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Ontogenia, Polietismo Fisiológico e Plasticidade de Ninhos em *Nasutitermes ephratae* (Homlgren, 1910) (Termitidae: Nasutitermitinae)

RICARDO AUGUSTO NINK

João Pessoa – PB

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Ontogenia, Polietismo Fisiológico e Plasticidade de Ninhos em *Nasutitermes ephratae* (Homlgren, 1910) (Termitidae: Nasutitermitinae)

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal da Paraíba, como parte dos requisitos para obtenção do título de Doutor em Ciências Biológicas – área de concentração em Zoologia.

Orientador: Dr. Alexandre Vasconcellos

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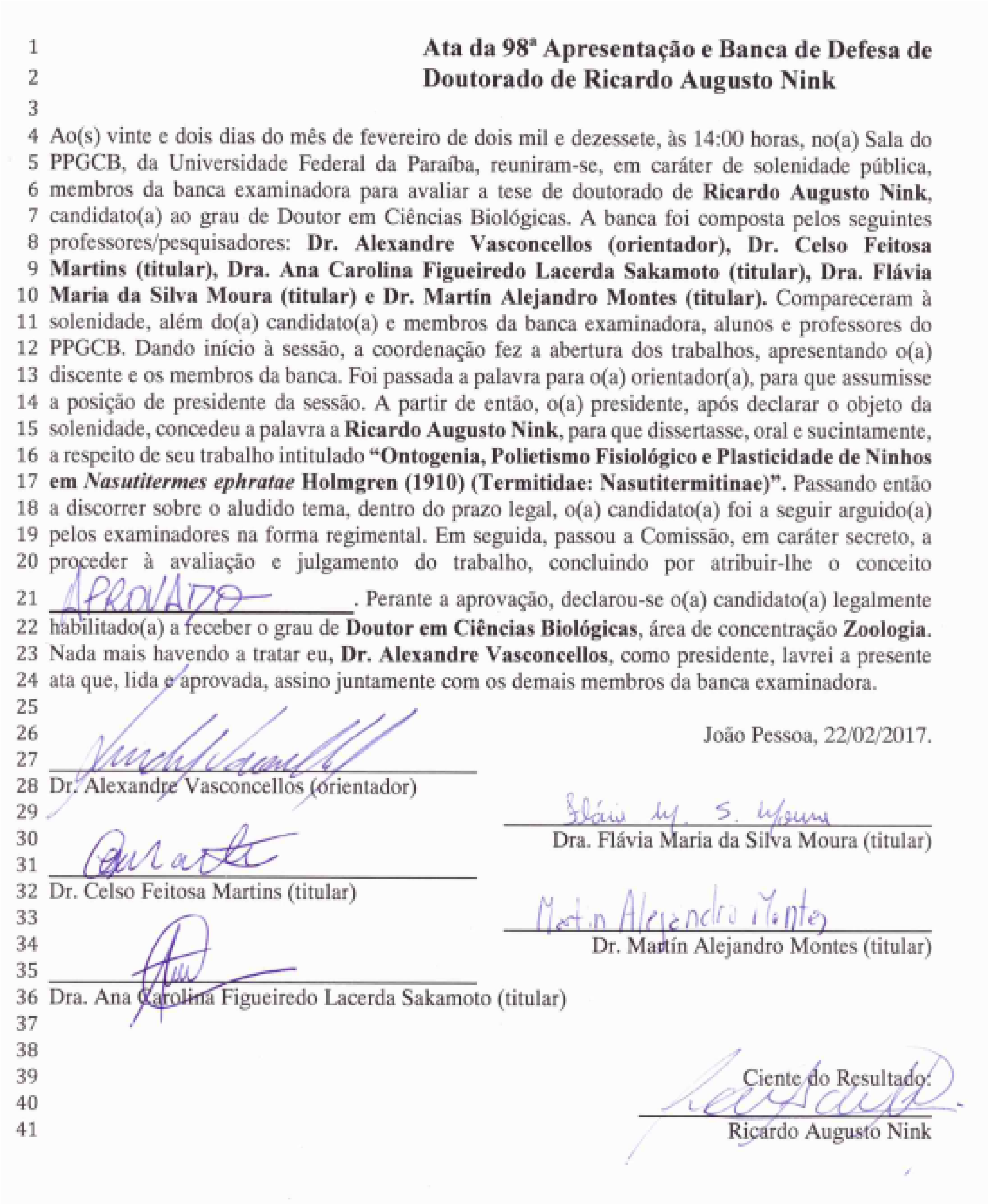

Ata da 98ª Apresentação e Banca de Defesa de Doutorado de Ricardo Augusto Nink

Ao(s) vinte e dois dias do mês de fevereiro de dois mil e dezessete, às 14:00 horas, no(a) Sala do PPGCB, da Universidade Federal da Paraíba, reuniram-se, em caráter de solenidade pública, membros da banca examinadora para avaliar a tese de doutorado de **Ricardo Augusto Nink**, candidato(a) ao grau de Doutor em Ciências Biológicas. A banca foi composta pelos seguintes professores/pesquisadores: **Dr. Alexandre Vasconcellos (orientador), Dr. Celso Feitosa Martins (titular), Dra. Ana Carolina Figueiredo Lacerda Sakamoto (titular), Dra. Flávia Maria da Silva Moura (titular) e Dr. Martín Alejandro Montes (titular).** Compareceram à solenidade, além do(a) candidato(a) e membros da banca examinadora, alunos e professores do PPGCB. Dando início à sessão, a coordenação fez a abertura dos trabalhos, apresentando o(a) discente e os membros da banca. Foi passada a palavra para o(a) orientador(a), para que assumisse a posição de presidente da sessão. A partir de então, o(a) presidente, após declarar o objeto da solenidade, concedeu a palavra a **Ricardo Augusto Nink**, para que dissertasse, oral e sucintamente, a respeito de seu trabalho intitulado **"Ontogenia, Polietismo Fisiológico e Plasticidade de Ninhos em *Nasutitermes ephratae* Holmgren (1910) (Termitidae: Nasutitermitinae)".** Passando então a discorrer sobre o aludido tema, dentro do prazo legal, o(a) candidato(a) foi a seguir arguido(a) pelos examinadores na forma regimental. Em seguida, passou a Comissão, em caráter secreto, a proceder à avaliação e julgamento do trabalho, concluindo por atribuir-lhe o conceito APROVADO. Perante a aprovação, declarou-se o(a) candidato(a) legalmente habilitado(a) a receber o grau de **Doutor em Ciências Biológicas**, área de concentração **Zoologia.** Nada mais havendo a tratar eu, **Dr. Alexandre Vasconcellos**, como presidente, lavrei a presente ata que, lida e aprovada, assino juntamente com os demais membros da banca examinadora.

João Pessoa, 22/02/2017.

Dr. Alexandre Vasconcellos (orientador)

Flávia M. S. Moura
Dra. Flávia Maria da Silva Moura (titular)

Dr. Celso Feitosa Martins (titular)

Martin Alejandro Montes
Dr. Martín Alejandro Montes (titular)

Dra. Ana Carolina Figueiredo Lacerda Sakamoto (titular)

Ciente do Resultado:
Ricardo Augusto Nink

DEDICATÓRIA

Aos meus amados filhos, Malu e Cadu.

Os melhores e mais belos "artigos" que ajudei a elaborar ao longo do doutorado!

A melhor coisa sobre a Ciência é que ela
é verdade, quer você acredite, quer não!

Neil deGrasse Tyson

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PREÂMBULO

Os térmitas são um dos grupos mais abundantes de invertebrados terrestres, tendo reconhecida importância nos processos de degradação da massa lignocelulósica vegetal, fertilização dos solos via movimentação vertical de nutrientes, conversão de nitrogênio atmosférico em nitrogênio orgânico e ciclo do metano, por exemplo. A despeito da sua riqueza e abundância – especialmente nas regiões tropicais – e da sua importância ecológica, diversos aspectos da ontogenia, fisiologia, comportamento e história natural dos térmitas, bem como seus efeitos sinergéticos, permanecem obscuros.

A presente tese foi elaborada com o objetivo de testar a hipótese de que diferentes morfotipos de operários e soldados em uma mesma colônia realizam diferentes funções dentro da colônia, especialmente em relação ao processamento do alimento. A fonte alimentar dos térmitas condiciona a estruturação da microbiota intestinal bacteriana, logo a análise da comunidade microbiana pode fornecer indícios acerca da ocorrência de polietismo nas castas neutras. O objetivo geral desta tese foi, portanto, correlacionar o desenvolvimento das castas de térmitas com os papéis desenvolvidos por cada instar na divisão do trabalho - em especial com relação à fixação de nitrogênio atmosférico e digestão de celulose realizada por bactérias intestinais - e na resultante organização social das colônias. A espécie *Nasutitermes ephratae* foi escolhida como modelo em razão de: 1. ser relativamente abundante em áreas antropizadas de Mata Atlântica e; 2. construir ninhos arbóreos facilmente identificáveis; 3. o gênero *Nasutitermes* apresentar operários geralmente polimórficos; e 4. a espécie ter sido objeto de investigações científicas anteriormente, e informações sobre a sua história natural e microbiota simbionte associada estarem disponíveis para consulta na literatura especializada.

A tese foi dividida em capítulos. A primeira parte da tese traz o estado da arte do conhecimento científico acerca da ontogenia, polietismo e microrganismos simbiontes em térmitas. O conhecimento produzido nesta tese é apresentado em três capítulos distintos, confeccionados em formato de manuscritos científicos escritos em inglês e formatados ao padrão de cada revista científica indexada as quais se pretende submetê-los. Ao final, são apresentadas as conclusões gerais acerca desta tese, sumarizando as informações geradas a partir deste estudo.

RESUMO

Ontogenia, Polietismo Fisiológico e Plasticidade de Ninhos em *Nasutitermes ephratae* (Holmgren, 1910) (Termitidade: Nasutitermitinae)

Nasutitermes ephratae, um térmita superior xilófago e arborícola da região Neotropical foi estudado sobre os seguintes aspectos i) via ontogenética de desenvolvimento de castas; ii) estrutura da comunidade bacteriana intestinal associada a seis instares distintos da linhagem neutra; iii) e plasticidade de seus ninhos. Sete medidas morfométricas foram tomadas de vários ínstares distintos de *N. ephratae* e analisados via Análise de Componentes Principais. A ontogenia de *N. ephratae* foi similar àquela previamente descrita para outras espécies do gênero *Nasutitermes*, com um primeiro instar larval sofrendo muda para a linhagem áptera ou a linhagem ninfal; a linhagem ninfal compreendendo cinco instares além dos imagos alados; a linhagem neutra foi bastante polimórfica, contendo dois ínstares larvais de 2ªordem, 5 ínstares operários, e pré-soldados e soldados monomórficos. A comunidade microbiana intestinal foi estudada via sequenciamento em larga escala do gene rRNA 16S e diferenças consideráveis foram observadas entre os instares operários jovens e velhos. Uma transição de bactérias degradadoras de madeira (Spirochaetes, Fibrobacteres) para bactérias degradadoras de húmus (Bacteriodetes, Firmicues, Proteobacteria) foi notada entre os operários jovens e velhos, sugerindo a ocorrência de polietismo por idade relativo ao processamento dos alimentos. A plasticidade de ninhos também foi documentada através da dissecação e caracterização da arquitetura de um ninho policálico coletado na Mata Atlântica e um ninho tipo montículo de *N. ephratae* coletado no Cerrado. A plasticidade de ninhos nesta espécie de térmita parece estar relacionada à qualidade e à disponibilidade de troncos-suporte adequados.

Palavras-chave: Isoptera; desenvolvimento de castas; simbiontes microbianos; divisão do trabalho; hábitos de nidificação.

ABSTRACT

Ontogeny, Physiological Poliyethism and Nest Plasticity in *Nasutitermes ephratae* (Homlgren, 1910) (Termitidae: Nasutitermitinae)

Nasutitermes ephratae, an arboreal wood-feeder higher termite from Neotropical region was analyzed regarding i) ontogeny pathway for caste development; ii) structure of gut bacterial community among six different instars of neuter lineage; iii) and policalism and nesting plasticity behaviors. Seven morphometric measurements were taken from several distinct instars of *N. ephratae* and analyzed by principal component analyses (PCA). The development system was similar to previously describe *Nasutitermes* genus, with a first instar larvae molt into neuter or reproductive lineages; reproductive line comprising five nymphal steps plus alates; a high polymorphic neuter lineage, within 5 worker instars and monomorphic soldier and presoldier. The microbial intestinal community was checked by high-throughput sequencing of 16S rRNA and remarkable differences were observed between young and old worker instars. A transition from wood-cellulolytic bacteria (Spirochaetes, Fibrobacteres) to humus-degrading bacteria (Bacteriodetes, Firmicues, Proteobacteria) was observed from young to old workers, suggestion the occurrence of age polyethism in food processing. Nest plasticity was also documented by the dissection and characterization of the peculiar architecture of both polycalic nest found in Atlantic Rain Forest and a mound nest of *N. ephratae* collected in Cerrado region. Nest plasticity in this termite species seems to be drive by the quality and availabity of adequate support tree trunks.

