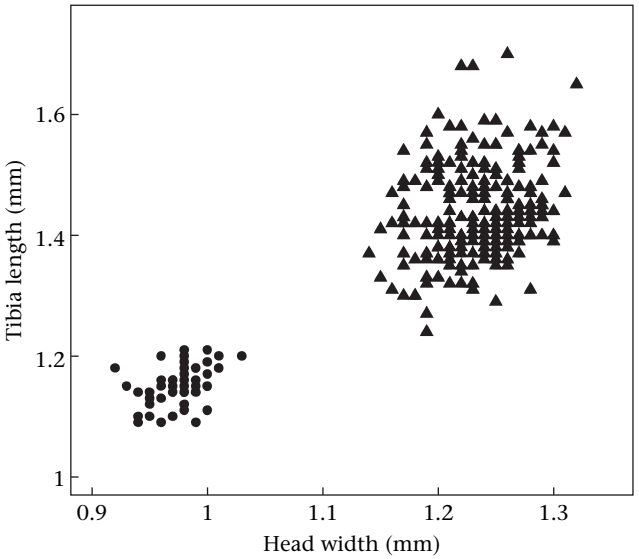

Figure 1. Size dimorphism in termite workers of *Velocitermes heteropterus* based on head width and tibia length. Solid triangles: majors; solid circles: minors.

head width was 1.23 ± 0.001 mm for major workers ($N = 251$) and 0.97 ± 0.002 mm for minor workers ($N = 49$). Mean $\pm$ SE tibia size was 1.44 ± 0.002 mm for major workers ($N = 251$) and 1.14 ± 0.002 mm for minor workers ($N = 49$). Number of antennal segments was 15 for both workers, but in approximately 12% of the individuals these structures were damaged. The minor workers were scarcer in the colonies, accounting for only 49 (16.33%) of the 300 randomly chosen workers analysed, with the other 251 being major workers (83.66%).

Our analysis revealed that there was a significant difference in network lengths between groups (mixed-effect model: $F_{2,116} = 108.47$, $P < 0.001$) and densities ($F_{1,116} = 71.43$, $P < 0.001$) of workers. The minor workers alone excavated less than the major workers. The mixed group had intermediate values between the majors and the minors (Fig. 2a, b). Similar results were found for network areas, which differed significantly between groups (mixed-effect model: $F_{2,116} = 43.45$, $P < 0.001$) and densities ($F_{1,116} = 45.09$, $P < 0.001$) of workers. The tunnel width analysis showed that the minor workers excavated significantly narrower tunnels than the majors and the mixed group (ANOVA: $F_{2,54} = 16.33$, $P < 0.001$) and that the density of workers significantly influenced the width of the tunnels, with wider tunnels at higher densities (ANOVA: $F_{1,54} = 17.41$, $P < 0.001$). Mean $\pm$ SE tunnel width of minor workers was 0.39 ± 0.01 cm at lower densities ($N = 454$) and 0.43 ± 0.009 cm at higher densities ($N = 565$), while that of major workers was 0.45 ± 0.01 cm ($N = 396$) and 0.51 ± 0.01 cm ($N = 438$), respectively. Given their head width, the termites could easily have crossed each other within the tunnels.

The global branching rates (Fig. 2c) also showed a highly significant effect between groups (mixed-effect model: $F_{2,117} = 50.49$, $P < 0.001$). There was no effect of density (mixed-effect model:

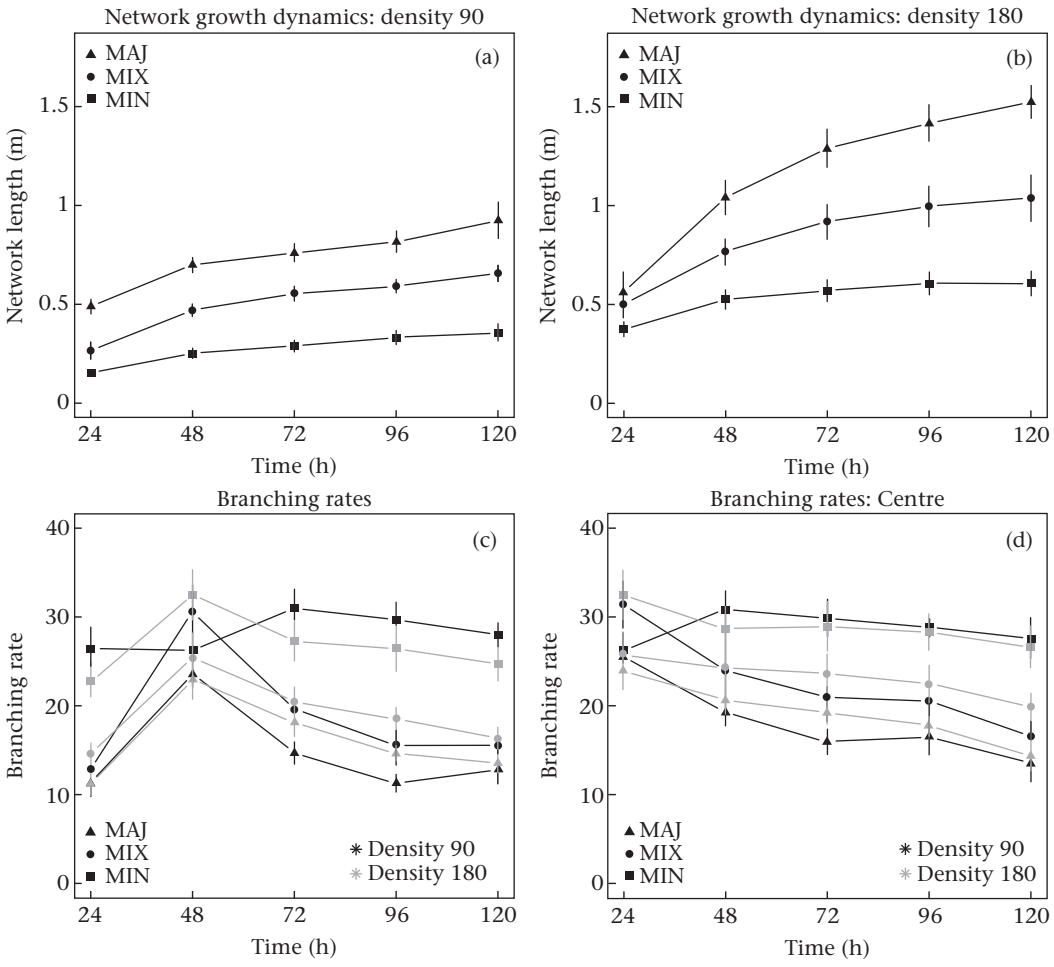

Figure 2. Dynamics of termite networks during the 5 days of the experiment. Mean $\pm$ SE length of networks at worker densities of (a) 90 and (b) 180. Branching rates of workers calculated from (c) the complete network and (d) the centre of the arena. MAJ: majors; MIN: minors; MIX: mixed group.

$F_{1,116} = 0.042$, $P = 0.8374$), however, indicating that the presence of one or two termites per 10 cm^2 did not influence branching rates. Minor workers showed the highest branching rates, followed by the mixed group and the majors. When we excluded the border effect of the networks (i.e. when we removed the tunnels dug around the arena perimeters and considered only the central tunnels to compute branching rates), we obtained similar results (Fig. 2d): there was a highly significant difference between groups of workers (mixed-effect model: $F_{2,117} = 32.06$, $P < 0.001$), but no effect across densities ($F_{1,116} = 0.3427$, $P = 0.5594$). The number of inactive branches (no growth during the previous 24 h) was less than 20% at all times, for all densities and groups of workers. The number of primary tunnels departing from the entry point did not differ significantly between densities (GLM: $P = 0.31$), but showed a worker group effect ($P = 0.044$), with minors excavating more primary tunnels (mean $\pm$ SE $= 2.55 \pm 0.28$, $N = 20$) than majors (1.8 ± 0.24, $N = 20$) and mixed groups (1.85 ± 0.31, $N = 20$).

The survival curve of the interbifurcation distances for each group fitted an exponential distribution (Fig. 3). The simplest model explaining such an exponential distribution is that, while tunnels are being dug, there is a constant probability per unit distance (which we will call a 'rate', although rate usually refers to probability per unit time) to start a new tunnel. The mean $\pm$ SE rate to start a new bifurcation, estimated from the interbifurcation distances in the final network architecture of each replication, significantly differed between groups of workers (minor workers: 56.09 ± 0.054 bifurcations/m, $N = 182$; major workers: 24.09 ± 0.07 bifurcations/m, $N = 206$; mixed group: 33.95 ± 0.05 bifurcations/m, $N = 160$; ANOVA: $F_{2,27} = 19.142$, $P < 0.001$). The minor workers bifurcated significantly more than the major workers (Tukey HSD: $P < 0.001$) and the mixed group ($P = 0.001$), but no difference was detected between the mixed and the major groups ($P = 0.125$).

DISCUSSION

Workers of *V. heteropterus* showed sexual polymorphism, with two classes: male minors and female majors. These results are consistent with the findings of Roisin (1996) for *V. barrocoloradensis* and confirm the sexual size pattern for workers in the genus *Velocitermes*.

Our results revealed a highly significant effect of worker group on tunnelling behaviour. The major workers dug more extensive networks than the minors. In contrast, the minor workers showed a greater branching rate than the majors, suggesting that this class of workers is connected with the ramification of the network. When both workers tunnelled together, the network length and the branching rate showed intermediate values between those found for the majors and the minors. Although the rate of starting a new bifurcation was higher for the mixed group, it did not differ significantly from that of the majors. Also, mean tunnel width of the mixed group was similar to that of the major group but significantly larger than that of the minor group. These results indicate that the majors may govern the tunnelling activities when both classes are together, excavating more extensive tunnels and therefore decreasing the branching rates compared to the networks of minors alone. This result supports the ideas of Crosland & Traniello (1997) and Campora & Grace (2004) that larger workers dig fewer and longer tunnels while smaller workers dig shorter and more ramified tunnels. In the species studied by these authors, the polymorphism of the workers is temporal and the difference between minors and majors depends on worker age. We observed the same pattern of tunnelling, but with worker polymorphism based on the sex of the individuals. The correlation between worker size and tunnel network patterns seems to be general for termites, independent of the basis of the polymorphism. Larger workers have larger mouthparts, enabling them to carry more soil particles, and they have larger legs, enabling them to move at higher speed, making it possible to excavate longer tunnels and explore a larger area. Smaller workers have smaller mouthparts and legs, and with a lower digging speed they have a higher probability to initiate a new tunnel, ramify the network and better explore a smaller area.

Yang et al. (2009) observed that only a small percentage of foragers participate in tunnel excavation. Later, Bardunias et al. (2010) showed that the contribution of the individuals involved in the tunnelling activity was not homogenous (polyethism). Similar mechanisms may explain our results concerning the mixed-group networks, which were much more similar to the majors' networks than to the minors' networks. In *V. heteropterus*, minor workers may excavate tunnels less often than major workers in natural settings if they are responsible for more of the intranidal activities, as is the case in *Odontotermes distans* (McMahan et al. 1984). In the presence of majors, minors may contribute less to tunnelling activity, so that networks of the mixed group more closely resemble those of the majors. In the absence of majors, the minors actively excavate tunnels and this fact may be explained by a behavioural plasticity, a phenomenon that is common in insect societies and that has already been observed in termite tunnelling (Crosland & Traniello 1997; Evans 2006). The study of Crosland & Traniello (1997) showed that when majors were experimentally removed from the arenas, the subsequent instars started to tunnel.

The social setting of the termites is an important external factor because tunnelling behaviour is not the result of a single individual's activity, but the effort of all nestmates together. In the laboratory, we tested two densities of termites for each group of workers. Despite the significant differences observed for total network lengths and areas, which are in accordance with the relationship between number of termites and volume of tunnels described by Su & Lee (2009) and Bardunias & Su (2010), there was no difference between densities for the branching rate of each group of workers. Moreover, our results showed a significant effect of density on tunnel width, with tunnels being wider at higher densities. These results are in accordance with the queue size effect on termite tunnel width studied by Bardunias & Su (2010).

The process that controls the branching rates along tunnels (the probability per unit distance) is important because it determines the complexity of the tunnel network structure (Bardunias & Su 2009). Interbifurcation distances of the networks from the different groups of workers fitted an exponential distribution, indicating that the branching rate of each group was constant per unit distance (Haccou & Meelis 1992). Initiation of a bifurcation in the network occurs randomly but at a constant rate (probability per unit distance), which means that bifurcation rate is independent of the distance from the last bifurcation. These behavioural processes can be modelled as Markov processes (Haccou & Meelis 1992) and follow a property called 'lack of memory' (Shimizu 1979). This property states that the survival of a determined individual is not affected by its age, and that the individual has the same probability of dying independent of its age. In the present case, it indicates that, regardless of the distance to the last bifurcation, the termites will always have the same probability of initiating a new bifurcation at each step.

In the present study, we found that sexual dimorphism of *V. heteropterus* workers was associated with different tunnelling networks. We confirmed the hypothesis that larger workers tend to excavate more extensive tunnels, whereas smaller workers tend to excavate more bifurcated networks. Moreover, our results show that the tunnelling behaviour of the termite *V. heteropterus* has a random bifurcation pattern that may emerge from simple probabilistic rules that follow the 'lack of memory' property. Future studies focusing on the direct observation of the individual digging behaviour would

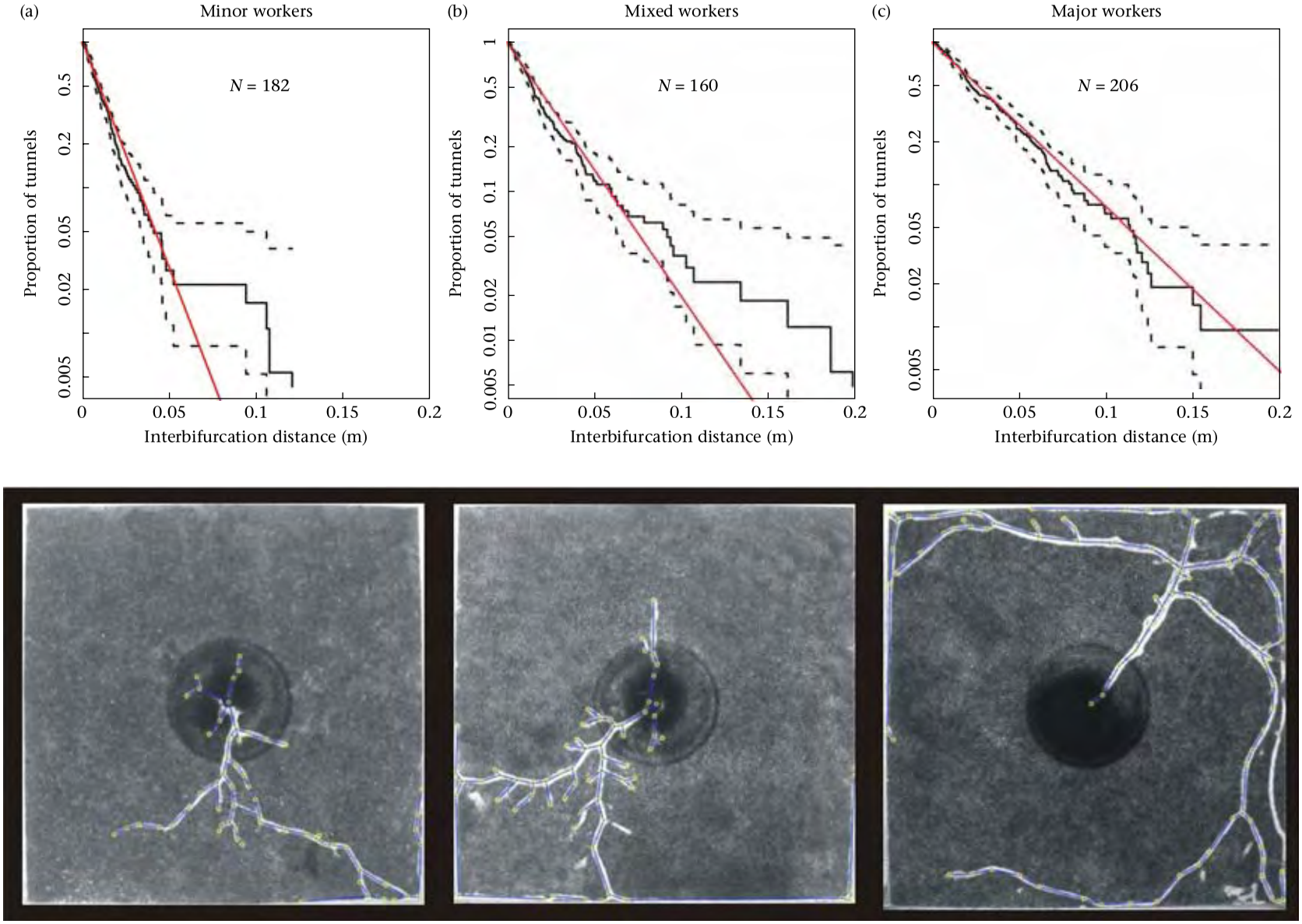

Figure 3. Survival curves of the interbifurcation distances, the fitted exponential distribution (in red) and an example of the final network topology for (a) minor workers, (b) the mixed group and (c) major workers. The exponential model was fitted using the survreg function in the R program (maximum likelihood estimation) after the minimum observed distance was subtracted from each distance to obtain a less biased estimate.

clarify whether there is a division of labour among workers during this behaviour.

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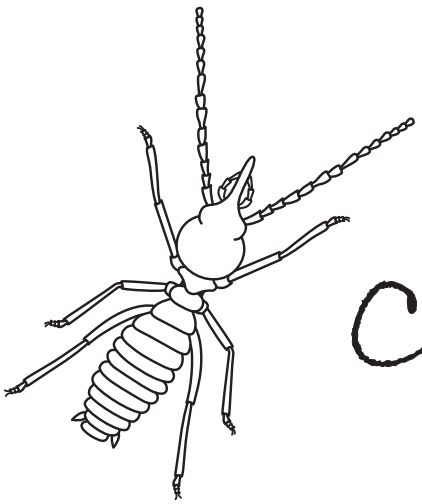

Capítulo 2

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On the Apterous Line of the Termite *Velocitermes heteropterus* (Isoptera: Termitidae): Developmental Pathways and Cellulose Digestion

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Termites are social insects with an extraordinary ability to digest cellulose. Termite societies are structured into castes, and patterns of postembryonic development vary between different termite species. The apterous line may exhibit polymorphism ("physical castes"), in which workers are dimorphic and soldiers can be either dimorphic or trimorphic. We examined the occurrence of polymorphism in the apterous line of *Velocitermes heteropterus* and determined the developmental pathways for this termite species. We also investigated the expression of the cellulase genes encoding β-glucosidase and endo-β-1,4-glucanase among the castes to determine whether there is a difference in digestion and, consequently, a possible division of labor with respect to this activity among the worker castes. The apterous line of *V. heteropterus* presents individuals of both sexes with two larval instars. The female larvae become major workers, and the male larvae become minor workers and soldiers. The expression of β-glucosidase was similar within the castes, but the expression of endo-β-1,4-glucanase was higher in workers than in soldiers. No significant differences were found between minor and major workers. These results suggest that there is no division of labor between the minors and majors with regard to cellulose digestion, with both workers contributing similarly to this process.

Key words: castes, β-glucosidase, endo-β-1,4-glucanase, polymorphism, real-time PCR, transcript

INTRODUCTION

Termites are one of the dominant insects in terrestrial habitats, where they play an essential role as primary consumers and decomposers due to their extraordinary ability to digest cellulose (Lee and Wood, 1971; Tayasu et al., 1997). Termites also form structured societies that are organized to optimize the energy obtained from their environment.

The individuals in a termite society are divided into castes and distributed into the alate-nymphal and the apterous lines, which are separated early and irreversibly during postembryonic development in species of the family Termitidae (Noirot, 1955; Roisin and Korb, 2010). These castes may vary in size according to the molting events and to the sex of the individuals, and these differences within a caste are commonly referred to as physical castes. Age and sex are the two principal characteristics responsible for the polymorphism observed among termites (Noirot and Bordereau, 1991; Roisin, 2000).

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The patterns of caste development have been studied extensively for the family Termitidae, from the pioneering studies of Noirot (1955, 1969) over the past few decades (Okot-Kotber, 1981; Noirot, 1985; Roisin, 1992; Roisin, 1996; Miura et al., 1998) to more recent studies (Hojo et al., 2004; Neoh and Lee, 2009; Moura et al., 2011). In the apterous line, the workers are generally the most abundant caste. According to Grassé (1986), workers are the mainstay of the colony. These individuals are responsible for nearly all activities including providing nutrition for the colony. The workers forage and collect food and may even digest it prior to feeding the larvae, soldiers, and the royal couple. A recent study showed that when workers are present, the levels of cellulase gene expression decrease in reproductives (Shimada and Maekawa, 2010), which highlights the importance of workers in food processing.

Efficient cellulose digestion in many termites appears to require both endogenous and intestinal microbial cellulases, but the contribution of endogenous cellulases to digestion in species of the family Termitidae, where cellulolytic flagellates are absent, is greater than for species of other families (Lo et al., 2010). There are three main types of cellulases, cellobiohydrolases, endo-β-1,4-glucanases, and β-glucosidases, but termites produce only the latter two (Lo et al.,

2010). Endo-β-1,4-glucanases hydrolyse the interior part of the cellulose chain, cleaving the beta (1–4) linkage to generate glucose (Lo et al., 2010; Watanabe and Tokuda, 2010). β-glucosidases degrade cellobiose and other cello-oligosaccharides to glucose (Tokuda et al., 2002).