Key-words: Isoptera; caste development; microbial symbionts; division of labor; nesting behavior

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CAPÍTULO I

POLIMORFISMO, POLIETISMO E SIMBIOSE EM TÉRMITAS SUPERIORES

POLIMORFISMO, POLIETISMO E SIMBIOSE EM TÉRMITAS SUPERIORES

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Resumo: Realizou-se uma revisão acerca do desenvolvimento ontogenético dos térmitas e suas implicações na divisão de trabalho e na associação com microrganismos intestinais. É descrito como a exploração de diferences nichos alimentares levou à associação dos térmitas com uma variada gama de micróbios intestinais que vêm evoluindo juntamente com seus respectivos hospedeiros. O desenvolvimento ontogenético e a eussocialidade em térmitas são revisadas e discutidas, culminando na complexidade da divisão de trabalho hierárquica que caracteriza a organização social dos térmitas. Os padrões de desenvolvimento de castas de diversas espécies de térmitas superiores são apresentados e comparados sob a ótica do polimorfismo variar de acordo com o grau de humificação das fontes alimentares acessadas por cada espécie. A divisão de trabalho dentro de uma mesma casta polimórfica – ora referida como polietismo de segunda ordem – e extensivamente caracterizada para diferentes tratos, desde forrageio ao processamento alimentar, resultando numa discussão sobre como o polietismo influencia as relações simbióticas entre os térmitas hospedeiros e seus micróbios intestinais.

Palavras-chave: Ontogenia; Termitidae; Divisão de trabalho; Microrganismos intestinais.

ESTADO DA ARTE

TÉRMITAS - IMPORTÂNCIA ECOLÓGICA, HÁBITOS ALIMENTARES E SIMBIOSE

Os térmitas ou cupins são os insetos eussociais mais antigos, cujos hábitos de vida em sociedade datam do período Cretáceo, de acordo com os registros fósseis (Thorne et al. 2000). A história evolutiva dos térmitas aponta que estes são filogeneticamente relacionados a baratas xilófagas subsociais da família Cryptocercidae (Inward et al. 2007). Os térmitas formam um clado monofilético caracterizado por duas sinapormofias: i) a evolução de soldados estéreis, caráter esse que confere efetivamente aos térmitas a classificação como insetos eussociais; e ii) a evolução de reprodução neotênica, em que a prole pode produzir reprodutores ápteros via uma única muda e permanecer no seu ninho de origem (Korb et al. 2012).

Térmitas são também um dos insetos sociais mais diversificados e abundantes do planeta. Excluindo-se espécies fósseis, há cerca de 2933 espécies de térmitas descritas, distribuídas em 282 gêneros e nove famílias, sendo elas: Mastotermitidae, Archotermopsidae, Kalotermitidae, Stolotermitidae, Hodotermitidae, Rhinotermitidae, Stylotermitidae, Serritermitidae e Termitidae (Krishna et al. 2013). Os membros das famílias mais basais são comumente designados térmitas inferiores, enquanto as espécies da família Termitidae são denominadas térmitas superiores. A família Termitidae é também a mais diversa, contabilizando mais de 75% das espécies até então descritas (Kambhampati & Eggleton 2000), distribuídas em 8 diferentes subfamílias, sendo elas: Macrotermitinae, Sphaerotermitinae, Foraminitermitinae, Apicotermitinae, Cubitermitinae, Termitinae, Syntermitinae e Nasutitermitinae (Krishna et al. 2013).

Os térmitas se distribuem predominantemente em regiões tropicais, sendo que os maiores índices de diversidades são observados nas florestas úmidas, onde certamente são os invertebrados decompositores de matéria orgânica ecologicamente mais importantes (Eggleton & Tayassu 2001; Ohkuma 2003). Esta habilidade permite que, em alguns ecossistemas degradados e com baixa disponibilidade de nutrientes, os térmitas sejam praticamente os únicos invertebrados capazes de colonizar o ambiente do solo e decompor a matéria orgânica (Quédraogo et al. 2004).

Esses insetos estão entre os invertebrados com maior biomassa e abundância em ecossistemas tropicais, com valores que podem exceder 100g/m^2 e 10.000 indivíduos/m^2, respectivamente (Eggleton et al. 1996). Térmitas podem corresponder até 95% de toda a biomassa de insetos que habitam o perfil do solo e até 82% de toda a biomassa de invertebrados nos troncos em decomposição (Watt et al. 1997; Bandeira & Torres 1985). Devido a essa elevada biomassa, estima-se que os térmitas sejam bastante relevantes para a manutenção da dinâmica dos processos de decomposição, assim como para os fluxos de matéria e energia nos ecossistemas (Holt & Lepage 2000).

Todas as espécies de térmitas se alimentam de material celulósico em diferentes estados de decomposição, mas suas fontes de alimento são bastante diversas, incluindo matéria orgânica presente no solo, detritos, serapilheira, madeira ou casca de árvores (Wood & Sands 1978). A história evolutiva dos térmitas mostra uma gradação dos térmitas que se alimentam de madeira seca (basais) para os térmitas que se alimentam de húmus (apicais), indicando uma relação entre o grau de humificação do alimento e a filogenia das espécies. Esta ideia é biologicamente intuitiva, uma vez que os térmitas basais se alimentam quase que exclusivamente de madeira, o que precede os hábitos humívoros de algumas espécies de térmitas superiores, sendo, portanto, um caractere plesiomórfico (Noirot 1992; Donovan et al. 2001). A história evolutiva que permitiu a exploração eficiente destes recursos pelos térmitas envolveu não somente a adaptações anatômicas, morfológicas e metabólicas das espécies, mas também a sua distribuição geográfica, a seleção de habitat e nichos, a evolução de padrões comportamentais e, sobretudo, a associação com diferentes tipos de microrganismos (Eggleton & Tayassu 2001).

Todas as espécies conhecidas possuem complexas relações mutualísticas com micróbios intestinais. A especialização trófica está diretamente relacionada à composição da comunidade microbiana associada aos ninhos e ao tubo digestivo dos térmitas. A estratégia de se alimentar de materiais ricos em lignocelulose, muito abundantes na natureza, permitiu que surgissem uma série de associações simbióticas entre térmitas e microrganismos, que habitam o tubo digestivo dos térmitas, proporcionando vias metabólicas de processamento de carbono e de fixação de nitrogênio (Correia et al. 2008).

Os térmitas possuem relações mutualísticas com variados microrganismos intestinais, incluindo protozoários flagelados celulolíticos associados aos térmitas filogeneticamente mais basais, além de fungos e procariotos predominantes no tubo digestivo dos Termitidae. Algumas tendências de associação mutualística são características de famílias inteiras, demonstrando haver uma coevolução entre os simbiontes microbianos e as espécies de térmitas hospedeiras (Eggleton & Tayasu 2001; Brune 2014).

As simbioses entre térmitas e microrganismos são conhecidas, ao menos parcialmente, desde 1923, ano em que L. R. Cleveland demonstrou a simbiose entre protozoários flagelados e térmitas inferiores. Os simbiontes ingeriam partículas de madeira previamente fragmentadas pelo hospedeiro, convertendo a celulose à glicose e, posteriormente, a glicogênio. Todos os metabólitos eram produzidos em excesso para as necessidades dos protozoários, permitindo que fossem utilizados pelos hospedeiros (Correia et al. 2008). Entre os térmitas superiores, os protistas flagelados foram substituídos por fungos e/ou procariotos simbiontes (Tokuda et al. 1997; Brune 2014).

Mesmo sendo os térmitas capazes de secretar suas próprias enzimas celulolíticas (endoglucanases e β-glucosidadese) em suas glândulas salivares e/ou no intestino médio, a degradação eficiente do material lignocelulolítico é o resultado de um sistema sinergético da ação das celulases endógenas combinadas a celulases de origem microbiana (Tokuda & Watanabe 2007; Warnecke et al. 2007).

Os térmitas são ainda um dos poucos artrópodes terrestres capazes de fixar nitrogênio atmosférico em simbiose com bactérias mutualistas que vivem em seu tubo digestivo, compensando dessa forma, a baixa concentração deste nutriente no alimento lignocelulósico do qual se alimentam. Esta associação com bactérias fixadoras de nitrogênio parece ter se desenvolvido há muito tempo no processo evolutivo dos térmitas, já que é observada nas famílias mais basais como Mastotermitidae e Kalotermitidae, além das subfamílias Macrotermitinae, Termitinae e Nasutitermitinae dos térmitas superiores (Yamada et al. 2007). Em geral, essa associação com microrganismos celulolíticos e fixadores de nitrogênio é mais proeminente em espécimes da linhagem operária que, após processar o alimento, se encarregam de nutrir os membros das demais castas – larvas, ninfas e soldados – presentes na colônia.

EUSSOCIALIDADE E ONTOGENIA EM TÉRMITAS

Insetos sociais apresentam sociedades altamente complexas nas quais poucos indivíduos podem se reproduzir (reis e rainhas) enquanto uma maioria dos indivíduos da colônia (soldados e operários) renuncia a reprodução, ao menos temporariamente. Esse comportamento altruístico das castas neutras é geralmente explicado pela teoria de seleção de parentesco, focalizando na propagação de alelos correspondentes através de parentes não descendentes (Hamilton, 1964). Em abelhas, formigas e algumas espécies de vespas o cuidado aloparental da ninhada emergiu como um fator comum que favoreceu a evolução do comportamento social nestas espécies (Bourke & Franks 1995; Crozier & Pamilo 1996; West-Eberhard & Turillazzi 1996; Strohm & Leibig 2008). Em térmitas, no entanto, o cuidado parental realizado pelos operários não é um pré-requisito necessário à eussocialidade, e os níveis de altruísmo dos operários variam grandemente a depender da espécie. Todos os térmitas possuem soldados estéreis altruísticos e vivem em colônias protegidas em regime de "defesa do forte" (*fortress-defenders*), indicando que a eussocialidade foi dirigida mais pela necessidade de aumentar as habilidades defensivas da colônia do que o cuidado aloparental cooperativo (Korb et al. 2012).

Todas as espécies de térmitas vivem em colônias que variam em tipo, tamanho e número de indivíduos, podendo conter de algumas centenas a milhões de indivíduos. As sociedades de térmitas apresentam uma elevada organização social e estrutura hierárquica baseadas na divisão de trabalho. Castas morfologicamente distintas - tais como larvas, ninfas, reprodutores, operários e soldados – são especializadas em um tipo de função, cada uma delas de vital importância para a manutenção e viabilidade da colônia (Korb 2008).

Nesta hierarquia social, o casal real é responsável pela reprodução e geração de novos indivíduos na colônia. Ninfas são responsáveis pelo desenvolvimento de novos alados reprodutores que irão propagar a espécie. Soldados são responsáveis pela defesa ou atividades correlatas. Operários, por sua vez, conduzem a maior parte das atividades intrínsecas à manutenção da colônia, tais como i) construção, reconstrução e expansão dos ninhos; ii) forrageamento e demarcação de trilhas; iii) construção de galerias e túneis; iv) cuidado aloparental de ovos e larvas; v) *grooming* e manutenção do casal real; vi) remoção de dejetos para fora do ninho; vii) auxílio na defesa da colônia; viii) digestão e processamento do alimento; ix) nutrição das demais castas da colônia via trofalaxe, dentre outras.

A divisão de trabalho ou polietismo é certamente o traço mais característico dos insetos eussociais, uma vez que, mediante a promoção de efeitos sinergéticos, ela aumenta a eficiência da atividade total da colônia e mantém sua integridade (Oster & Wilson 1978). Em térmitas, a divisão de trabalho é um subproduto do seu complexo sistema de desenvolvimento de castas (Noirot & Pasteels 1987).

Nos insetos sociais holometábolos (Hymenoptera), abelhas, vespas e formigas só assumem papéis sociais após a transição da fase larval para a fase adulta via empupação. Os térmitas, entretanto, sendo insetos hemimetábolos, assumem papéis nas atividades sociais muito antes de – ou sem nunca sofrer – a muda imaginal. Os únicos imagos presentes nas colônias são os alados, os quais, após as revoadas, tornam-se os reprodutores primordiais, reis e rainhas. Logo, as linhagens neutras – soldados e operários – responsáveis pela manutenção da colônia, podem ser consideradas produtos de divergências do desenvolvimento normal que origina os imagos (Noirot & Pasteels 1987).

Os Isoptera têm demonstrado padrões de diferenciação de castas que variam bastante entre famílias, gêneros e espécies (Miller 1969; Noirot 1969; Roisin 2000). Essa diversidade existe especialmente devido ao seu desenvolvimento hemimetabólico pós-embrionário, cujo número de eventos de mudas que podem ocorrer não é constante (Noirot & Pasteels 1987). O desenvolvimento de castas também pode variar de acordo com a idade da colônia e fatores ambientais externos (Noirot 1969; Matsumoto & Hirono 1985; Clarke 1993; Henderson & Delapane 1994).