Fujita et al. (2008) analyzed the gene expression level of the cellulase endo-β-1,4-glucanase for different castes of the termite *Nasutitermes takasagoensis* and found that minor workers express higher levels of the cellulase gene and are more involved in cellulose digestion.

Velocitermes heteropterus is a neotropical termite species of the family Termitidae that occurs in the Brazilian Cerrado. This termite forages open-air in dried grass at night, but the trails are covered with frequent use (Mathews, 1977). It has been suggested that workers of the genus *Velocitermes* are dimorphic, while soldiers can be dimorphic or trimorphic, depending on the species (Mathews, 1977; Roisin, 1996; Haifig et al., 2011). In this study, we examine polymorphism and the developmental pathways of the apterous line of the termite *V. heteropterus*, and investigate the relative expression levels of the endogenous cellulases endo-β-1,4-glucanase and β-glucosidase within the castes.

MATERIALS AND METHODS

Termites

Nests of *Velocitermes heteropterus* were collected in Santa Rita do Passa Quatro, SP, Brazil (21°42′S, 47°28′W). The nests were carried to the laboratory where they were placed in chambers containing moistened sterilized sand. The termites were fed with dried grass collected from the same location as the nests. Sample termites were taken from the nests and either fixed in FAA (formaldehyde: acetic acid: ethanol, 1:1:3) for morphometric analyses and sex identification or kept at –80°C for RNA extractions. A total of seven nests were used in this study.

Developmental pathways

Morphometry: Samples of 100 soldiers and 100 larvae of *V. heteropterus* were collected at random from three different nests (totaling 300 individuals of each type) and fixed in FAA for 24 h before being transferred to 70% ethanol. The individuals were observed under a Zeiss Stemi SV6 stereomicroscope connected to a Motic-CAM camera. Motic Images Plus 2.0 software was used to analyze the images and to perform measurements. For each larva, the maximum width of the head and the length of the hind tibia were measured, following the classical study of Noirot (1955) on Termitidae species. The separation of larvae into groups was performed by a correlation of these two measurements, and the differences among the resulting groups were analyzed using one-way ANOVA ($\alpha = 0.05$), considering each variable separately, and multiple comparisons were performed using the Tukey HSD test. For each soldier, thirteen measurements were extracted in order to classify the soldiers into groups, following Koshikawa et al. (2002, 2004) and Moura et al. (2011), with some modifications. The *Velocitermes* soldiers seem to present temporal polymorphism (Noirot and Bordereau, 1991), and required a more detailed morphometric analysis. The extracted measures were: 1) head length, including the nasus (HL); 2) maximum head width (HW1); 3) head width at the base of the mandibles (HW2); 4) maximum head height, excluding the postmentum (HH); 5) pronotum length (PnL); 6) pronotum width (PnW); 7) mesonotum width (MsW); 8) mesonotum length (MsL); 9) metanotum width (MtW); 10) metanotum length (MtL); 11) tibia length (TL); 12) femur length (FL); 13) femur width (FW). We did not measure the labrum width (LW) or the mandible length (ML) as did Koshikawa et al. (2002, 2004) and Moura et al. (2011), as *V. heteropterus* is a nasute-soldier species and their mouthparts are vestigial. In place of these two missing measurements, however, we did measure the width of the mesonotum and the width of metanotum. The differences among soldiers were analyzed using principal component analysis (PCA), considering the thirteen variables. The significances on the PCA-resulting groups were analyzed using one-way ANOVA ($\alpha = 0.05$), considering the scores of the first principal component, and multiple comparisons were performed using the Tukey HSD test. Data were analyzed using the R software package (version 2.14.0, The R Foundation for Statistical Computing, Vienna, Austria, 2011). Additionally, the number of antennal segments that were fully developed in each insect was recorded for both left and right antennae. The worker caste was previously analyzed and classified into minor and major workers (see Haifig et al., 2011).

Sex Identification: Sex identification was carried out according to the method described by Noirot (1955). The abdomens of 30 soldiers and 30 larvae were cut across the frontal plane. For each individual, the dorsal half of the abdomen and the gut were removed. The specimens were stained with alcoholic haematoxylin for 45–60 min and then rinsed in hydrochloric alcohol (0.5% HCl in 80% ethanol). Females were characterized by the rudiments of oviducts at the rear margin of sternite VII, rudiments of spermatheca on sternite VIII and vestigial colleterial glands on sternite IX. Males were characterized by spermiducts, which reached a vestigial structure corresponding to the seminal vesicles and ejaculatory duct, on the posterior margin of sternite IX.

Molting individuals: Individuals in molt provided evidence of the caste differentiation pathways as the cuticle of the mandibles and the antennae of the next instar are visible within the older mandibles and antennae from the previous instar. Therefore, the mandibles of 30 molting workers (15 minors and 15 majors) and the antennae of 30 molting larvae were removed and analyzed under a Zeiss microscope. In addition, to compare the presoldier mandible with that found beneath the mandible of the minor worker molting to a presoldier, we extracted a mandible of a presoldier molting to a soldier and stained with methylene blue, as these individuals are unsclerotized, therefore presenting no contrast in a simple preparation.

Differential cellulase expression

RNA extraction and cDNA synthesis: Total RNA was isolated from minor and major workers and soldiers of *V. heteropterus* using a QIAGEN RNAeasy Mini Kit according to the manufacturer's instructions. Aliquots of approximately 1 μg of total RNA were treated with 1 U DNaseI (Invitrogen Corp., Rockville, MD, USA) for 30 min at 65°C and subsequently with EDTA for 10 min at 65°C to stop the reaction. The treated RNA was reverse transcribed utilizing SuperScript® II Reverse Transcriptase (Invitrogen, Corp., Carlsbad, CA, USA), primer Oligo dT [5′-GGCGGCCGCACAACTTTGTAA-CAAGAAAGTTGGGT$(T)_{19}$-3′].

DNA sequencing: cDNAs were amplified using degenerate primers designed from sequences available on Genbank in PCR reactions. The products were sequenced using an ABI 3500 Genetic Analyzer (Applied Biosystems). The cDNA sequences of *V. heteropterus* genes for β-glucosidase, endo-β-1,4-glucanase, α-tubulin and β-actin were deposited in GenBank's dbEst database (Accession numbers JK265129, JK265130, JK815761, JK815762).

Real-time PCR - relative gene expression: The cDNA samples from the bodies of minor and major workers and soldiers were quantified using a Nanodrop spectrophotometer (ND-1000; Nanodrop Technologies Inc., Wilmington, DE, USA). Synthetic oligonucleotide primers were designed to amplify cDNA for the two genes encoding the cellulases β-glucosidase and endo-β-1,4-glucanase (Primer Express TM, Applied Biosystems, Foster City, CA, USA). The α-tubulin and β-actin genes were used to normalize variations of cDNA concentrations. Gene expression analyses were performed with the geNorm software (Vandesompele et al., 2002). The

primer sequences and concentrations are shown in Table 1. All samples were assayed in a 12-μl volume containing 60 ng cDNA (3 μl), 6 μl SYBR Green Master Mix PCR (Applied Biosystems) and 3 μl specific primers in a MicroAmp Optical 96-well reaction plate (Applied Biosystems) using the Step-One-Plus Real-Time PCR System (Applied Biosystems). Two replicates of each preparation were run on the plate, and the data presented are the mean values. Ten preparations were performed for each class of worker. The results are expressed as the mRNA levels of each gene studied, normalized according to α-tubulin and β-actin expressions. The differences among the castes were analyzed using an ANOVA ($\alpha = 0.05$), and Tukey HSD when necessary. Data were analyzed using R software package (version 2.14.0, The R Foundation for Statistical Computing, Vienna, Austria, 2011).

RESULTS

Developmental pathways

Larvae: The apterous line of *V. heteropterus* follows the ontogeny described for nearly all species of the family Termitidae, with two distinct larval instars, L1 and L2 (with a notable exception being the Macrotermitinae, which undergo three larval instars). The correlation of the variables showed three distinct groups of larvae, which were significant different among them considering the head width (ANOVA: $F_{2,297} = 11141$, $P < 0.001$) and the hind tibia length (ANOVA: $F_{2,297} = 11574$, $P < 0.001$). The L1 larvae were composed of only one group with males and females, undistinguishable in size. The L1 head width varied between 0.45–0.50 mm and the tibia length between 0.32–0.39 mm. All the individuals presented 12 antennal segments. The L2 larvae were composed of two distinct groups that followed the sexual division. The smaller L2 were males (hereafter ML2), and the larger L2 were females (hereafter FL2). The head width of the ML2 varied between 0.66–0.72 mm and the tibia length between 0.64–0.73 mm. The head width of the FL2 varied between 0.81–0.88 mm and the tibia length between 0.78–0.89 mm (Fig. 1A). All L2 presented 14 antennal segments.

Table 1. Primer sequences and concentrations used for the real-time PCR. Orientation: F = forward, R = reverse.

Primers	Sequence 5′-3′	Concentration (nM)
Endo-β-1,4-glucanase F	ATCAGAAAGTCACGTGGAGGAAG	70
Endo-β-1,4-glucanase R	TCACCAGCGTCATAGTATCCTCC	70
β-glucosidase F	GCTCGCATCCTGCCTGAAG	70
β-glucosidase R	CGTTGTCTAGAAGCGCGTTTAT	70
α-tubulin F	CCCTCTCCTTCGCCTTCAA	150
α-tubulin R	GAGGATCTCGCTGCTCTGGA	150
β-actin F	GGACAGTGTTGCGCGTAGAGA	150
β-actin R	CTTGGGTATGGAATCCTGCG	150

Soldiers: The soldiers of *V. heteropterus* were divided into three classes, minor, intermediate and major soldiers. Two measured individuals did not fit in any group (Fig. 1B). The first principal component (PC1) was responsible for 74.20% of the separation among the groups and was positively correlated with all the thirteen variables. The second principal component (PC2) was responsible for 5.95% of the separation among the groups and was positively correlated with the variables HW2, PnL, PnW, MsL, MtW, TL, FL, and FW and negatively correlated with HL, HW1, HH, MsW, and MtL. The variables TL and FL had the greatest influence on PC1, whereas PC2 was most strongly influenced by HW2, PnW, and MtW. The graph result from the PCA analysis in Fig. 1B showed three groups of soldiers that were significantly different among each other considering the PC1 values (ANOVA: $F_{1,2} = 265.79$, $P < 0.001$). The major soldier group, composed of only three individuals, was a rare physical caste, corresponding to 1% of the total sampled individuals. All soldiers presented 14 antennal segments.