Em térmitas eussociais basais, o desenvolvimento pós-embrionário é linear, similar ao observado em insetos hemimetábolos não-sociais. Instares larvais mais velhos ou ninfas são responsáveis por conduzir as atividades pertinentes à casta operária. A plasticidade fenotípica associada ao desenvolvimento ontogenético, tais como mudas estacionárias ou mudas regressivas, possibilitou a alguns se desviarem do caminho linear que resulta na produção dos imagos e permanecerem por longos períodos como operários funcionais sem, necessariamente, abrir mão das suas potencialidades ontogenéticas. Postula-se que, no decorrer da evolução, esse "desvio" tenha ocorrido cada vez mais cedo durante o desenvolvimento pós-embrionário, resultando no padrão de castas bifurcado observado nos membros mais apicais da família Termitidae (Noirot & Pasteels 1987).

Nos Termitidae, a linhagem áptera diverge da linhagem ninfal após a primeira muda das larvas de primeiro instar e, exceto pela ocorrência de reprodutores secundários ergatoides (originários de operário), não há transição entre as linhagens ou mudas regressivas, conforme observado em térmitas inferiores. Os padrões de desenvolvimento da linhagem sexual são notavelmente constantes entre as espécies de Termitidae, com cinco diferentes instares e um alado (imago) resultante; enquanto que as linhagens ápteras variam de acordo com o gênero ou espécie (Roisin 2000).

Em regras gerais, a linhagem neutra desta família comporta um estágio larval de segundo instar, operários ativos e soldados, originados via pré-soldados após duas mudas sucessivas das larvas de segundo instar ou dos operários. A maioria dos Termitidae apresenta operários sexualmente dimórficos, e soldados geralmente de um único sexo (Noirot 1951), havendo, no entanto, bastante variação em relação ao polimorfismo e especialização das castas neutras.

Na subfamília Macrotermitinae, mais basal dentre os Termitidae, a linhagem áptera é altamente plástica. Em espécie do gênero *Odontotermes* já foram documentadas tanto castas operárias sexualmente monomórfica (*Odontotermes formasanus*) (Li et al. 2015a; Li et al. 2015b) quando linhagens sexualmente dimórficas (*O. distans*) (MacMahan et al. 1984). Dentre os soldados, um polimorfismo por tamanho é observado, a depender do instar operário

(1º ou 2º) que tenha originado o pré-soldado. No gênero *Macrotermes* o polimorfismo das castas neutras é mais elaborado, havendo a ocorrência de sucessivos instares larvais antes da muda definitiva para operário. Os operários são sexualmente dimórficos, resultando em operários grandes (machos) e pequenos (fêmeas). Os soldados são sempre fêmeas, mas apresentam dimorfismo por tamanho, a depender do instar larval que o tenha originado. Larvas de 3º instar resultam em soldados pequenos, enquanto larvas de 4º instar resultam em operários grandes (Neoh & Lee 2009).

Na subfamília Termitinae, o estudo ontogenético mais proeminente envolve a espécie *Drepanotermes perniger*, a qual apresenta uma única linhagem operária dotada de dois a três instares sucessivos que resultam em um leve polimorfismo por tamanho. Os soldados são estritamente monomórficos e oriundos de operários de 2º instar (Watson 1974). O sistema de castas de espécies de *Amitermes* segue a mesma tendência de *Drepanotermes*, apresentando dois traços reconhecidos como primitivos: i) a presença de indivíduos de ambos os sexo dentre os operários e soldados; ii) a ausência de dimorfismo sexual dentre a linhagem neutra (Noirot 1955).

O sistema de castas de *Microcerotermes championi*, outra espécie de Termitinae, foi caracterizado por apresentar operários sexual e morfometricamente dimórficos. Cada linhagem apresentava ainda sucessivas mudas que ocasionam numa maior esclerotização e um incremento no tamanho de cada instar, tornando a casta operária altamente polimórfica. Soldados foram igualmente dimórficos quanto suas dimensões morfométricas, sendo o maior originado pelos operários machos de 1º instar e o menor pelos operários fêmeas de 1º instar (Rasib & Akhtar 2012).

Para espécies da subfamília Syntermitinae, a análise ontogenética revelou que a linhagem neutra pode ser monomórfica e monossexuada (*Cornitermes cumulans* e *C. walkeri*); conter operários monomórficos de ambos os sexos, mas apenas soldados fêmeas

(*Silvestritermes holmgreni*); ter operários e soldados sexualmente dimórficos (*Embiratermes chagresi* e *Syntermes wheeleri*); ou ainda possuir exclusivamente operários machos e soldados fêmeas (ambos com elevado grau de polimorfismo), a exemplo de *Rhynchotermes perarmatus* (Noirot 1989; Roisin, 1992; Constantino 1995; Barbosa et al. 2012).

A subfamília Nasutitermitinae é certamente aquela com o maior número de espécies cuja ontogenia já foi estudada, principalmente as do gênero *Nasutitermes*, a exemplo de *N. arborum*, *N. maculiventris* (Noirot 1955), *N. lujae* (Pasteels 1965), *N. exitiosus* (McMahan & Watson 1975), *N. princeps* (Roisin & Pasteels 1986), *N. novarumhebridarum* (Roisin & Pasteels 1987) e *N. corniger* (Lima et al. 2014).

O padrão de castas descrito para o gênero *Nasutitermes* é similar àquele registrado para o Termitinae *M. championi*, em que, após a primeira muda da linhagem áptera, uma nova bifurcação ocorre, resultando em duas linhagens operárias sexualmente dimórficas, com machos e fêmeas vestigiais originando operários menores e maiores, respectivamente (Noirot 1955; Roisin & Pasteels 1987; Rasib & Akhtar 2012).

Em *N. exitiosus* e *N. corniger*, além de castas operárias polimórficas, frequentemente são descritos também dois tipos morfologicamente distintos de soldados em relação ao tamanho, sendo um menor e outro maior, provavelmente oriundos de linhagens operárias também morfologicamente distintas. Para essas duas espécies, também foram descritos operários polimórficos em relação à pigmentação da cápsula cefálica e do aparelho intestinal. Dois estágios distintos de operários menores e quatro de operários maiores foram descritos para *N. exitiosus*; e, em *N. corniger*, foram identificados três diferentes estágios de operários pequenos e quatro de operários grandes (McMahan & Watson 1975; Lima et al. 2014)

Velocitermes barrocoloradensis, por outro lado, apresenta polimorfismo sexual na casta operária, mas não na casta soldado. Os soldados são morfometricamente dimórficos, porém de apenas um único sexo: machos. Diferenças entre o tamanho dos soldados são atribuídos ao instar que o originou: larva pequena de 2º instar ou operários pequenos de 1º ou 2º instar (Roisin 1996). Padrão extremamente similar foi descrito para V. *heteropterus*, mas evidenciando diferenças morfométricas também entre os soldados originados a partir de operários de 1º (origina soldado intermediário) ou 2º instars (resulta em soldado grande), perfazendo três tamanhos distintos de soldados, quando somados ao soldado pequeno oriundo da larva macho de 2º instar (Haifig et al 2012). Em *Hospitalitermes medioflavus*, os operários são uma linhagem sexualmente dimórfica, há dois instares dentre os operários grandes e somente um único em operários pequenos, que apenas podem sofre muda para pré-soldado, gerando assim os soldados monomórficos (Miura & Matsumoto, 1998)

Outras espécies de Nasutitermitinae, no entanto, podem apresentar um esquema bastante simplificado de organização das linhas ápteras, contendo tanto operários quanto soldados estritamente monomórficos e de ambos os sexos, a exemplo de *Coatitermes clevelandi*, *Subulitermes denisae*, (Roisin 1996) e *Constrictotermes cyphergaster* (Moura et al. 2011).

A elevada plasticidade observada no desenvolvimento ontogenético entre subfamílias de Termitidae evolutivamente distantes aponta claramente que a determinação das castas não é uma característica geneticamente condicionada (Roisin 2000), portanto não sujeita a seleção natural e sem valor filogenético. No entanto, as características de cada casta, e as sugestões e os limiares de resposta que orientam os indivíduos para uma determinada casta são geneticamente determinados e, obviamente, sujeitos à seleção natural (Roisin 2000). A abordagem mais indicada é buscar entender não apenas como as castas são determinadas, mas por que um dado instar se envolve em uma dada via de desenvolvimento ontogenético (Higashi et al 1991; Higashi & Yamamura 1994). Os diversos fatores envolvidos na determinação de castas em térmitas foram extensamente revisados e sumarizados por Roisin (2000).

Noirot (1992) observou que térmitas humívoros apresentam um sistema de castas simplificado, havendo geralmente uma redução no número de instares operários, e sugeriu que as tarefas realizadas por operários humívoros não diferem grandemente de atividades como construção do ninho, escavação e defesa de galerias, o que favorece a formação de soldados e operários com tamanho e pigmentação homogêneos. Hipótese similar foi aventada por Roisin (1996), ao considerar que a elevada variabilidade intracastas observada dentre operários e soldados de *R. perarmatus*, poderia ser resultado do forrageamento na serrapilheira das florestas, um ambiente muito mais heterogêneo do que as galerias subterrâneas empregadas por outras espécies de Syntermitinae humívoros monomórficos.

Essa hipótese é aparentemente consistente ao considerarmos a maior parte das castas de Termitidae ora descritas, mas três incongruências sugerem um sistema regulatório mais elaborado na determinação de castas polimórficas do que somente a estratégia de forrageamento: i) Membros da família Macrotermitinae que empregam as mesmas estratégias de forrageamento e similares fontes alimentares podem apresentar tanto duas linhagens operárias sexualmente dimórficas (*Macrotermes*) quanto linhagens monomórficas contendo indivíduos de ambos os sexos (*Odontotermes*); ii) Termitinae xilófagos, a exemplo de *Amitermes*, apresentam padrão de castas neutras estritamente monomórfico, assim como Termitinae que se alimentam de serrapilheira (*Drepanotermes*), uma fonte alimentar com um maior coeficiente de humificação, ao invés de se assemelhar ao padrão altamente polimórfico de outros térmitas xilófagos como *Nasutitermes* e *Microcerotermes*; iii) Nasutitermitinae comedores de liquens e que forrageiam em ambientes abertos podem apresentar tanto linhagem operária monomórfica (*Constrictotermes*) quanto linhagens sexualmente dimórficas e instares polimórficos (*Hospitalitermes*).

Ainda que os hábitos alimentares possam ter alguma influência sobre o desenvolvimento de castas, estes não são determinantes para a ontogenia dos térmitas. As evidências apontam para condicionantes espécie-específicas estritamente reguladas, uma vez que mesmo espécies de um mesmo gênero (e.g. *N. exitiosus* e *N. novarumhebridarum*; *O. distans* e *O. formosanus*) podem apresentar um desenvolvimento da linhagem neutra relevantemente distinta.

O conhecimento acerca dos padrões ontogenéticos em térmitas avançou muito com base em diversos estudos conduzidos ao longo dos últimos cinquenta anos, mas pontos importantes sobre como esse processo evolui ainda são incertos. Ainda que as evidências apontem que o desenvolvimento de castas é influenciado pelas fontes de alimento, pelas estratégias de reprodução e defesa, e até mesmo pelos hábitos de nidificação das espécies, o porquê de os térmitas terem atingido um grau de complexidade e organização social tão elevado via dimorfismo e especialização sexual ainda é uma incógnita.

POLIMORFISMO E POLIETISMO EM TÉRMITAS SUPERIORES

A divisão de trabalho dentre as castas morfologicamente distintas de térmitas é um dos fatores-chave para entender a evolução da eussocialidade neste táxon. A especialização em uma de três atividades básicas – reprodução, defesa e manutenção da colônia – associada à aquisição de uma morfologia específica a cada tarefa, resultou na mais complexa organização social já documentada entre os insetos sociais. Evolutivamente, conduziu os térmitas de meros colonizadores de troncos de madeira a condição de construtores de ninhos altamente complexos em termos de arquitetura e hierarquização dos espaços sociais. Também contribui, certamente, em sua capacidade de explorar nichos e recursos alimentares cada vez mais elaborados.

Essa intrigante divisão de trabalho não se restringe somente à castas distintas, como também envolve tipos morfologicamente distintos de indivíduos pertencentes a uma mesma casta. Esse segundo nível de polietismo é frequentemente relacionado a um polimorfismo sexual, morfométrico, temporal ou uma combinação destes fatores (Miura & Matsumoto 1995).

O trabalho pioneiro de Pasteels (1965) evidenciou a existência de polietismo por tamanho e por idade em operários de *Nasutitermes lujae*. Observou-se que os instares mais velhos de operários maiores eram mais facilmente recrutados em atividade de forrageamento. Essas observações foram posteriormente corroboradas e ampliadas com a documentação de polietismo entre operários de *N. corniger* para as atividades de reparo e reconstrução de ninhos. Operários grandes (fêmeas) de 3º instar superavam em muito qualquer outro instar durante as atividades de reparo. Por outro lado, operários pequenos (machos) participavam mais intensivamente da reconstrução do ninho quando cessavam as condições de distúrbio (MacMahan 1970).