Molting individuals: The analysis of the worker mandibles showed that the major workers always molt into another major worker, while minor workers can molt either into a presoldier, with a cushion-like mandible within the mandible (Fig. 2A), or another minor worker, in which the formation of the next instar teeth is visible (Fig. 2B). The Fig. 2C shows a mandible of a presoldier molting to a soldier.

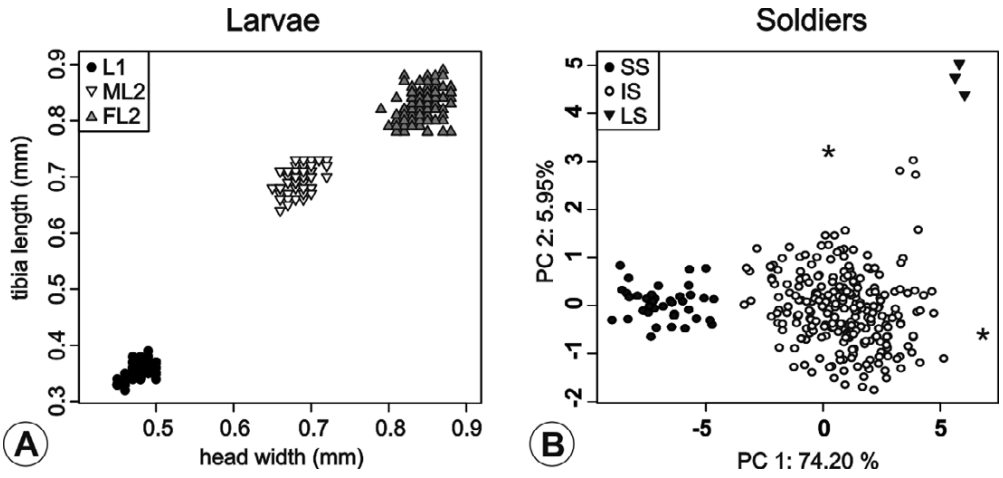

Fig. 1. Polymorphism in the apterous line of *V. heteropterus*. **(A)** Head width versus tibia length is shown for larvae, with a group first larval instar (L1) composed of males and females, and two groups of second larval instars, one composed of males (ML2) and the other of females (FL2). **(B)** Projection of the first and second principal components showing the polymorphism of soldiers, with minor (SS), intermediate (IS) and major (LS) individuals. The two single points marked with * were not considered to the analysis.

The third segment of the antennae of molting L1 showed a division into three new segments (Fig. 2D). These data support the finding that the first larval instar presents 12 well-defined segments, while the second larval instar presents 14 segments.

The data concerning the size and the sex of the apterous individuals provided here and from the study of Haifig et al. (2011), in addition to the mandible analysis of molting individuals, allowed us to schematize the developmental pathways of the apterous line in *V. heteropterus* (Fig. 3). The apterous line exhibited a sexual division, which promoted the sexual dimorphism of the L2 and the workers as well as a temporal division of the individuals by instar, which influenced the number of physical castes of soldiers. The workers presented at least two instars, but they did not differ in size.

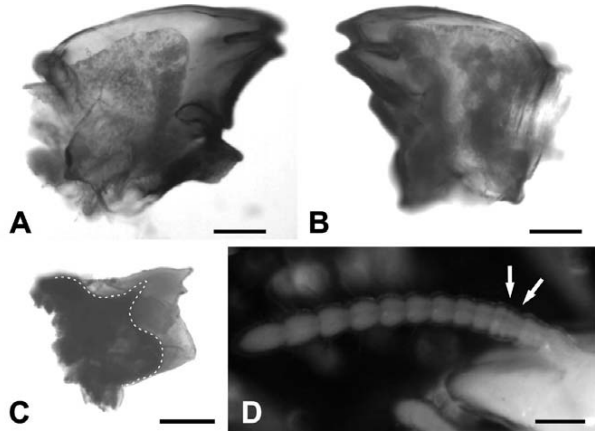

Fig. 2. Evidence from the molting individuals. Mandibles of a minor worker molting to a presoldier **(A)** and to another worker **(B)**. **(C)** Mandible of a presoldier molting to a soldier, evidenced by a white dashed line. **(D)** Antenna of a first larval instar molting to the second instar. Note the division of the third segment into three (white arrows). Scale bars = 0.1 mm.

Differential cellulase expression

We investigated the relative expression levels of two genes encoding the endogenous cellulases β-glucosidase and endo-β-1,4-glucanase. Significant differences were found among the castes for endo-β-1,4-glucanase (ANOVA: $F_{2,27} = 12.041$, $P = 0.0001$), with majors expressing around 33 times and minor expressing around 18 times more than soldiers. The expression was higher for major workers, but did not differ statistically when compared to minors (Tukey HSD: $P = 0.082$). No significant differences were found among the castes for β-glucosidase gene expression (ANOVA: $F_{2,27} = 0.1357$, $P = 0.8737$). The relative expression comparison is shown in Fig. 4.

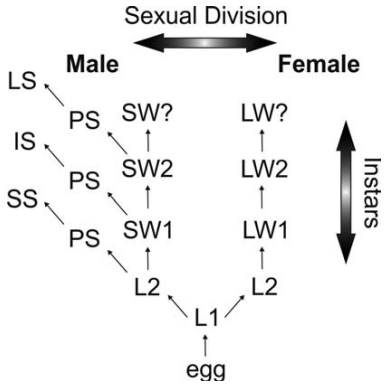

Fig. 3. Developmental pathways of the apterous line in *V. heteropterus* based on morphometric analysis and molting evidences. The male and female L1, which are undistinguishable in size, hatch from the egg. The L2 male molts to a minor worker (SW1) or to a presoldier (PS). The SW1 molts either to another minor worker (SW2) or to a PS. The PS from the L2 molts to minor soldier (SS), the PS from SW1 molts to intermediate soldier (IS) and the PS from SW2 molts to major soldier (LS). The L2 female molts to a major worker (LW1). The evidence from the molting individuals shows that there are even two instars of minor workers (SW1 and SW2) and two of major workers (LW1 and LW2), and it remains unknown whether development continues (SW? and LW?).

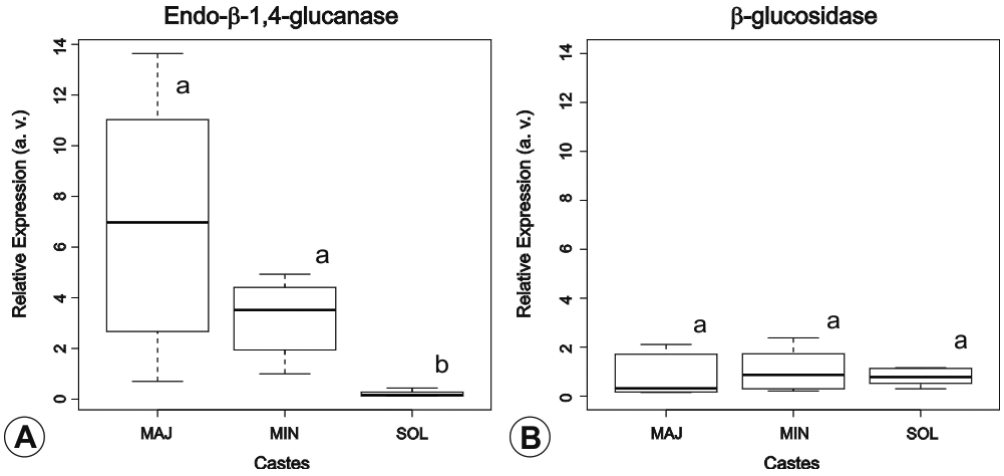

Fig. 4. Relative expression of the genes encoding the enzymes endo-β-1,4-glucanase **(A)** and β-glucosidase **(B)** in *V. heteropterus* castes. Columns marked with different letters represent statistical differences ($P < 0.05$). a.v. = arbitrary values, MAJ = majors, MIN = minors, SOL = soldiers.

DISCUSSION

In *V. heteropterus*, male and female larvae emerge from eggs and continue onto a second larval instar, in which sexes are distinguishable by the size of their heads. The female line originates major workers, while the male line originates both minor workers and soldiers. The developmental pathways of the apterous line in *V. heteropterus* are similar to that of *V. barrocoloradensis* as described by Roisin (1996), with only males differentiating into soldiers. One difference is that in *V. heteropterus* we found two or more instars of minor workers, with little or no intermolt growth. These individuals give rise to the soldier caste, which is polymorphic and composed of minor, intermediate, and major soldiers. The minor soldiers likely originate from the ML2, while the intermediate soldiers, the most abundant form found in the colonies, derive from the first instar of minor workers. The major soldiers may derive from the next instar of minor workers (SW2), or possibly successive instars (SW?). These results are consistent with the suggestion by Noirot (1969, 1985) that soldier polymorphism is related to their origins from individuals of different developmental instars.

Samples of soldiers from *V. heteropterus* were previously measured by Mathews (1977) and Fontes (1998). Both studies suggested the presence of three types of soldiers. However, the measurements of the individuals were quite different between the studies. The minor soldier described by Mathews (1977) had a head length of 1.57 mm and was based on a single specimen, while the minor soldiers described by Fontes (1998) had a head length of 1.26 mm. Our results showed minor soldiers with a head length varying between 1.39 and 1.54 mm, which differs from the earlier data. These individuals are more conspicuous when we observe a sample. The challenge of identifying the soldier caste in *V. heteropterus* increases when we consider the larger soldiers, which were separated into two groups, intermediates and majors, by Mathews (1977) and by Fontes (1998), with different measurements for the groups in both studies. Our data showed that the differences among the soldiers are result from a multivariate analysis, and it is not possible to identify the soldier physical castes considering only a single or few variables. The PnW and MtW seem to be the best variables to distinguish intermediates from majors, but not intermediates from minors. These variations among individuals in addition to the two individuals sampled did not fit in any of the groups continue the discussion started by Roisin (1996) concerning the actual number of soldier morphs in species of *Velocitermes*, which needs to be addressed.

Roisin (1992) hypothesized that high intra-caste variability, leading to continuous polymorphism in some termites such as *Rhynchotermes*, may represent an adaptation to foraging in the heterogeneous environment of the litter-covered forest floor. The genus *Velocitermes* is known for open-air grass foraging (Mathews, 1977), and the high variance in the size of soldiers in *V. heteropterus* shown in the present study may be a consequence of this adaptation, as inferred by Roisin (1996) for *V. barrocoloradensis*. Additionally, Matsuura (2006) proposed that sexual differences in sensitivity to hormones and pheromones may explain the skewed sex ratio in soldier differentiation. In the case of *V. heteropterus*, the soldiers are all male and originate from larvae and minor workers, which differ in morphology from the major workers. However, the differences between male and female workers in *V. heteropterus* may include not only sexual and morphological differences, but behavioral variations as well (Haifig et al., 2011).