Em *N. exitiosus* verificou-se também que operários exibiam polietismo temporal na reparação dos ninhos, sendo o é o instar mais esclerotizado entre os operários maiores o mais ativo na reparação imediata de danos provocados na superfície do ninho (MacMahan 1977) assim como anteriormente registrado para *N. corniger*. Estudos adicionais sobre polietismo em *N. exitiosus*, revelaram ainda que os operários raramente trocam de atividade dentro da colônia, como por exemplo, passando de forrageadores para construtores ou vice-versa (Evans 2006).

Outros membros da subfamília Nasutitermitinae também apresentam padrões típicos de polietismo morfológico e temporal. No gênero *Hospitalitermes*, operários de primeiro instar são completamente pigmentados e participam de expedições de forrageamento, onde realizam a maior parte da atividade de trituração do material lignocelulósico (Miura & Matsumoto 1995). Em *H. medioflavus*, operários menores são sempre do sexo masculino e oriundos de larvas de segundo instar; eles agem como trituradores durante as atividades de busca e coleta de alimentos. Operários menores podem ainda sofrer muda para soldados (via estágio de pré-soldado), mudando drasticamente de papel e passando agora atuar na defesa da colônia (Miura & Matsumoto 1998).

Por outro lado, larvas fêmeas de segundo instar de *H. medioflavus* dão origem aos operários medianos, que atuam tanto como trituradores ou como carregadores de alimento, compondo parte de um vasto e eficiente sistema de forrageamento em benefício da colônia. Operários medianos podem mudar para operários maiores, cujos membros atuam exclusivamente no carregamento de comida. Assim, uma mudança de atividade durante o desenvolvimento também é observada em operários do sexo feminino, apesar de não ser uma mudança tão profunda quanto aquela observada em machos. Pode-se dizer então que a divisão de tarefas em *H. medioflavus* é realizada bidimensionalmente: primeiramente, baseada no sexo e, secundariamente, baseada no instar; ou, em resumo, apresentando polietismo sexual e temporal (Miura & Matsumoto 1998).

Estudos mais recentes têm demonstrando ainda a ocorrência de polietismo em reação à digestão de celulose entre três morfotipos de operários – erroneamente caracterizados por Hojo et al (2004) como pequenos, médios e grandes – de *Nasutitermes takasagoensis*. Tanto a expressão gênica endógena da enzima celulolítica endo-β-1,4-glucanase, quanto a atividade celulolítica desta enzima e das β-glucosidades foram mais acentuadas em operários menores do que em operários maiores e soldados, sugerindo uma maior participação dos operários pequenos na degradação da celulose (Fujita et al 2008).

Outro Nasutitermitinae, *Velocitermes heteropterus*, apesar de apresentar um marcado polietismo em atividade de tunelamento e escavação de galerias, demonstrou, no entanto, não comportar um sistema similar de divisão de trabalho em relação à digestão do material celulósico, pois os níveis de expressão gênica de endo-β-1,4-glucanase entre diferentes tipos de operários e soldados não apresentaram diferenças significativas (Haifig et al 2011; Haifig et al 2012).

Em Nasutitermitinae, o polietismo de segunda ordem não está restrito à casta operária. Em *N. exitiosus*, o soldado maior (fêmea) demonstra um comportamento não combativo, recuando imediatamente ao ninho em qualquer sinal de perigo. Esse comportamento é completamente distinto daquele dos soldados pequenos (machos), altamente combativos e agressivos. Postulou-se que os soldados grandes funcionam como um tipo de batedor, retornando ao ninho para alarmar os demais indivíduos (Kriston el al., 1977).

Polietismo em castas polimórficas não é restrito a táxons apicais do Termitidae, mas também já foi documentada em diversas espécies da subfamília basal de térmitas cultivadores de fungos, Macrotermitinae. *Odontotermes distans* apresenta apenas polietismo por tamanho, onde 96% dos operários menores permanecem na câmara real prestando assistência para a rainha, e 99% dos operários maiores, ao contrário, tomam parte no forrageamento, sendo que 70% deles podem ser encontrados pululando próximo às cavidades de saída na superfície do ninho (MacMahan et al. 1984). Já operários de *O. formosanus* apresentam um interessante polietismos temporal em relação ao processamento de alimento que é fartamente documentado também para várias espécies de *Macrotermes*. Operários mais velhos são responsáveis por coletar o alimento e levá-lo até o ninho, mas se nutrem ingerindo não o material celulósico coletado, mas favos fúngicos maduros ricos em proteína. Já operários jovens ingerem a lignocelulose provida pelas suas contrapartes mais velhas, juntamente com nódulos fúngicos (conídios) e utilizam suas fezes ricas em esporos fúngicos para construir novos favos. Operários jovens também são responsáveis pela nutrição das larvas e de operários recém-mudados via trofalaxe estomodeal de secreções salivares (Badertscher et al. 1983; Leuthold et al. 1989; Hinze et al. 2002; Li et al. 2015).

Uma vez que a qualidade da dieta muda significativamente durante a vida útil dos operários em térmitas cultivadores de fungos, estes se tornaram modelos adequados também à realização de estudos para investigar como a microbiota intestinal dos térmitas responde ao substrato alimentar e, obviamente, ao poliestimo no processamento alimentar. Hongoh e colaboradores (2006) documentaram uma evidente e relevante diferença nos perfis microbianos entre operários de 1º e 2º instares em *Macrotermes gilvus*. Em *O. formasanus*, Li et al. (2015b) também documentaram uma maior abundância dos filos bacterianos Firmicutes e Spirochaetes (reconhecidos por seu metabolismo lignocelulolítico) em operários mais jovens (reponsáveis por digerir a celulose coletada por operários mais velhos), enquanto que em operários velhos houve uma maior abundância de Bacterioidetes, frequentemente mais copiosas em térmitas que ingerem alimentos com um grau de humificação mais elevado (neste caso, favos fúngicos).

Estes estudos ressaltam não apenas a importância da microbiota simbionte intestinal dos térmitas nos processos de degradação da celulose, mas demonstram que a estrutura das comunidades bacterianas está intrinsecamente relacionada à ontogenia e ao papel que cada um dos instares operários polimórficos desempenha dentro da colônia.

CONCLUSÕES

Os térmitas são insetos com a mais elevada plasticidade ontogenética. A determinação de castas *per se* não se apresenta como um caráter filogenético sujeito à seleção natural, mas como o subproduto de fatores biológicos intrínsecos a cada espécie de térmita, talvez de origem epigenética. A relação de causalidade entre polimorfismo/polietismo e a eussocialidade – quem é derivado do quê – ainda carece de evidências científicas para ser estabelecida.

Linhagens polimórficas estão geralmente associadas a um polietismo secundário, resultando em uma complexa rede de divisão de tarefas codependentes ou sinergéticas dentro de uma mesma casta. Esse elaborado polietismo exerce influência não somente nas relações sociais dos térmitas como também condiciona a estruturação das comunidades de micróbios simbiontes intestinais.

A necessidade de digerir lignocelulose recalcitrante pode ter sido um dos fatores que dirigiram a divisão de trabalho e contribuíram à eussocialidade em térmitas, uma vez que a presença de indivíduos dedicados e especializados em processar eficientemente essa fonte alimentar pode ter despontado como uma vantagem evolutiva.

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CAPÍTULO I

ONTOGENETIC DEVELOPMENT SYSTEM IN THE WOOD-FEEDER TERMITE *Nasutitermes ephratae* HOLMGREN (TERMITIDAE: NASUTITERMITINAE)

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ONTOGENETIC DEVELOPMENT SYSTEM IN THE WOOD-FEEDER TERMITE *Nasutitermes ephratae* HOLMGREN (TERMITIDAE: NASUTITERMITINAE)

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ABSTRACT

The ontogenetic pathway of the arboreal wood-feeder termite *Nasutitermes ephratae* were studied in natural colonies collected in Atlantic Rain Forest northeastern Brazil. A total of 7 morphometric measurements were taken of the head, tibia, femur, pronoto e mesonoto from larval, neuter and nymphal lineages. The measurements were analyzed by principal component analyses (PCA). A first instar larvae are common for both neuter and reproductives lineages. Winged line comprises five nymphal steps plus alates, as the pattern observed for all Termitidae species. The apterous line included two 2nd larval instars (small and large) that originate small and large workers, respectively. 1st small worker could molt to 2nd small worker or soldier, via presoldier molt. Large worker line comprised 3 different instars. All small workers and soldiers were male, while large workers were female. Soldiers were monomorphic. The development pathway of *N. ephratae* is similar to the general pattern previously described for *Nasutitermes*, except for a lower number of large worker instars and the absence of dimorphic soldier line.

Keywords: Caste system; Morphometry; Polymorphism; Nasutitermitinae

INTRODUCTION

In higher termites (family Termitidae), the caste development diverges at first molt between neuters and sexual lines. The neuter or apterous line comprises second (and sometimes a third) larval stage, presoldiers, and active workers and soldiers. The sexual or nymphal line includes five different instars - characterized by growing wind pads - and imagoes (Noirot 1969; Roisin and Pasteels 1987). Among Termitidae species, the development patterns of the nymphal lineage are very constant, while the apterous lineage may comprises a variable number of categories of individuals which may differ by size, morphology, pigmentation and behavior, according genera-species patterns (Roisin 2000).

For subfamily Nasutitermitinae, it has been shown that after the first molt of the neuter caste, a new bifurcation occurs that gives rise to two lines of workers (Noirot 1955). Male larvae molt to minor workers and female larvae to major workers, characterizing a sexual dimorphic ontogeny (Roisin and Pasteels, 1986). In some species, like *Velocitermes heteropterus* and *Nasutitermes corniger*, two or three size-different soldiers and presoldiers morphotypes are also described (Haifig et al. 2012; Lima et al. 2013). However, has been shown that some Nasutitermitinae, as *Subulitermes denisae*, *Coatitermes clevelandi* and *Constrictotermes cyphergaster*, have no polymorphic worker and soldier lineages (Roisin 1996; Moura et al 2011), indicating plasticity in ontogeny development for the subfamily.

Considering *Nasutitermes* genus, a temporal polymorphism is also observed in workers that can go over several successive instars, each of them separated by a molt. These molts normally produce a small size increase, but there is always an increase in pigmentation and sclerotization (Noirot 1989; Roisin 2000). By these criteria, generally are described two or three different instars of minor workers and 3-5 of major workers.

Studies regarding the ontogenetic development in termites have been shown important for phylogenetic relationship determination (Roisin 1992; Miura et al. 1998) and as a crucial step for polyethism analysis (Miura et al. 1998; Haifig et al. 2012). In this paper, we characterize the caste development system of *Nasutitermes ephratae*, a Neotropical wood-feeder Nasutitermitinae, and identify the polymorphic stages for the non-reproductive lineage. The conclusions will use as basis for analysis size/sex and age physiological polyethism in this species that will appear elsewhere.

MATERIAL AND METHODS

Termite Sampling

Natural arboreal colonies of *N. ephratae* were collected in the Atlantic Forest fragment that comprises the Buraquinho Complex Forest, located in João Pessoa, State of Paraíba – Brazil (7°08'18.9"S; 34°50'34.7"W). Before collection, nest measurements were taken and volumes were calculated using the formula for an ellipsoid: $V = 4/3\ \pi\ hDd$, where h = ½ nest height, D = ½ widest diameter, and d = ½ narrowest diameter.

The colony samples were collected in a quarterly basis approximately, every three months, during one year, from May/2013 to March/2014 to sample all of the instars and to analyze any possible seasonal influences on their production. Two nests were sampled in each expedition, comprising a total of eight nest: A) 25 L; B) 84 L; C) 14 L; D) 56 L; E) 13 L; F) 62 L; G) 78 L; H) 20 L.

The nests were packaged in plastic bags and taken intact to the Laboratory of Termitology, where they were carefully dissected, broken into pieces and termites separated from nest debris by successive water flotation. Collected individuals were fixed in FAA solution (37% formalin : acetic acid : ethanol = 1:1:3) and subsequently stored in 70% ethanol.

Morphometric Analyzes

The morphometric analyses were performed by measuring the most relevant body parts to separate Nasutitermitinae instars, following Koshikawa et al. (2002) and Moura el al (2011). Eight measurements were taken: **1.** HL (head length), from the distal tip of the mandible to the posterior margin of the head; **2.** HW (maximum head width), including the eyes, when present; **3.** HH (maximum head height), excluding the postmentum; **4.** PL (pronotum length); **5.** PW (pronotum width); **6.** MW (mesonotum width), including wing buds, when present; **7.** TL (tibia length), of the left anterior tibia; and **8.** FL (femur length), of the left anterior femur. A logarithmic transformation was made to equalize proportional increases from one instar to the next and to linearize allometric relationships, as suggested by Roisin and Pasteels (1986).

The measurements were taken using a stereomicroscope Leica® S8APO with a digital camera DFC295, using the software *LAS Interactive Measurements,* according the manufacturer instructions; standardizations were based on the study by Roonwal (1970). A total of 613 individuals were measured.

Morphometric differences among the instars were analyzed using principal component analysis (PCA), employing the morphometric data from all of the individuals measured. The significances of these differences were evaluated separately for individuals of the apterous and nymphal lineages using ANOVA (one-way), with the scores of the first principal component with *a posteriori* Tukey test. All calculations were realized using the software Statistica 13.0 (StatSoft, Inc. 2016).

Rearing Experiments

In the laboratory studies of developmental pathways, termites were reared in closed 9 cm Petri dishes containing filter paper and moisture for nutrition and some inner mound material. Larval and worker instars were classified and separated based on head measurements. Different instars of larvae, workers and soldiers were combined to form ten unique sets, as listed in Table 1. Two replicates for each set were prepared, totalizing twenty rearing plates. Petri dishes were kept in the dark at room temperature during 22 consecutive days and checked daily.

Sex Determination

Sex determinations were carried out on workers and soldiers as described by Noirot (1955). Their abdomens were cut in the frontal plane and the dorsal half of the abdomen and the gut were removed. The abdominal cuts were prepared for staining by dehydration in absolute alcohol (PA, pro analysis) (5 min) and cleared with xylol (5 min); excess xylol was then removed in absolute alcohol (PA) (5 min). Specimens were stained with chloride carmine for 60 min and then cleared in hydrochloric alcohol (0.5% HCl in 80% ethanol) for 5 min.

Caste Proportion Estimation and Seasonality

Three subsamples of fixed termites with 5 g each were taken from each colony sample. The termites were separated according their respective caste and counted to estimate the proportion among instars. Due to the difficult of separation of larval and the first two nymphal instars, they were excluded of the caste proportion analysis, but used to check seasonality on instar production along the year. Developmental stages of polymorphic worker caste were determined by head pigmentation/sclerotization and size taken by morphometry, as described above. Seasonality was verified combining the presence/absence of each instar along the year and rainfall accumulation between May/2013 and March/2014. The dry and rainy seasons were defined as the complete rainfall accumulation between January/1997 and December/2014 (Proclima, 2016).

RESULTS

The results of the PCA were plotted using the first and second principal component axes (Fig. 1). The first principal component (PC1) explained 88.34% of the total variance, was negatively correlated with all of the variables and thus gives a general measure of the individual's size; the second principal component (PC2) explained 9.43% of the total variance, was positively correlated with the variables HH, HL, HW, FL and TL and opposes individuals with wing buds (nymphs) to those with a frontal projection (nasus) on the head, as presoldiers and soldiers.

The variables that had the greatest influence on the PC1 were HW, FL and TL while HL, PW and MW had the greatest influence on the PC2. The remaining five components together represent only 2.23 of the total variance and are hereafter neglected.

A total of 16 instars were observed (Fig. 2). The nymphal line in *N. ephratae* comprises five instars plus the alate, as occurs in other members of Termitidae. ANOVA performed on the scores of the PC1 demonstrated that there were significant morphometric differences between nymphal and apterous lineages (F = 133.9; P < 0.05; Tukey, P < 0.05) were observed. Among castes of apterous lineages, there was also significant morphometric differences (F= 3435; $P < 0.05$), with all of the instars being significantly different among themselves (Tukey, $P < 0.05$). This was also true for castes among nymphal lineages (F= 35565; $P < 0.05$; Tukey, $P < 0.05$).

The second instar larvae are polymorphic, sorting into two size classes, corresponding to an expected sexual dimorphism, being small larvae (SL2) male and large larvae (LL2) female. Worker caste has not only a size but also an age polymorphism, easily recognizable by the level of sclerotization of the cephalic capsule. Two age morphotypes were observed for small workers (SW1 and SW2) and three for large workers (LW1, LW2 and LW3) (Fig. 3). Pre-soldiers (PS) and soldiers (S), in the other hand, are monomorphic.

The rearing experiment corroborated the results of morphometric analysis regarding the proposed ontogeny for *N. ephratae*. SL2 and LL2 always molted to SW1 and LW1, respectively. Seven LW1 changed to LW2 via new molts, however no LW2 molted to LW3. Three SW1 from plates E, F and J molted into pre-soldiers (Fig. 4); and three other SW1 from plate I molted into SW2. Regarding sex determination, a rudiment of spermatheca was present on the eighth sternite of large workers, indicating they were female. This structure was absent in small workers and soldiers, indicating these instars were formed by males corroborating the results of rearing experiment about the origin of presoldiers from small workers.

All larval and neuter instars were present into colonies over the entire year. Alates were collected only in May.2013, which correspond to the middle of rain season to the region (Proclima, 2016); and 3º, 4º and 5º nymphal instars were found from December.2013 to March.2014. We also verified a reduction in the number of soldiers during production of alate nymphal instars. No pattern among caste proportion along the year was observed (Table 2). Regarding the reproductive lineage, *N. ephratae* queens were encountered in 7 of the 8 nests collected, with two queens being found in one of them. Only one king was recovered from all colonies. All nests had only one queen (monogyny), except for one where two queens were present, indicating the occurrence of polygyny for this species. No microimagoes or secondary reproductives - as nymphoids, adultoids or ergatoids - were found.

DISCUSSION

The caste system of *N. ephratae* resembles those of other species of *Nasutitermes* that have been studied in detail, particularly *N. arborum, N. maculiventris, N. lujae* (Noirot 1955; Pasteels 1965), *N. exitiosus* (McMahan and Watson 1975), *N. princeps* (Roisin and Pasteels 1986) and *N. corniger* (Lima et al. 2014), with a sole L1 instar, a dimorphic L2 (small and large) and also dimorphic worker caste. Similar to other species of *Nasutitermes*, those morphometric differences between large and small workers are the expression of a sexual dimorphism, being the large workers female, and the small workers male.

Differences on morphometric measurements and the level of head pigmentation among both small and large worker lines were considerate as an indicative of age polymorphism. For some other species (i.e.: *N. exitiosus*) several more LW instars, as LW4 and LW5 were suggested (McMahan and Watson 1975), but for *N. ephratae* the separation among morphotypes was not clear enough to enable the identification of more than three LW instars.

In other species of *Nasutitermes*, additional SW3 and LW3-LW5 instars were described as a product of new molts (Noirot 1955; Roisin and Pasteels 1986). Noirot (1955) has indicated that in *N. arborum,* the SW2 can molt, producing a small presoldier or possibly, a SW3. However, the evidence for the existence of SW3 was tenuous, and Noirot (1969) omitted the stage in a later account of the development of *N. arborum.*

During our rearing experiment, we could not observe any SW2 or LW2 molting into SW3 and LW3, respectively; or LW3 molting to LW4-LW5. MacMahan and Watson (1975) made a long-time rearing experiment with *N. exitiosus* and these authors also could not observe any LW2 molting into LW3-LW5 or SW2 molting to SW3.

Despite several studies regarding caste systems in *Nasutitermes* could clearly separate by morphometry several SW and LW instars, we speculate, based in the absence of an observable molting in SW2 and LW2 instars, that both are very persistent instars that rarely molt to larger forms.

The molts which insure the eventual growth of the insect without notable structural modification and mark the development of workers, as happen with SW1 to SW2 and LW1 to LW2, were qualified as stationary molts by Noirot and Pasteels (1987). This kind of molts can lead to a misinterpretation of the real ontogenetic system in termites with polymorphic neuter lines. Hojo et al (2004) described for *N. takasagoensis* (Shiraki) a trimorphic worker lineage, being small, medium (MW) and large workers, where MW and LW were female ones. The same trimorphic pattern is also described for other Nasutitermitinae species, as *Hospitalitermes medioflavus* (Miura et al 1998).

Our data suggest a slight increase in body size after each molting, ergo, for trimorphic worker lineages, we consider that the medium worker is in fact the SW2 or LW1 and the considered large workers are just LW2-LW5 later instars. The rearing experiment corroborates the idea of non-trimorphic worker lineages in Nasutitermitinae, because LL2 always molted to LW1 and, additionally, we found several depigmented LW intermediate instars, resulting from new molts of LW1 to LW2.

We also verified that SW1 can molt into presoldier; and SW2 is a very inconstant and rare instar in all collected nests. Similar results were reported for *N. exitiosus* and *N. corniger* (McMahan and Watson, 1975; Lima et al, 2014). SW2 as an uncommon instar in natural colonies indicates that SW1 remains longer in that stage as a reservoir for soldiers reposition, via presoldier molt.

Regarding soldiers, only one morphotype was observed. Polymorphic soldier caste was described for several species of *Nasutitermes* mentioned above, but evidences of monomorphic soldiers were also observed for *N. novarumhebridarum* (Roisin and Pasteels, 1987). In *N. ephratae,* however, only SW1 was able to molt to presoldier, not SL2, as in *N. novarumhebridarum*.

The monomorphic soldiers present in *N. ephratae* could reflect the adoption of different strategies for defense and/or foraging tasks. In *N. exitiosus*, large soldiers show a non-combative behavior, flee when attacked and possibly act as messengers, raising an alarm in the nest (Kriston el al., 1977). This strategy could particularly efficient for open-air foraging strategies, especially before workers recruitment. But *N. ephratae* build tunnels as trails and are rarely found outside it during foraging activities, making unnecessary the presence of scout-large soldiers.

Roisin (1992) suggested that a high intracaste variability – resulting in a continuous polymorphism – can represent a secondary adaptation of termites to heterogeneous foraging environment, as litter in the forest floor. A phylogenetic clade comprising *Nasutitermes*, *Trinervitermes*, *Velocitermes*, and *Hospitalitermes* that all demonstrate dimorphisms and sexual specializations in their apterous castes was proposed by Miura et al. (1998).

The most recent termite phylogenies (Inward et al., 2007; Bourguignon et al, 2014), however, do not corroborate monophyly among these genera solely based on ontogeny, because non-polymorphic Nasutitermitinae genera as *Subulitermes*, *Coatitermes* and *Constrictotermes* (Roisin, 1996; Moura et al, 2011) form intermediate branches between *Velocitermes-Hospitalitermes* and *Hospitalitermes-Trinevitermes-Nasutitermes* clades. Thereby, ontogenetic polymorphism seems not to be a remarkable phylogenetic character and that high plasticity in the number and type of instars can be due to specie-specific factors or suffer external influences from the environment.

Considering the proportion among castes and seasonality, we could not verify a fixed proportion among castes or morphotypes along the year. The numbers vary with the nest volume and the presence/absence of nymphal lineages. During nymphs production period, a reduction in the number of soldiers was evident, suggesting a strategy to save energy in defense to be used in reproduction.

A high intraspecific variability in numerical neuter castes ratios was observed for *N. corniger* and *N. ephratae* by Thorne (1985). This author pointed that colony size, age, proximity to resources, individual history, season and vulnerability to predators could all simultaneously affect caste composition. In our study, the reduce number of nests sampled and the high variation in nests volumes also may have interfered in the precision of caste proportion estimation.

All instars except reproductive lineage were collected over along the year. Alates were found only inside nests sampled during the middle of rainy season (May/2013). A similar result was observed by Thorne (1983) for *N. corniger*, being the alates liberate from the middle to the end of rainy season.

In the other hand, later nymphal lineages were present in all nests collected between December/2013 and March/2014, but the first one collected in December. This absence of nymphs can be due to the reduced volume of this nest (12,83 L), indicating an immature colony that was investing energy in nest growing instead reproduction. Maturation of nymphs during the interval comprising the end of dry and beginning of rainy seasons (Dec/2013 to Feb/2014), corroborates our finds regarding alates production and maturation from the middle to the end of rainy season.

Queens were always found inside royal chamber or close to that. A polygynyc colony was also dissected, though *N. ephratae* is a predominant monogynic species, as *Nasutitermes coxipoensis*. This observation of rare polygyny in *N. ephratae* confirms earlier observations of this phenomenon to this termite species (Becker, 1961, Mathews, 1977).

The Neotropical termite *N. ephratae* has a polymorphic ontogeny as other Nasutitermitinae, but with a monomorphic soldier lineage. Differences in size among workers are a result of sex polymorphism and variations in sclerotization of head capsule are the principal character to define age polymorphism in this lineage. Polymorphism in neuter caste also can be an indicative of polyethism in colonies of *N. ephratae*. The characterization of the caste system in close-related *Nasutitermes* species, as *N. coxipoensis*, could help to understand the importance of ontogeny for the termite phylogeny, ecology and evolution.

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Table 1. Scheme of rearing experiment using *Nasutitermes ephratae*.