Termites initiate the digestion of cellulose material with a mechanical degradation of the substrate using their mandibles. This small processed material allows for rapid breakdown using digestive enzymes. The endogenous enzymes of *V. heteropterus* play an important role in food digestion because this termite species does not harbor cellulolytic flagellates in its gut. The apterous of *V. heteropterus* presented similar amounts of β-glucosidase. However, the expression of the gene encoding the endo-β-1,4-glucanase transcript, which is produced in abundance in termites (Tokuda et al., 1997; Watanabe et al., 1998), differed significantly among the castes, with higher levels for workers compared with the soldiers.

Fujita et al. (2008) compared the relative levels of endo-β-1,4-glucanase expression within the workers of *Nasutitermes takasagoensis* and found that majors presented lower levels when compared with minor and medium workers. These authors also analyzed enzymatic activity and found significantly higher activity in minors. They suggested that these differences in the cellulase expression and activity might contribute to a division of labor among the workers, with minor workers having a central role in cellulose digestion. In the case of *V. heteropterus*, the major workers seem to play an important role in the digestion of food, dominate tunneling (Haifig et al., 2011) and possibly other foraging activities, while the minor workers remain in the nest and may represent transitory individuals, acting as a soldier reserve.

In this study, we presented the developmental pathway of the apterous line in *V. heteropterus*, revealing specific differences when compared with another species of the genus *Velocitermes*, *V. barrocoloradensis* (Roisin, 1996). The concept of various instars as the basis for multiple morphs found in one caste is referred to as temporal polymorphism (Noirot and Bordereau, 1991), and appears to be the case for the soldiers of *V. heteropterus*, a finding supported by Roisin's foraging hypothesis. Additionally, the results of cellulase expression in the castes do not support a system of cellulose-digestion division of labor between the worker castes, which differs from previous studies on foraging behavior of this species (Haifig et al., 2011). Further investigations should clarify the relation between polymorphism and the division of labor in *V. heteropterus*.

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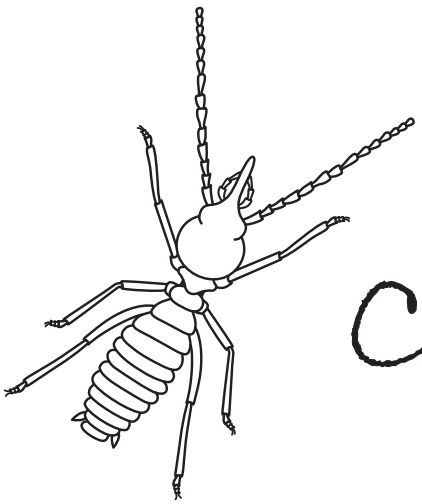

Capítulo 3

DINÂMICA DAS TRILHAS DE FORRAGEAMENTO NO CUPIM NEOTROPICAL *Velocitermes heteropterus* (ISOPTERA: TERMITIDAE)

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RESUMO

A busca do alimento em algumas espécies de cupins ocorre por meio de uma trilha que conecta o ninho ao alimento. Poucas espécies de cupins no mundo forrageiam a céu aberto, e a dinâmica de forrageamento foi estudada em apenas algumas delas. O presente estudo objetivou estudar a dinâmica das trilhas de forrageamento no cupim *Velocitermes heteropterus*, que forrageia a céu aberto, visando responder as seguintes questões: Quais os indivíduos responsáveis pela exploração da nova área? A atividade de forrageamento apresenta diferentes fases? Existe uma taxa constante entre as castas durante o forrageamento? A velocidade dos cupins varia conforme a fase do forrageamento? Os cupins apresentam uma organização espacial nas trilhas de forrageamento? Para tanto, as expedições de forrageamento foram filmadas sob condições laboratoriais e os vídeos resultantes foram analisados manualmente e automaticamente com auxílio de um programa desenvolvido na plataforma Matlab®. Os dados foram analisados estatisticamente por meio de um modelo de efeitos mistos e por ANOVA. As trilhas de forrageamento foram compostas por operários pequenos, operários grandes e soldados. O fluxo de indivíduos aumentou gradativamente desde o início da exploração da nova área até o encontro do alimento, e um recrutamento massivo de indivíduos ocorreu nos primeiros 40 minutos a partir da exploração da nova área. A razão de castas foi constante durante todo o processo de forrageamento, sendo encontrados oito operários grandes para cada operário pequeno e soldado (8:1:1). A velocidade individual foi significativamente diferente em relação às castas, sendo os operários grandes e os soldados mais rápidos do que os operários pequenos. A trilha de forrageamento foi fracionada em 9 raias, de acordo com o fluxo de indivíduos, o qual ocorreu preferencialmente na raia central da trilha, tanto para os indivíduos que deixavam o ninho como para os indivíduos que retornavam da fonte alimentar. O processo de forrageamento de *V. heteropterus* é dinâmico e apresenta três fases principais, caracterizadas pelo fluxo diferencial e velocidade dos indivíduos.

Palavras-chave: Castas, polimorfismo, exploração, recrutamento

INTRODUÇÃO

As atividades de forrageamento são dependentes dos hábitos alimentares das diferentes espécies de cupins, e podem ocorrer por meio de túneis subterrâneos, galerias cobertas na superfície do solo ou mesmo a céu aberto, sendo esta última ocorrência restrita a poucas espécies de cupins no mundo (MIURA; MATSUMOTO, 1998a). O forrageamento a céu aberto facilita o estudo da dinâmica deste processo, uma vez que todas as etapas envolvidas podem ser visualizadas, desde a exploração da área para a busca de fontes alimentares até o recrutamento dos indivíduos após a escolha do recurso.

As trilhas de forrageamento podem apresentar uma organização espacial que facilita o fluxo de indivíduos que estão saindo e retornando ao ninho (FOURCASSIÉ et al., 2010). Os cupins que estão retornando ao ninho parecem ter uma tendência em utilizar o centro da trilha, enquanto os indivíduos que estão saindo do ninho utilizam as laterais. Esta padrão foi observado em diferentes espécies de cupins nasutos que forrageiam a céu aberto, como *Hospitalitermes medioflavus* (MIURA; MATSUMOTO, 1998a) e *Longipeditermes longipes* (MIURA; MATSUMOTO, 1998b). Contudo, os graus de organização espacial podem variar de acordo com as espécies, como observado em diferentes espécies de formiga do gênero *Atta* (BURD *et al.*, 2002; DUSSUTOUR, 2004).

Velocitermes heteropterus é uma espécie de cupim que ocorre no Cerrado e apresenta ninhos piramidais (MATHEWS, 1977; COLES DE NEGRET; REDFORD, 1982). Os indivíduos ápteros desta espécie são polimórficos, com soldados machos de três tamanhos distintos, pequenos, médios e grandes, e operários de dois sexos, sendo os pequenos machos e os grandes fêmeas (HAIFIG *et al.*, 2011, 2012). Os operários grandes parecem predominar em atividades extranidais como o tunelamento (HAIFIG *et al.*, 2011) e contribuem, junto aos pequenos, para os processos de digestão de celulose (HAIFIG *et al.*, 2012). As atividades de forrageamento desta espécie ocorrem durante a noite e a céu aberto, sendo que suas trilhas são cobertas gradativamente com a utilização contínua (MATHEWS, 1977).

A dinâmica das castas nas trilhas de forrageamento dos cupins sugere uma divisão de trabalho e uma organização entre os indivíduos no processo

comportamental. Em vista disso, o presente estudo objetivou entender a organização e a dinâmica dos cupins nas trilhas formadas durante o processo de forrageamento de *V. heteropterus*. Para tanto, foram respondidas as seguintes questões: Quais os indivíduos responsáveis pela exploração da nova área? A atividade de forrageamento apresenta diferentes fases? Existe uma taxa constante entre as castas durante o forrageamento? A velocidade dos cupins varia conforme a fase do forrageamento? Os cupins apresentam uma organização espacial nas trilhas de forrageamento?

MATERIAL E MÉTODOS

Cupins

Ninhos do cupim *Velocitermes heteropterus* Silvestri foram coletados na cidade de Santa Rita do Passa Quatro, SP, Brasil (21˚42'S, 47˚28'O), e levados ao laboratório, onde foram dispostos em recipientes plásticos contendo areia esterilizada e umedecida. Os ninhos foram mantidos a 25˚C, sob um ciclo de 12:12 h luz:escuro. Os cupins receberam água *ad libitum*, e o alimento foi fornecido durante os experimentos, sempre após um período de três dias em jejum. Um total de cinco ninhos foram utilizados neste estudo.

Forrageamento

O forrageamento de *V. heteropterus* foi analisado em laboratório sob as seguintes condições experimentais. Uma ponte contínua de 40 cm de comprimento e 2,5 cm de largura foi disposta entre o recipiente contento o ninho e o recipiente contendo o alimento. Esta ponte foi feita com papelão mousse e vedada com fita plástica para evitar o consumo pelos cupins. A ponte foi suspensa por duas colunas de madeira de 35 cm cada, uma disposta no recipiente do ninho e outra disposta no recipiente que apresentava a fonte alimentar (Fig. 1). O recipiente do alimento continha folhas secas de capim *Brachiaria* e uma placa de Petri de 9 cm de diâmetro com vermiculita umedecida. No total, foram realizados 5 experimentos, todos com diferentes ninhos.

As trilhas de forrageamento foram filmadas com uma câmera Sony® (modelo DCR-TRV120) na função nightshot. A área filmada da ponte foi de

aproximadamente 6,5 cm. Após o primeiro cupim atravessar a parte visível da ponte (área filmada), os indivíduos foram contados durante intervalos de 3 minutos, a cada 10 minutos, por 3 horas. Os cupins foram discriminados em operários, pequeno e grande, e soldados, seguindo a classificação proposta por Haifig *et al.* (2011, 2012). Além do número de indivíduos, o tempo de cada indivíduo para atravessar a área filmada e o número de encontros com outros indivíduos foram determinados. Estes dados permitiram calcular a velocidade média de cada indivíduo em diferentes etapas do forrageamento. A velocidade e o número de encontros foram determinados para 1076 indivíduos. Adicionalmente, a trilha de forrageamento foi fracionada em 9 raias e o número e o sentido dos cupins que passaram por cada uma delas foi registrado. A trajetória dos cupins que atravessaram a ponte no sentido do alimento foi marcada de vermelho, enquanto a trajetória dos indivíduos no sentido oposto (alimento -> ninho) foi marcada de verde. As imagens obtidas foram importantes para visualizar a dinâmica das trilhas de forrageamento de *V. heteropterus*. Estes últimos registros foram realizados com o auxílio de um programa específico desenvolvido na plataforma Matlab®.