Plate Identification	Instars		
A	10 Small Larvae	25 Large Workers	-----------
B	10 Small Larvae	25 Large Workers	5 Soldiers
C	10 Large Larvae	25 Large Workers	-----------
D	10 Large Larvae	25 Large Workers	5 Soldiers
E	10 Small Larvae	15 Small Workers	-----------
F	10 Large Larvae	15 Small Workers	-----------
G	30 Small Workers 1	-----------	-----------
H	30 Small Workers 2	-----------	-----------
I	20 Small Workers 1	10 Large Workers	5 Soldiers
J	20 Small Workers 1	10 Small Larvae	10 Large Larvae

Table 2. Proportions among castes from *Nasutitermes ephratae* collected along over one year in Atlantic Rain Forest, Brazil.

Date of Collection	Nest Volume (L)	Workers	Soldiers	Presoldiers	Alates	Nimphs (N3-N5)	SW1	SW2	LW1	LW2	LW3
May.07.2013	25.13	648.7 (34.4)	86.0 (10.4)	14.3 (5.1)	44.3 (6.8)	0.0	140.4 (7.5)	27.3 (1.4)	201.5 (10.7)	163.8 (8.7)	115.7 (6.1)
May.13.2013	84.45	424.3 (7.1)	81.0 (21.7)	9.3 (1.5)	49.3 (4.0)	0.0	40.7 (0.7)	30.8 (0.5)	105.0 (1.8)	114.0 (1.9)	133.9 (2.2)
Aug.27.2013	13.82	497.0 (24.2)	73.3 (15.3)	9.7 (5.1)	0.0	0.0	38.2 (2.3)	23.5 (1.4)	123.5 (7.4)	158.8 (9.5)	152.9 (9.1)
Aug.30.2013	55.61	587.0 (34.0)	109.7 (1.5)	10.7 (6.5)	0.0	0.0	74.2 (5.3)	11.9 (0.8)	220.0 (15.6)	139.1 (9.9)	141.8 (10.1)
Dec.06.2013	12.83	652.7 (18.6)	77.3 (18.5)	19.3 (7.2)	0.0	0.0	120.4 (4.2)	18.2 (0.6)	233.6 (8.2)	155.3 (5.4)	125.2 (4.4)
Dec.08.2013	62.20	559.0 (21.2)	28.7 (9.7)	42.0 (10.8)	0.0	73.0 (4.0)	114.7 (5.3)	20.0 (0.9)	209.5 (9.7)	128.4 (6.0)	66.3 (4.0)
Feb.10.2014	78.13	475.0 (13.9)	35.7 (7.5)	4.3 (1.2)	0.0	45.3 (7.6)	96.9 (3.5)	33.5 (1.2)	129.5 (4.6)	127.6 (4.6)	87.5 (3.1)
Feb.14.2014	19.90	564.3 (37.4)	21.0 (4.4)	7.7 (1.5)	0.0	23.3 (4.7)	117.7 (9.5)	9.4 (0.8)	250.9 (20.4)	140.6 (11.4)	45.8 (3.7)

*Numbers in parenthesis indicate standard-deviation.

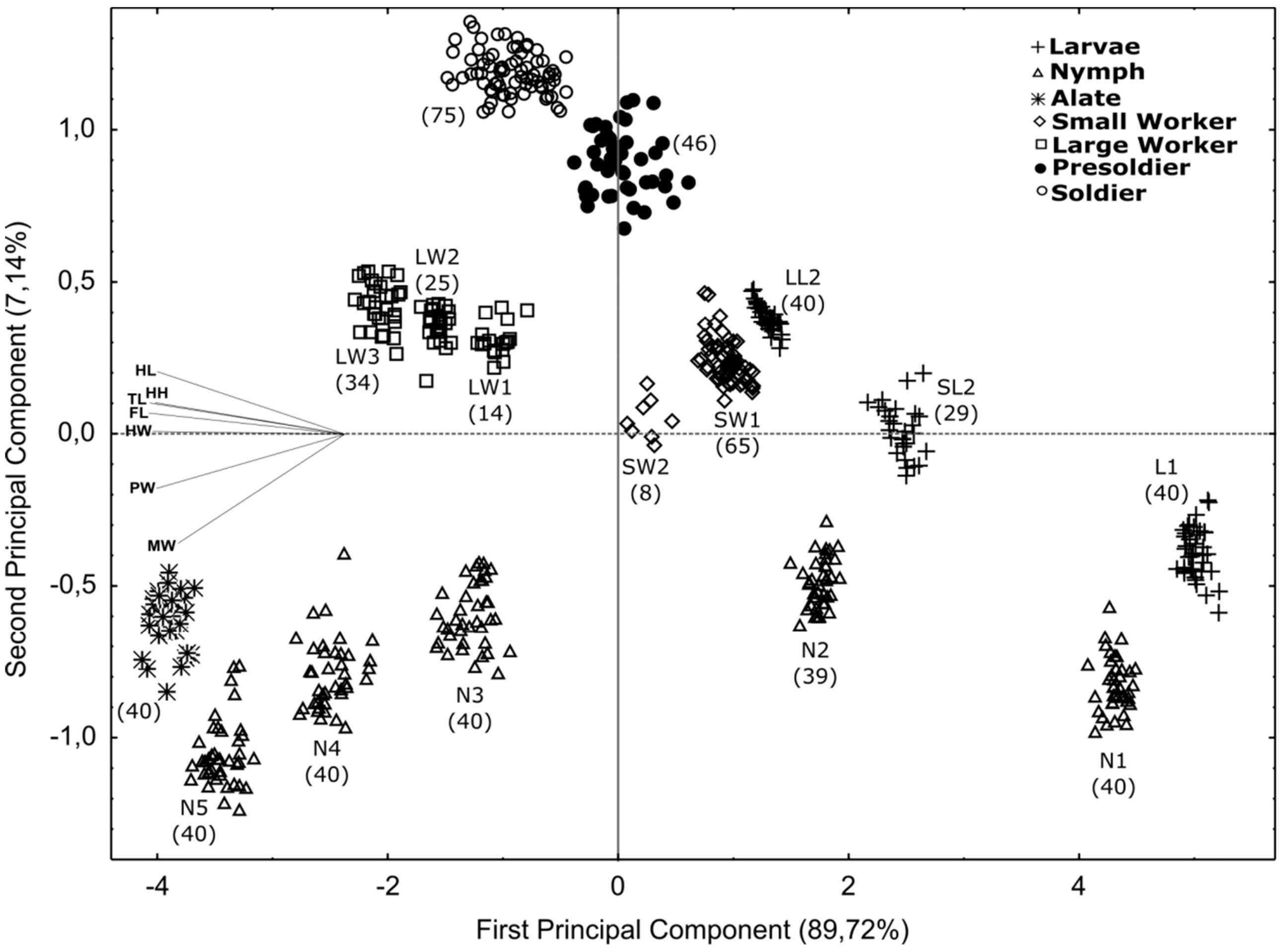

Fig. 1 - Principal component analysis, plotted from the first and second principal component axes, evaluating morphometric changes among instars of *Nasutitermes ephratae*. The values within parentheses indicate the numbers of measured individuals per instar. See text for information about abbreviations.

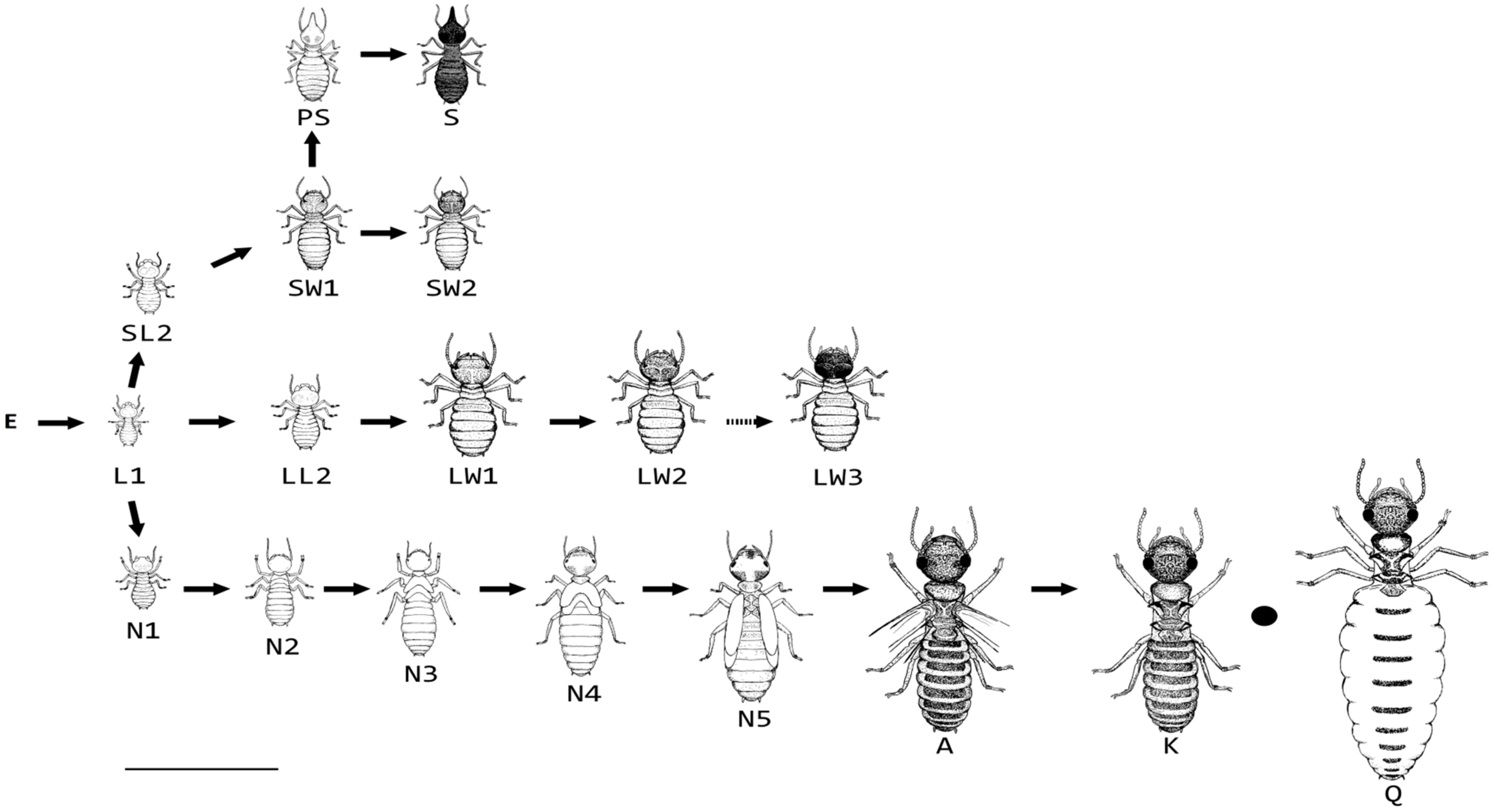

Fig. 2 - Schematic representation of the caste developmental pathways in *Nasutitermes ephratae*. E: Egg; L1, SL2 and SL2: larval instars; SW1, SW2, LW1, LW2 and LW3: worker instars; PS: presoldier; S: soldier; N1-N5: nymphal instars; A: alate; K and Q: Royal couple. Each *arrow* symbolizes a molt. The *dotted arrow* indicates an unobserved path. *Scale bar*: 5 mm.

Fig. 3 - Polymorphic worker types in *Nasutitermes ephratae*. A: Small Worker 1 (SW1); B: Small Worker 1 (SW2); C. Newly-molted Large Worker 2 (LW2); D: Larger Worker 1 (LW1); E: Larger Worker 2 (LW2); F: Larger Worker 3 (LW3).

Fig. 4 – Small worker 1 molting into presoldier. The nasus has already emerged and the presoldier was in process of ecdysis.

CAPÍTULO II

CHANGES IN COMMUNITY STRUCTURE OF THE BACTERIAL GUT MICROBIOTA INDICATE THE OCCURRENCE OF AGE POLYETHISM IN THE WOOD-FEEDER HIGHER TERMITE *Nasutitermes ephratae*

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Changes in Community Structure of the Bacterial Gut Microbiota Indicate the Occurrence of Age Polyethism in the Wood-Feeder Higher Termite *Nasutitermes ephratae*

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Abstract

Division of labor, i.e., polyethism, is one of the most important traits in social insects, since, by promoting the synergetic effect, it increases the efficiency of total colony activity, and maintains colony integrity. In higher termites (family Termitidae) several polyethism traits were already documented – as size, sex and age polyethism or a combination of these factors – regarding a broad range of tasks, as foraging, defense, nest construction and food processing. The quality of the diet is known to be a driver for the structure of the gut microbiota community in termites. Here we investigate the intestinal symbionts bacteria in five worker instars of different size and age and the soldier of a wood-feeder termite *Nasutitermes ephratae*. The bacterial succession across stages was analyzed by 16S rRNA gene deep sequencing. A remarkable change in the abundance and species richness was observed in the transition from young to old instars, independent of size. Spirochaetes and Fibrobacteres, dominant in the young workers, are harshly reduced in the older workers, followed by an increase in the Bacteriodetes, Firmicutes and Proteobacteria being consistent with the previously observations that these last three phyla are more abundant according to the humification content of the diet. The taxonomy-to-phenotype mapping also supported the hypothesis of diet segregation among workers of *Nasutitermes*. Regarding food processing, we postulate that older instars are responsible only for food collection, while younger workers are more involved in cellulose digestion and N2 fixation, being responsible to fed the older ones. Our findings also provide support for the concept that changes in diet are important determinants of community structure due to provide new niches to be explored by microbial symbionts.