Análises e Modelagem

Os dados foram analisados estatisticamente com auxílio do programa R, versão 2.14.0 (The R Foundation for Statistical Computing, 2011). Os dados de velocidade individual foram analisados por meio de um modelo com efeitos mistos (MEM) utilizando a função *lme* do programa R. As variáveis fixas utilizadas foram a velocidade individual (transformada por raíz quadrada para atingir homocedasticidade), as castas, o tempo, o tempo ao quadrado (efeito quadrático) e o número de encontros com outros cupins. As variáveis aleatórias utilizadas foram as unidades experimentais e o tempo de forrageamento. Os dados referentes ao fluxo de cupins em cada raia foram analisados estatisticamente por meio de uma ANOVA: dois critérios, sendo o primeiro critério a raia e o segundo o tempo.

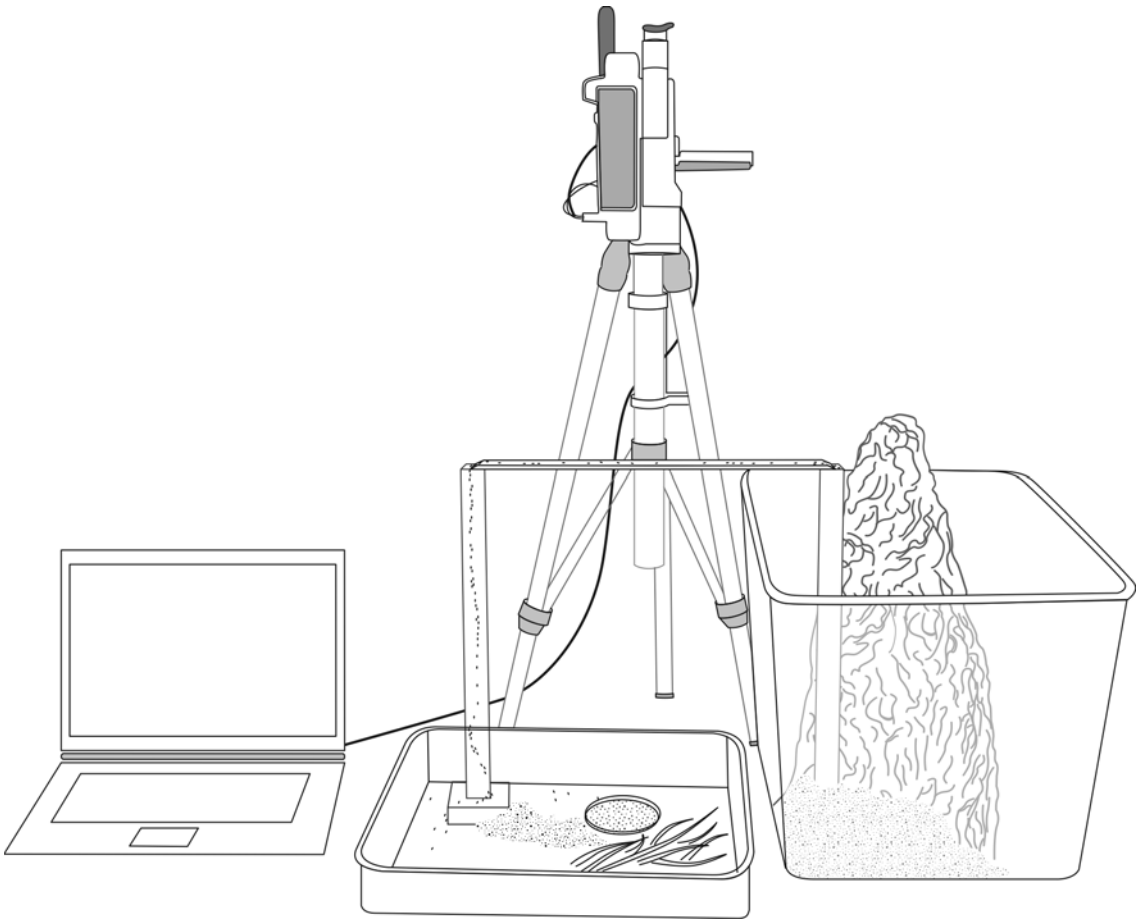

Figura 1. Representação esquemática do delineamento utilizado nos experimentos.

RESULTADOS

As trilhas de forrageamento são iniciadas com a exploração da área por um grupo de cupins forrageiros. Ambos operários (pequenos e grandes) e soldados caminham para frente e retornam repetidas vezes, com uma velocidade média inicial de aproximadamente 3,5 cm/min. No início da exploração, os operários e soldados orientam-se por uma trilha previamente marcada por um ou mais indivíduos antecessores, os quais ultrapassam pouco a pouco a delimitação antiga, marcando a nova área e retornando ao grupo. Como pode ser observado na figura 2, os forrageiros de *V. heteropterus* não utilizam toda a área disponível da ponte para a procura do alimento. Os primeiros indivíduos que escolhem o caminho são seguidos pelos demais indivíduos. Uma vez que a trilha é estabelecida, os cupins utilizam uma faixa de aproximadamente 1 cm de largura para o fluxo de indivíduos em ambos os sentidos (ninho-alimento e alimento-ninho).

O fluxo de cupins durante o forrageamento variou de acordo com o tempo. O início do forrageamento foi caracterizado por um grande número de cupins que saem do ninho em direção a fonte alimentar e por alguns indivíduos que retornam ao mesmo. Após aproximadamente 40 minutos, esta situação se inverte e ocorre um decréscimo no número de indivíduos que deixam o ninho, além do aumento de cupins que retornam da fonte alimentar (Fig. 3). Entre 60 e 80 minutos o fluxo de cupins saindo e retornando para o ninho é similar, e após este período, há mais indivíduos no sentido alimento-ninho.

A composição das castas manteve-se estável durante todo o processo de forrageamento, sendo observada uma razão constante de oito operários grandes para cada operário pequeno e soldado (8:1:1) nas diferentes fases, tanto para os indivíduos que deixavam o ninho como para os indivíduos que retornavam da fonte alimentar (Fig. 4).

A velocidade individual variou significativamente de acordo com as castas ($F = 21{,}489$; $P < 0{,}0001$). Os operários grandes e os soldados foram significativamente mais rápidos do que os operários pequenos (MEM, $P < 0{,}001$). Contudo, não houve diferença significativa de velocidade entre operários grandes e soldados (MEM, $P = 0{,}715$). A velocidade individual dos cupins foi crescente

nos primeiros 25 minutos de experimento, atingindo uma assíntota após esse período (Fig. 5). A leve diminuição na velocidade, observada após os 30 minutos, não foi significativa. O modelo sugerido para descrever a variação da velocidade consta da fórmula abaixo:

$$\textbf{Velocidade} = a_0 + a_1 \,.\, \textbf{Tempo} + a_2 \,.\, \textbf{Tempo}^2$$

Para cada casta, o modelo foi definido como:

$\textbf{Velocidade}_{\text{op. grande}} = 0.9061733 + 0.0247129 \,.\, \textbf{Tempo} - 0.0004539 \,.\, \textbf{Tempo}^2$
$\textbf{Velocidade}_{\text{op. pequeno}} = 0.8391828 + 0.0247129 \,.\, \textbf{Tempo} - 0.0004539 \,.\, \textbf{Tempo}^2$
$\textbf{Velocidade}_{\text{soldado}} = 0.9026671 + 0.0247129 \,.\, \textbf{Tempo} - 0.0004539 \,.\, \textbf{Tempo}^2$

As trilhas de forrageamento foram fracionadas em nove raias, de acordo com o fluxo de indivíduos, o qual variou significativamente entre as raias durante os intervalos analisados, tanto para os cupins que saíram do ninho (Raia: $P < 0,001$, $F = 47,54$; Tempo: $P < 0,001$, $F = 2,93$) como para os que retornaram da fonte alimentar (Raia: $P < 0,001$, $F = 51,77$; Tempo: $P < 0,001$, $F = 3,06$). A raia central, onde a marcação química deve ser mais forte em razão do número de indivíduos que passaram e contribuíram com a deposição feromonal, foi preferencialmente utilizada pelos indivíduos saindo e retornando ao ninho, com aproximadamente 50% dos indivíduos em ambos os sentidos (Fig. 6). Contudo, o maior fluxo de cupins retornando da fonte alimentar ocorreu a partir dos 70 minutos, o que permitiu uma menor taxa de colisões durante o processo (Fig. 2).

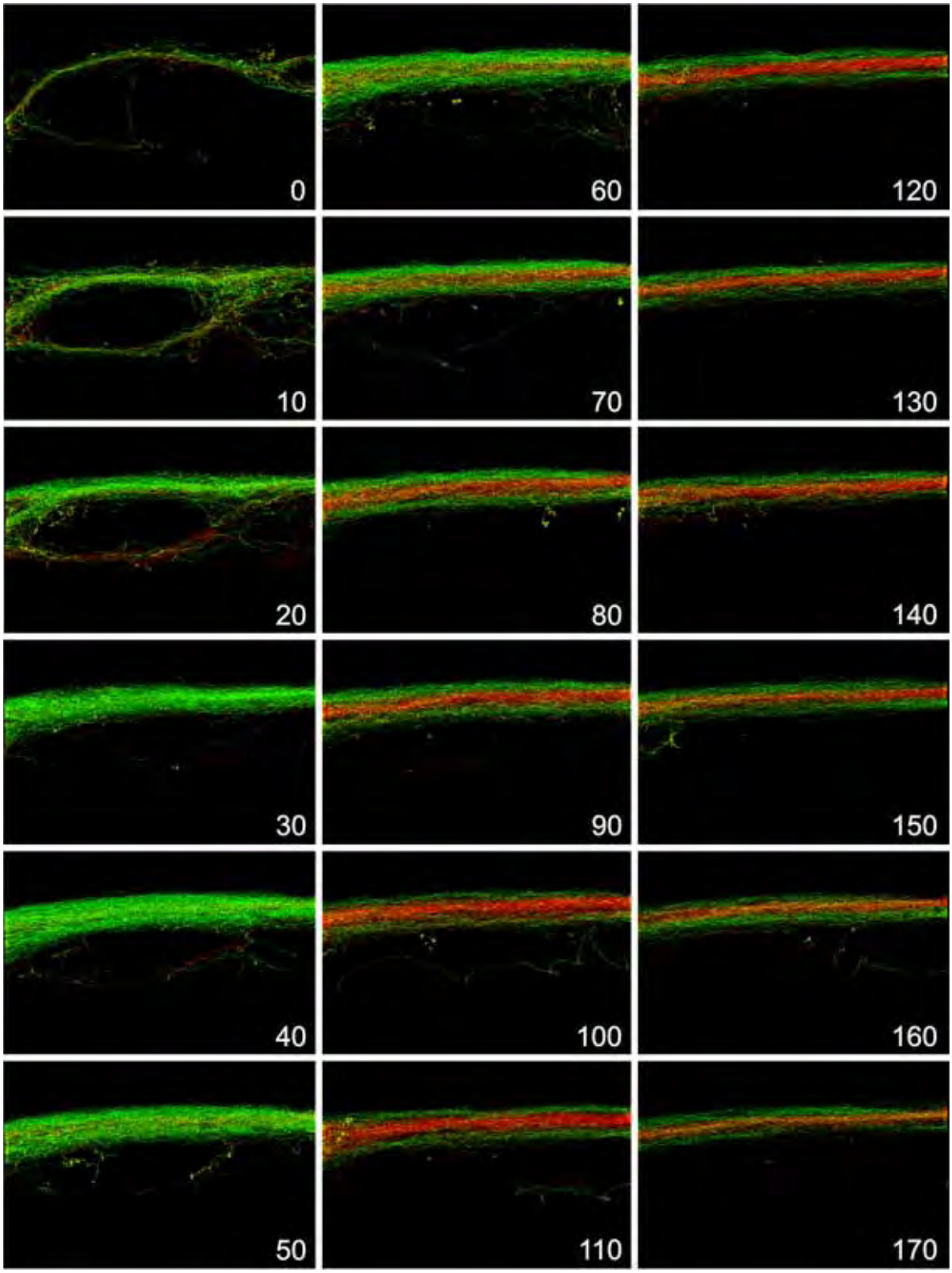

Figura 2. Fluxo de cupins nas trilhas de forrageamento nos diferentes intervalos de tempo, de 0-3 até 170-173 minutos. As trajetórias dos cupins no sentido ninho-alimento estão marcadas em verde e as do sentido oposto (alimento-ninho) estão marcadas em vermelho.