Introduction

Termites host an abundant and diverse gut bacterial community, which plays essential roles in their carbon and nitrogen metabolism [1-4]. Termite guts are highly structured microenvironments and characterized by quite distinct physicochemical gradients, providing different niches for a diverse intestinal microbiota. As a counterpart, symbionts allow termites to process recalcitrant lignocelluloses, comprising a large scale of diets, ranging from wood-feeders - passing by grass, dung and humus-feeders - to strict soil-feeders [5-6].

On the last decade, several studies significantly advanced our knowledge regarding the gut microenvironment, microbial community structures and functional roles of the gut microbiota in several termite species [4, 7-9]. Despites the majority of gut bacteria in termites are uncultivable so far, their complex community structure and have been gradually revealed using culture-independent approaches.

Comparisons between colonies, locations and host termite species were made to determine the bacterial community structure in termite guts. A basic similarity of the community structure among congeneric hosts was observed for the genera *Reticulitermes*, *Microcerotermes* [7], *Cubitermes* [10] and *Nasutitermes* [11]. Adaptations of gut symbionts to their respective host lineages (co-evolution) was postulated as to be reflect of the vertical transmission of the gut microbiota by trophallaxis – or coprophagy [7,11,12,13,14].

In higher termites, the diversifications of diet and feeding strategies have created new niches for microbial symbionts. Dietary diversification apparently involved certain adaptations in mandible morphology [15,16], intestinal anatomy [17,18] and physicochemical gut conditions [10,11,19-21]. Because dietary diversification is part of the evolutionary history of higher termites, the influence of diet on gut community structure cannot be easily separated from that of host phylogeny [22,23].

These studies regarding structure of microbial communities in termite guts, however, assumed that the gut samples from several worker termites represent, at least, all worker morphotypes in the whole colony. This may be true when the food, tasks and morphologies are not much different among individuals within a colony. However, some termite groups are known to employ a highly elaborate system of division of labor, or polyethism, accompanying changes in food among castes and ages [24].

Those features add new levels of complexity to gut colonization and successional changes during the life cycle of an individual coincident with changes of role within the colony. Despites the distinct patterns in the microbial communities of the major termite groups clearly reflect the evolutionary history of the host [13,14], the factors driving community structure in the individual groups remain unclear.

Polyethism in food processing has been shown as an important secondary driver of microbial community structure in species of Macrotermitinae [24,25]. In subfamily Nasutitermitinae, the most apical subfamily of higher termites, studies also have suggested the occurrence of polyethism regarding food processing among different neuter instars of *Nasutitermes* spp., i.e., as the nitrogen source [26] and cellulose digestion [27], but nothing is known about the importance of polyethism for microbial community structure in *Nasutitermes* genus.

In this paper we investigate whether the composition of the gut microbiota changes in response to size and age of different worker instars of *Nasutitermes ephratae*, a wood-feeder Nasutitermitinae, and draw conclusions regarding the functional role of individual lineages in lignocelluloses digestion and nitrogen fixation.

Materials and Methods

Termite sampling

Three nests of *N. ephratae* were collected in the Atlantic Forest fragment that comprises the Buraquinho Complex Forest, located in João Pessoa, State of Paraíba – Brazil (7°08'18.9"S; 34°50'34.7"W), with the permission of Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio – Brazil; permission number: 49720-2). This field study did not involve endangered or protected species. Nests were involved in plastic bags, transported to the Laboratory of Termitology (Universidade Federal da Paraíba) and processed immediately after collection.

The nest was partially broken to sample worker and soldier castes. Five worker instars (Small Worker 1 - *SW1*; Small Worker 2 - *SW2*; Large Worker 1 - *LW1*; Large Worker 2 - *LW2*; Large Worker 3 - *LW3*) and one soldier instar (*Sd*) were distinguished by morphometric measurements, as well the sclerotization level of the cephalic capsule as described previously [28].

Gut dissection and DNA extraction

Mixed samples from all three nests were prepared using representatives of each instar. A total of 30 individuals of each stage (10 from each nest) were used. Termites were degutted with sterile fine-tipped forceps, and the hindguts were separated from the crop and midgut with a scalpel. No remarkable differences were observed in gut external morphology besides of the gut size between small and large workers. After dissection, hindguts were preserved in RNAlater solution and kept frozen until DNA isolation. Hindguts were pooled for each sample in 1.5 ml sterile microtubes and then extracted using the NucleoSpin® Soil Kit (Macherey-Nagels) in accordance with manufacturer's instructions.

Library preparation and processing of 16S rRNA gene sequences

Gut bacterial communities in all six instar from *N. ephratae* were analysed using Ilumina MiSeq System sequencing. We used gene-specific primers targeting the V3-V4 region of 16S rRNA [29] contained proper adapters and indexes, as recommended by the manufacturer. Amplicons were mixed in equimolar amounts and sequenced commercially (GATC Biotech, Konstanz, Germany).

The resulting sequences were analyzed using *mothur* v.1.33.3 [30] using the method described by Schloss [31]. Sequences were denoised to reduce sequencing error, and low-quality sequences (minimum length 200 bp, allowing 1 mismatch to the barcode, 2 mismatches to the primer, and homopolymers no longer than 8 bp) were removed. Sequences were then trimmed to keep only high quality reads (Q_35).

Chimeras were removed using the c*himera.uchime* command implemented in *mothur*. Singletons were removed from the analysis. Sequences were aligned and classified according to the SILVA bacterial SSU reference database v.102 [32]. They were then assigned to genus-level phylotypes using the naive Bayes classifier implemented in *mothur* and clustered into operational taxonomic units (OTU) using the average neighbor algorithm and a sequence identity cutoff of 97%.

DictDb reference database v.3.0 [33] were used for the taxonomic assignment of OTUs. The Candidate phylum TG3 was manually grouped in a single phylum together with

Fibrobacteres, following the classification proposed by Rahman et al [34]. UPGMA distances between all communities at the genus level were calculated, subjected to hierarchical cluster analysis and visualized as a dendrogram with the PVCLUST package [35] in the R statistical software suite [36].

In each library, the relative abundance of an OTU was assigned as the percentage of reads included in this OTU with respect to the total number of reads. The relative abundance of each taxon in a given sample is the sum of abundances of all the OTUs included. The shared OTUs (97% sequence identity) between and among instars were determined using the web-based tool InteractiVenn [37] that allows to compare up to six samples at same time. Resulting data were ploted and exported directly from InteractiVenn.

Metabolic Profiles

OTUs were automatically taxonomy-to-phenotype mapped using 16 different metabolic phenotypic categories using the online software METAGENassist [38]. Differences among the instars were analyzed using principal component analysis (PCA) considering the abundance of OTUs at phylum-level and the metabolic profiles for 13 unique microbial traits, using the PCA tool implemented into METAGENassist.

Results

MiSeq sequencing produced a total of 240646 raw reads for the six samples. After quality filtering and removal of chimera, 63761 high quality reads were recovered. In order to avoid bias attached to an unbalanced library size, the data were normalized based on the size of soldier library that had the lower number of sequences (1416). The same number of sequences was, therefore, randomly subsampled in the remaining instars. The resulting sequences from these three stages were assigned to 662 OTUs unequally distributed depending on the stage. After the removal of singletons, the 931 remaining sequences per sample were clustered at 97% sequence identity into 355 OTUs and classified into 16 bacterial phyla. OTUs per sample ranged from 115 (Sd) up to 175 (SW2). Classification success with the termite-specific reference database (DictDb) ranged from 100% at the phylum level to 80-100% at genus level (Supplementary Table 1).

Most of the OTUs fell within the following phyla: Spirochaetes (52%), Fibrobacteres (20.9%), Bacteroidetes (12.7%), Firmicutes (8.2%), Proteobacteria (2.2%) and Candidate phylum TM7 (1.86%). The remaining OTUs fell within 10 minor phyla, which their sum accounting for 1.98% of the reads belong to the phyla Acidobacteria, Actinobacteria, Chlorobi, Chloroflexi, Deferribacteres, Elusimicrobia, Planctomycetes, Synergistetes and Verrucomicrobia (Fig 1).

The abundance of Spirochaetes and Fibrobacteres was higher in younger instars than in older ones. The transition from younger to older instars was followed by a remarkable increase in the abundance of Bacteroidetes and Firmicutes, and a slight increase in the abundance of Proteobacteria and Candidate phylum TM7. The soldier microbiota appears to be more similar to SW1 microbiota, except for an increase in Spirochaetes (c.a. 10%), the absence of Candidate phylum TM7 and a remarkable reduction in the abundance of Fibromonadacae.

Among Spirochaetes, the taxon was dominated by *Treponema* If, *Treponema* Ic and *Treponema* Ia clusters. *Treponema* If was the most abundant genera in all instars sampled, especially in soldiers in which this taxon alone corresponded for circa 34% of sequences recovered. In the transition from SW1 to SW2 was observed a reduction of 5% in the abundance of *Treponema* If and 7% in *Treponema* Ic. From LW2 to LW3, we noted a reduction of 7.5% the abundance of *Treponema* If and 8.5% in *Treponema* Ic. *Treponema* Ie was present only in LW2; and *Treponema* Ih was absent in LW1 and LW2 instars (Fig 2).

Considering Fibrobacteres phylum, it corresponded for c.a. 17-25% of all sequences obtained, depend on instar. SW1 was the instar with the major presence of total Fibrobacteres. Intriguingly, after SW1 molt to SW2, occurs a reduction in the amount of Fibrobacteria (8%) but an increase in Chitinivibrionia (c.a. 3.5%). A similar reduction in Fibrobacteria is also noted after SW1 molt into Sd. LW instars appear to have a relatively constant abundance of Fibrobacteres, having been observed an increase in the abundance of Chitinivibrionia (around 4-5%) after LW1 molt into LW2 and LW3 (Fig 3).

Bacterioidetes, the third most abundant phylum, ranged from 20% of total microbial OTUs in SW2 and 16% in LW3, to less than 11% in the other instars. SW1, LW1 and LW2 demonstrated similar abundance and OUT richness for Bacterioidetes. The moults of SW1 into SW2 and LW2 to LW3 is followed by an increment of Rikenellaceae and all Porphyromonadaceae clusters, while in the soldier only a gain of Rikenellaceae is noted (Fig 4).

Regarding Firmicutes, all instars were dominated by the families Lachnospiraceae, Ruminococcaceae and Peptococaceae 1, except soldier, in which Streptococcaceae was more abundant than Peptococcaceae 1. The transition of SW1 to SW2 shows a slight enhance in the abundance and species richness of all Clostridiales clusters and Lactobacilales. The moult of LW1 in LW2 results in a decrease in the abundance of Lachnospiraceae and Peptococaceae 1. However, after LW2 moult into LW3, we can observe an augmentation of the two families plus Ruminococcaceae and unclassified Lactobacilales. OTUs belong to families Peptococaceae 2 and Eubacteriaceae 1 were retrieved only from LW1 and LW3, respectively (Fig 5).

Proteobacteria OTUs responded for 1% of total microbial community in LW2 until more than 3% in SW2. Desulfovibrionales was the most abundant representative in all instars, but LW2, in which Myxococcales, Desulfarculales and members of F-1404R cluster were more copious. Transitions between instars seem to be similar to that observed for Firmicutes. Myxococcales and Desulfovibionales are more plenty in SW2 than in SW1. LW1 is the richest in the number of species when compared with any other instar, but a reduction in abundance and richness is observed after molt in LW2, followed by a remarkable increase in the abundance of Desulfovibionales and F-1404R OTUs after molt into LW3. Several Proteobacteria OTUs were found to exclusive to specific instars, as Bdellovibrionales were recovered only from SW1; Pseudomonadales from SW2; representatives of an unclassified Deltaproteobacteria and TA18 cluster from LW1; Syntrophobacterales from LW2; and Xanthomonadales from LW3 (Fig 6).

OTUs representing the phylum Actinobacteria accounted for less than 0.03% of all sequences recovered. However, a surprisingly rise in species richness was observed in older instars when compared to younger ones. In SW1, LW1 and LW2 only sequences belong to family Sanguibacteriaceae was observed, while for SW2 and LW3 members from 5 and 7 different families were retrieved, respectively. For the old workers, Actinobacteria respond for around 0.65% of all SW2 sequences; and for 1.3% of LW3 total OTUs. None Actinobacteria OUT was recovered from soldier sample (Fig 7).