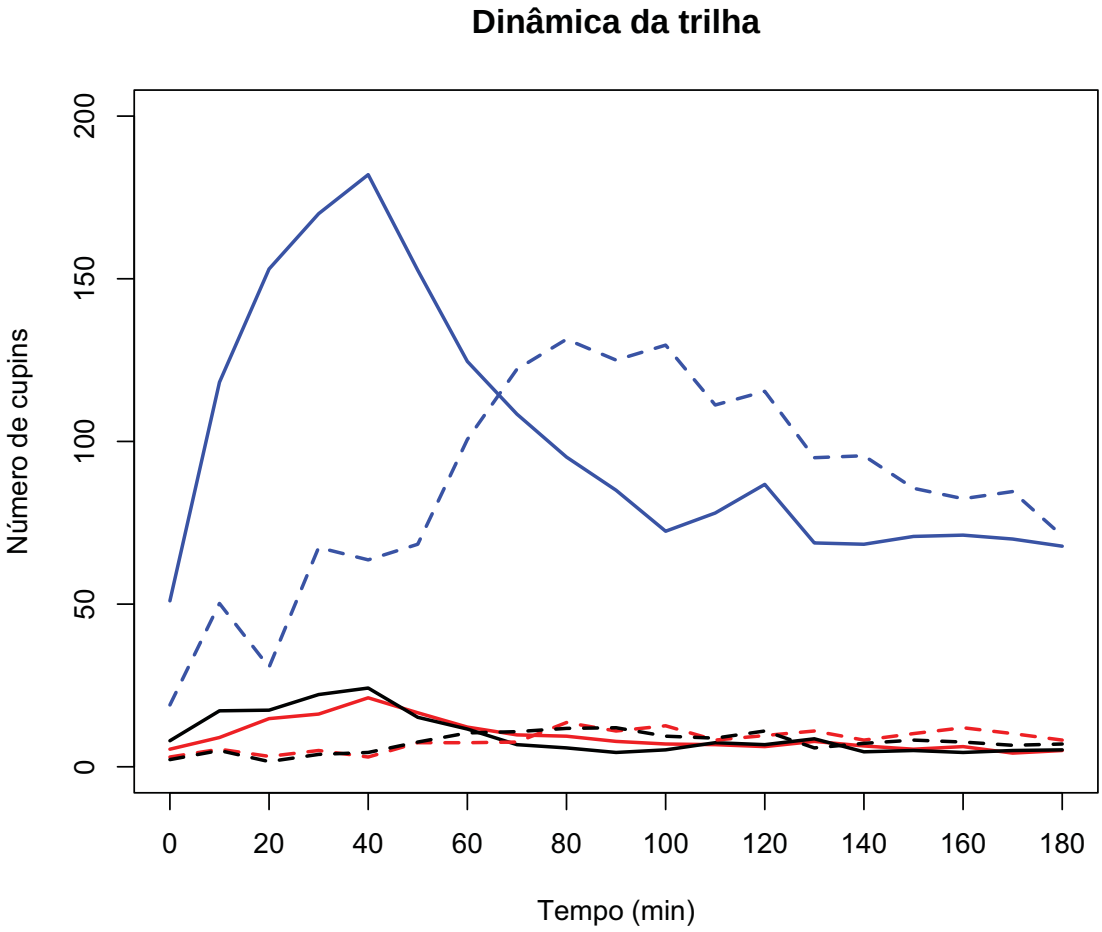

Figura 3. Fluxo de cupins nas trilhas de forrageamento. As linhas contínuas correspondem aos indivíduos que estão saindo do ninho em direção a fonte alimentar e as linhas tracejadas correspondem aos indivíduos trafegando no sentido oposto. Azul = operários grandes, vermelho = operários pequenos, preto = soldados.

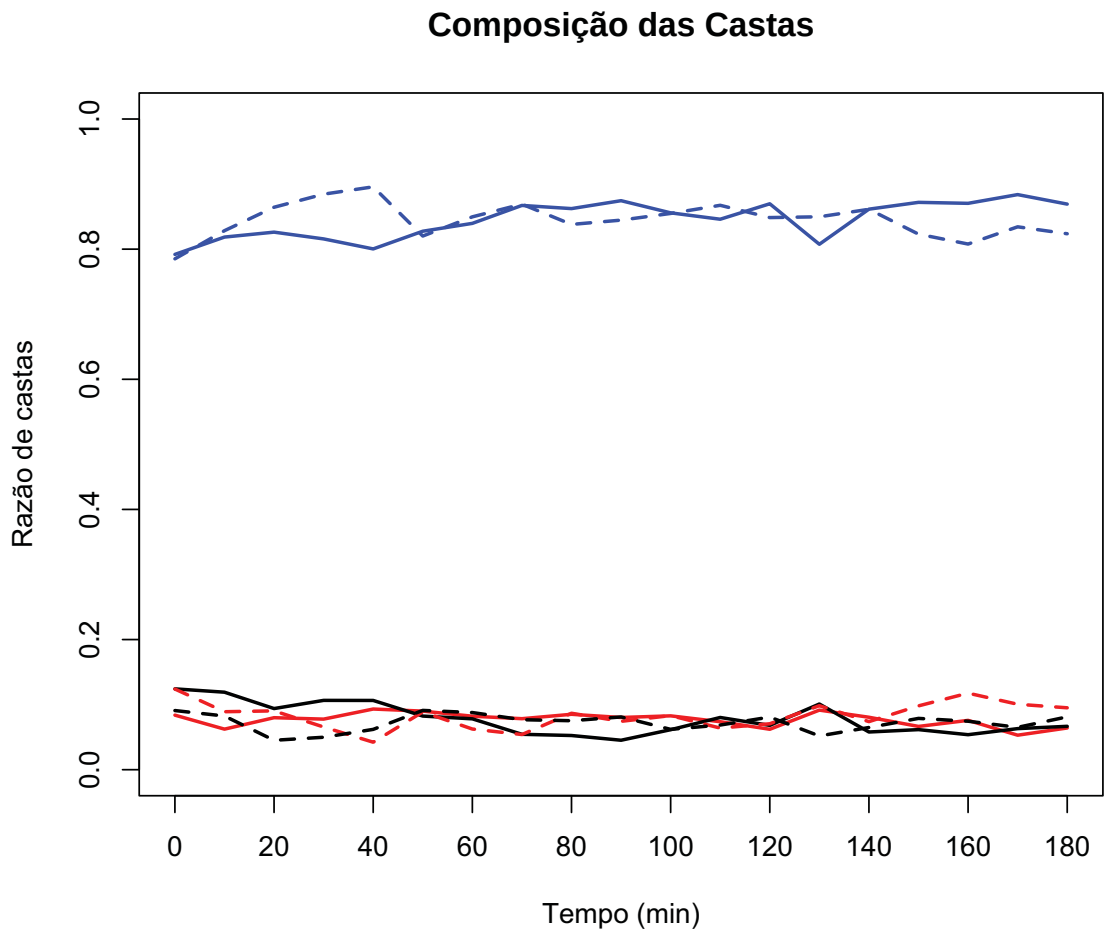

Figura 4. Razão das castas de cupins nas trilhas de forrageamento. As linhas contínuas correspondem aos indivíduos que estão saindo do ninho em direção a fonte alimentar e as linhas tracejadas correspondem aos indivíduos trafegando no sentido oposto. Azul = operários grandes, vermelho = operários pequenos, preto = soldados.

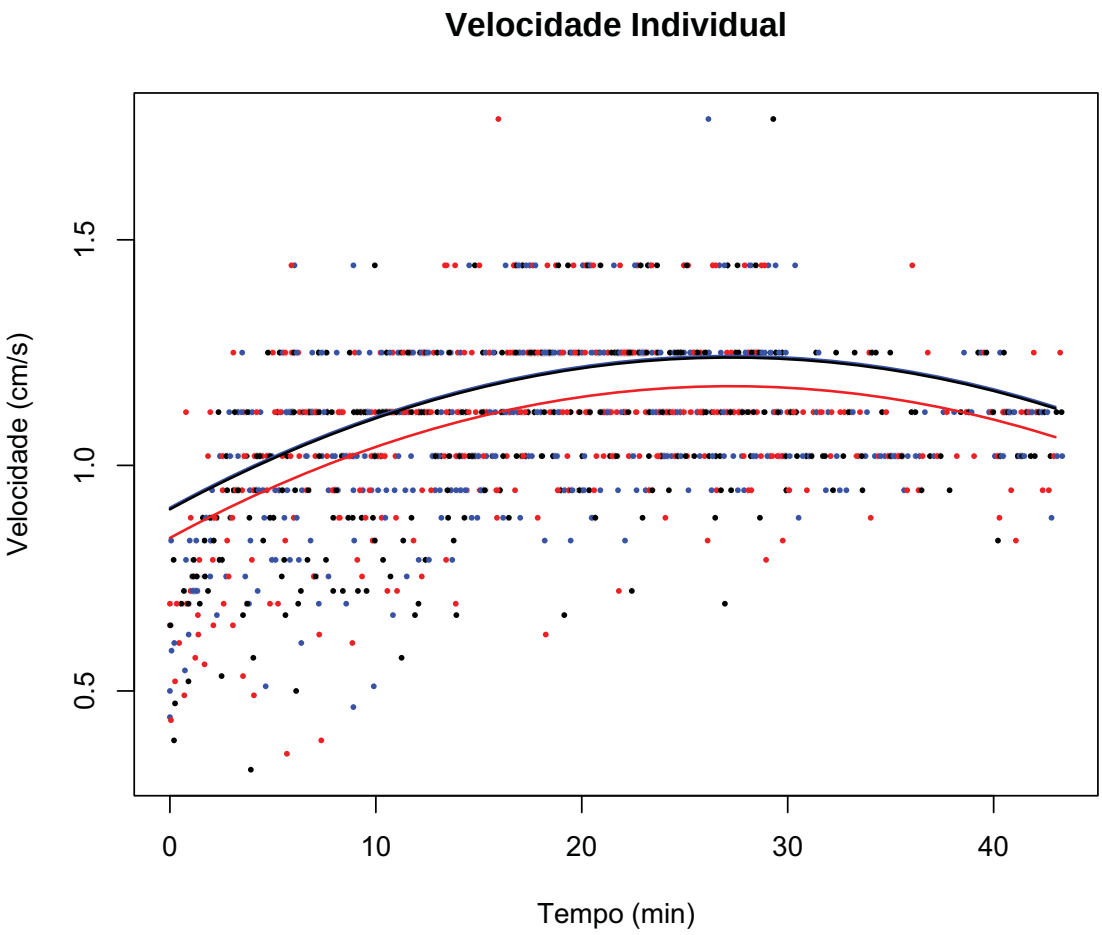

Figura 5. Velocidade individual dos cupins durante os primeiros 40 minutos de forrageamento. As linhas contínuas correspondem às predições do modelo de efeito misto para cada casta. Azul = operários grandes, vermelho = operários pequenos, preto = soldados.

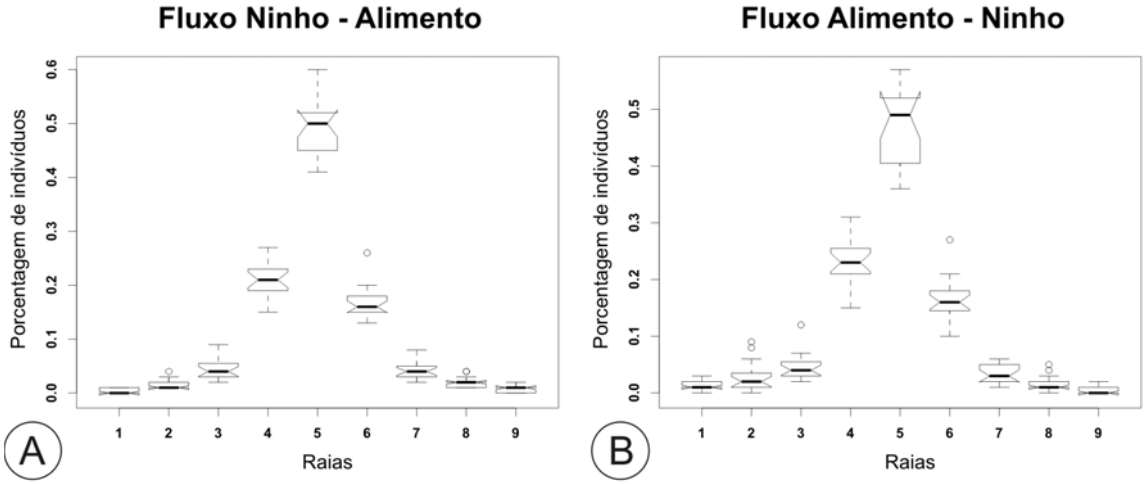

Figura 6. Fluxo dos cupins nas diferentes raias da trilha de forrageamento. **A.** Sentido ninho – alimento. **B.** Sentido alimento – ninho. Notar que, em ambos os sentidos, aproximadamente 50% dos cupins tiveram uma preferência por utilizar a raia central (número 5).

DISCUSSÃO

As trilhas de forrageamento de *Velocitermes heteropterus* formadas nos experimentos laboratoriais apresentaram uma largura de aproximadamente 1 cm, o que é consistente com a largura das trilhas cobertas (galerias sob o solo) observadas ao redor dos ninhos em áreas naturais.

No processo de forrageamento de *V. heteropterus*, a exploração da nova área é realizada por operários e soldados simultaneamente. A exploração da área é caracterizada inicialmente pela saída e retorno dos cupins, os quais avançam gradativamente na área desconhecida. A velocidade individual aumenta com o tempo até atingir um equilíbrio, e isto deve ocorrer concomitantemente à marcação química da trilha que orienta os cupins que estão saindo do ninho.

Durante a exploração da área, a trilha de forrageamento parece ser estabelecida ao longo da trajetória dos primeiros indivíduos. A casta áptera de *V. heteropterus* é cega e provavelmente utiliza a marcação feromonal dos primeiros indivíduos para se guiarem. Este mesmo comportamento foi observado nas lagartas sociais *Malacosoma disstria*, as quais seguem o primeiro indivíduo que deixou uma trilha química em direção ao alimento (COLASURDO; DESPLAND, 2005). No caso das lagartas, os indivíduos mais jovens parecem ser os pioneiros na exploração da nova área, tendo em vista o risco de predação. Já em algumas espécies de cupins, a casta de soldados é que explora a área desconhecida em busca de alimento (TRANIELLO, 1981).

Traniello e Busher (1985) descreveram três fases de organização no forrageamento em *Nasutitermes costalis*. No estágio inicial, os soldados saem do ninho para explorar o ambiente e informam a outros soldados sobre os recursos alimentares encontrados. Relativamente poucos operários aparecem nas trilhas de forrageamento ou no alimento neste período (menos de uma hora da descoberta da fonte alimentar). O recrutamento de um grande número de operários caracteriza a segunda fase do forrageamento, e a razão de castas nas trilhas tende a operários. Na terceira fase, o recrutamento de soldados diminui e o recrutamento de operários pequenos e grandes aumenta. Com um aumento do fluxo bidirecional de operários, os soldados posicionam-se nas bordas ao longo da trilha. O recrutamento diferencial de soldados e operários sugere aos soldados

um papel essencial na exploração (“scouts”) e comunicação da localização do alimento aos operários. Esta comunicação parece ser predominantemente química e as diferenças nas respostas comportamentais entre soldados e operários parecem estar ligadas à sensibilidade na percepção das substâncias da trilha (Traniello, 1981).

O processo de forrageamento de *V. heteropterus* é dinâmico e também pode apresentar três fases principais: uma de exploração da nova área, uma de recrutamento de indivíduos e outra de retorno dos indivíduos com alimento ao ninho, as quais são caracterizadas pelo fluxo diferencial e velocidade dos indivíduos. Diferentemente de *N. costalis*, a razão de castas de *V. heteropterus* é constante durante estas fases. Os soldados de *V. heteropterus* movimentam-se por todo o tempo pela trilha de forrageamento e, eventualmente, posicionam-se nas regiões laterais da trilha.

Os forrageiros de *V. heteropterus* não apresentam uma organização espacial com uma formação de raias nas trilhas de forrageamento, como já foi relatado por Miura e Matsumoto (1998a) para o cupim *Hospitalitermes medioflavus*. Desta forma, ambos os fluxos de saída e retorno ao ninho ocorrem preferencialmente na raia central, o que resulta em muitas colisões entre os indivíduos que trafegam em sentidos opostos. Provavelmente, estas colisões devem ser úteis para transferência de informações entre os cupins em tráfegos opostos. Processo similar parece ocorrer na formiga cortadeira *Atta cephalotes*, conforme sugerido por Burd et al. (2002).

Quanto aos forrageiros em trânsito nas trilhas de *V. heteropterus*, cerca de 50% dos indivíduos utilizaram a raia central. No presente estudo, os indivíduos para os quais as trajetórias foram analisadas não foram distintos por castas. Como a razão de castas é constante nas trilhas e cerca de 80% dos indivíduos forrageiros são operários grandes, futuros estudos serão necessários para esclarecer se existe uma preferência de determinada casta de *V. heteropterus* trafegar por alguma raia específica da trilha.

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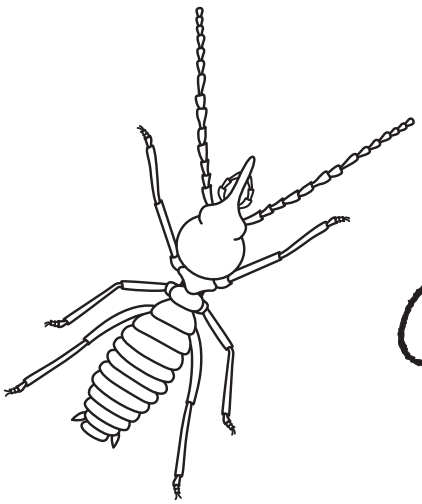
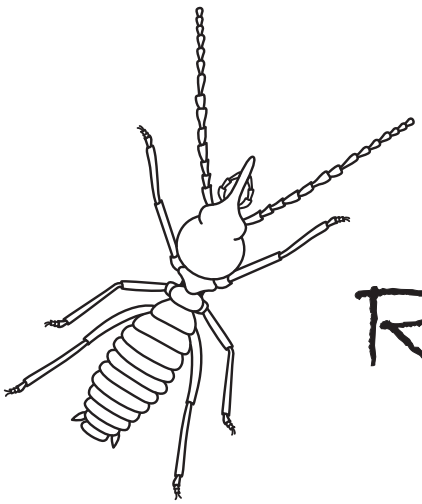


Conclusões

CONCLUSÕES

A linhagem áptera de *Velocitermes heteropterus* apresenta um polimorfismo que está intimamente vinculado ao sexo e aos ínstares dos indivíduos. O polimorfismo dos componentes desta linhagem está relacionado a uma contribuição diferencial das subcastas nos processos de tunelamento e forrageamento, mas não parece influenciar a digestão de celulose, que é realizada principalmente pela casta operária. Os resultados da presente pesquisa permitem inferir que:

- ✓ A casta áptera do cupim *Velocitermes heteropterus* é composta por operários dimórficos e soldados trimórficos. O operário pequeno é macho e o grande fêmea, enquanto os soldados são todos do sexo masculino. Estes últimos podem se originar tanto de larvas de segundo instar do sexo masculino como de operários pequenos de diferentes ínstares.
- ✓ Os operários grandes de *V. heteropterus* dominam as atividades de tunelamento, enquanto os operários pequenos apresentam uma tendência para bifurcação dos túneis escavados.
- ✓ O comportamento de tunelamento de *V. heteropterus* apresenta um padrão de bifurcação aleatório que segue a propriedade de “falta de memória”, ou seja, os cupins mostram sempre a mesma probabilidade de iniciar uma nova bifurcação, independentemente da distância da última bifurcação.
- ✓ A digestão de celulose é realizada principalmente pela casta operária, e operários pequenos e grandes parecem contribuir igualmente para o processo.
- ✓ Os operários grandes contribuem quantitativamente mais do que os operários pequenos e soldados em todo o processo de forrageamento, uma vez que a proporção de castas encontradas foi de oito operários grandes para cada operário pequeno e soldado (8:1:1).



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Rio Claro, 15 de abril de 2013

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