The bacterial communities in the hindgut of younger worker termites (SW1, LW1 and LW2) were different from those associated with older instars (SW2 and LW3) at phylum level. LW1 and LW2 had a similar gut microbiota, making a group that clustered together with SW1. Older instars SW2 and LW3 also showed a congruent microbial community, forming a separated branch from younger workers. Gut bacteria from soldier were the most dissimilar within the instars, forming an exclusive branch that clustered with younger workers (Fig 8).

Venn diagram grouped all six *N. ephratae* instars and showed that 17 up to 53 OTUs were exclusive to any specific instar. 43 OTUs were shared by every instar. 81 OTUs were shared between SW1 and SW2; 60 between SW1 and Sd; 75 between SW1 and LW1; and 57 among younger instars SW1, LW1 and LW2. Young large workers shared 72 OTUs between them; and 64 among LW1, LW2 and LW3. Old instars SW2 and LW3 had 93 similar OTUs; and when soldiers were included, the total number of shared OTUs was 59 (Fig 9).

The taxonomic-to-phenotypic identify 10 different microbial metabolic traits assumed to be present in, at least, 1% of all mapped OTUs (an exception was made to keep the trait “cellobiose degradation”, even it been present in less the 1% of all OTUs). Four traits were relative to carbon degradation, three to nitrogen metabolism, two relatives to sulfur processing and a dehalogenation pathway. 70% to 80% of OTUs could not be mapped to any specific metabolic phenotype (Fig 10).

SW1 was the instar with the highest abundance of taxa able to digest several carbon sources, including cellulose, xylan, chitin and cellobiose. The same is true for N2 fixating microbes. However, microbes involved in carbon and nitrogen metabolism were present in all instars, including soldiers, even though it showed a remarkable less abundance. In all instars, ammonia oxidizer bacteria were more common than N2 fixing ones. SW1, SW2 and LW3 also contained a relatively higher abundance of bacteria able to dehalogenate xenobiotic halogenated hydrocarbons by fermentative, oxidative, and/or reductive pathways (not specified).

PCA analysis from both taxonomy and phenotype (metabolism) were congruent, always grouping the young instars together (Fig 11). In both PCAs, however, the most closed instar to the young ones was LW3, instead Sd, as observed in the UPGMA cluster dendrogram.

Discussion

The gut bacterial community here described for *N. ephratae* seems to be similar to those previously described for workers of *Nasutitermes* species [4,11,39,40]. The higher abundance of Spirochaetes and Fibrobacteres phyla observed in wood-feeder *Nasutitermes* is related to their role in fiber digestion in P3 intestinal segment [41]. The abundance of the remaining phyla varies according to the termite species. Firmicutes was more common in the gut of *N. arborum* [40] and *N. corniger* than Bacterioidetes, most due to the amount of Lachnospiraceae attached to the midgut wall [11]. Here, we used only the hindgut portion, ergo Bacterioidetes was the third most abundant phyla. Also, Actinobacteria and Acidobacteria seem to be more copious than TM7 in both previously described termites. In *N. ephratae*, TM7 was more common due to an increase in its OUTs number associated to the moult of young workers to older instars.

We observed that the structure of bacterial community changes according to the age of the workers of *N. ephratae*: Fibrobacteres and Spyrochaetes give way to comprise an increment of Bacterioidetes, Firmicutes, Proteobacteria and Candidate phylum TM7.

Spirochaetes and Fibrobacteres are known to be niche-specific and are associated to the hindgut paunch of wood-feeder higher termites, living as free-cells in the lumen content or attached to the fiber-fraction of ingested lignocelluloses, respectively [41]. The reduction in the abundance of these two phyla – plus an increase of Bacterioidetes, Firmicutes and Proteobacteria - was associated to changes in *N. takasagoensis* diet [42], in which the food source ranged from pure wood to sole “soft” carbons (as xylan, xylose, cellobiose and glucose).

A similar result relative to worker age in *N. ephratae* indicates that young and old instars access different food sources. Coprophagy, nest material-feeding and consumption of pre-digested food regurgitated from the crop were postulated to explain the vertical transmission of the gut microbiota among termite lineages [11;40]. Those hypotheses also can help to explain the changes in the bacterial communities associated to a probable diet transition according to termite age.

The use of different food sources depend on age of the termite was exhaustively demonstrated in Macrotermitinae species [43-46]. The origin of the food source also has influence on the gut microbiota of the termites belong to this subfamily (specifically *Macrotermes* and *Odontotermes*) [24,25]. For *Nasutitermes* species, a foraging polyethism is also documented [47-49]. Evidences pointed to a very complex division of labor in *Nasutitermes*, in which foraging termites (commonly older SW2 and LW3 or later instars) gather the food, bring it to the nest, than passed it to be digested by younger small and large worker instars [27,47].

The fact that older workers are recruited faster for foraging – and because of that, they are more exposed to infection by pathogens – can be related to the increase in abundance and richness of Actinobacteria and Proteobacteria phyla observed in those instars. Actinobacteria species related to termites are known to act as defensive symbionts, producing antimicrobial metabolites that inhibits the growing of entomopathogenic fungal [50,51]. Some Betaproteobacteria, on the other hand, have been reported to protect leaf-cutting ants also against entomopathogenic fungi [52], but a similar case was not yet documented in termites. This defensive team can be also complemented by Chitinivibionia specimens, which could degrade chitin, an important structural component of fungal cell wall [53,54].

Based on the gut microbiota, workers of *N. ephratae* can easily be classified into two groups, according to age. However, the metabolic profile suggested that all instars retain a core microbial community that allows them to realize most part of the mapped tasks, as depolymerization (cellulose, xylan), nitrogen fixation, ammonia oxidation, sulfate reduction, dehalogenation and chitin degradation.

Fibrobacteres, Spirochaetes and also Clostridiales are recognized by their ability to metabolize carbon polymer as cellulose and xylan. Nitrogen fixing genes, in the other hand, are spread in a wide range of different bacterial taxa. In *Nasutitermes*, a metagenomic approach pointed that recovered NifH genes belong to both Spirochaetes and Fibrobacteres representatives [4], and several N_2 fixing genes were also associated to termite intestinal Clostridiales (Firmicutes) [57]. The fact that ammonia oxidizer bacteria were more copious than N_2 fixing, suggest that *Nasutitermes* instars are able to use alternative nitrogen sources eventually ingested with the food, making them not so dependent on nitrogen fixation to supply the necessary N stock.

The major abundance of all metabolic traits in SW1 suggests that this instar could be the main responsible for food processing and colony nourishment. This idea is convergent with the fact that small workers of *Nasutitermes* are rarely found carrying out risky activities, as foraging outside nest or being recruited for defense or nest reconstruction after disturbance [48]. We could not infer, yet, the way how the microbial genes are expressed and regulated inside termite gut solely based on bacteria abundance of traits. Depend on host intestinal physiology, even a low abundant group of bacteria can show a higher metabolic activity.

SW2 and Soldiers, on the other hand, were mapped as the less able to degrade carbohydrate polymers and fixate nitrogen. The results for both traits were quite intriguing, especially regarding chitin depolymerization, as was observed an increase in Chitinivibrionia after moult for these two lineages. All isolates belong to Chitinivibrionia are described as able to degrade chitin [54,56]. Soldiers also presented the highest proportion of Spirochaetes among all instars. As mentioned above, members of this phylum are characterized by their dual ability to metabolize cellulose and to fixate nitrogen in *Nasutitermes* guts. Not only that, it was demonstrated that *Nasutitermes* soldiers are able to fixate up to 10 times more N_2 than workers [26,57]. Those observations plus the fact that a high proportion of taxa could not be mapped for metabolic pathways, indicate a bias in ability of the software to correctly associate phenotypes to specific taxa, but also seems to be a consequence of the lower number of bacterial isolates obtained so far from termite guts.

The Veen diagram showed the only a small fraction of all recovered OTUs is shared among all neuter instars. This fact brings up question regarding bias in microbial composition depend on how worker termites are chosen for dissection. If we consider SW2 – a very rare instar that probably is not regularly sampled for the description of the gut community of *Nasutitermes* species – that had a total of 53 unique OTUs, important information regarding bacterial species richness and abundance may also be absent. Several OTUs also seem to be instar-specific, being exclusive from a given level and changing according to the termite moult to older instars However, some of those bacteria also could only represent fortuitous microbes acquired from the environment during food gathering, tunneling or nest construction, which do not regularly colonize termite guts.

Conclusions

Polymorphic castes of *Nasutitermes* have a quite distinct intestinal microbiota in both species richness and abundance context. To our knowledge, this the first time that age polymorphism is described as a secondary driver of microbial community structure in

Nasutiterminae termites. Based in our analysis, we strongly suggest that a pool of worker instars must be employed when describing the intestinal bacterial diversity of termites, or specific worker instars must be chosen and this decision explained in methods. Our findings add support to the concept that the quality of the ingested food and the gut microenvironment are strong drivers of microbial community structure in termite guts. However, studies regarding the specific role of abundant particular bacterial lineages in host hindgut metabolisms are still necessary to stabilize the hypothesis of polyethism in food processing among neuter instars of *N. ephratae*.

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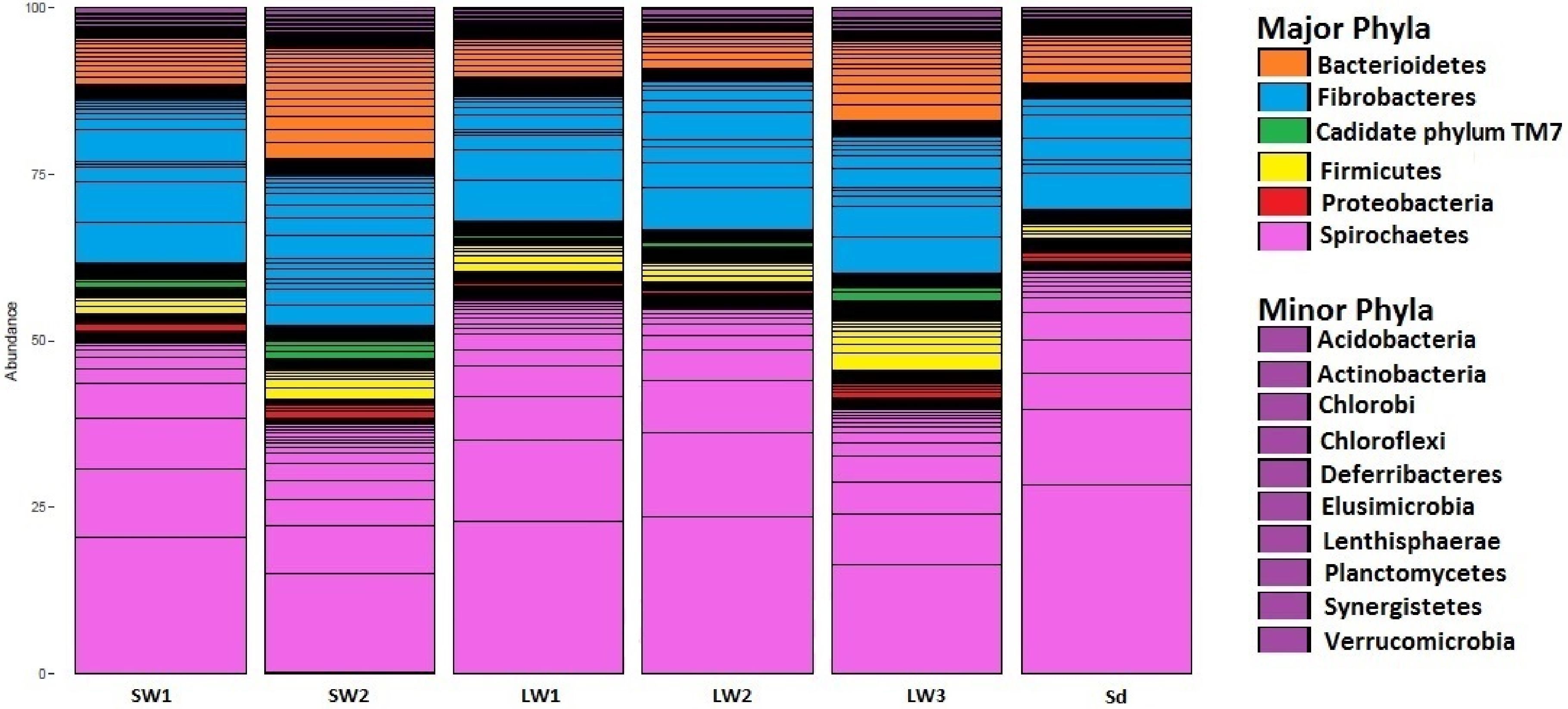

Fig 1. Phylum-level distribution of bacterial taxa from Illumina MiSeq libraries based on the 16S rRNA gene from the hindgut of neuter instars of *Nasutitermes ephratae*. “Minor phyla” correspond to the pool of all phyla with <1% of reads in good sequenced libraries. See main text for information about abbreviations.

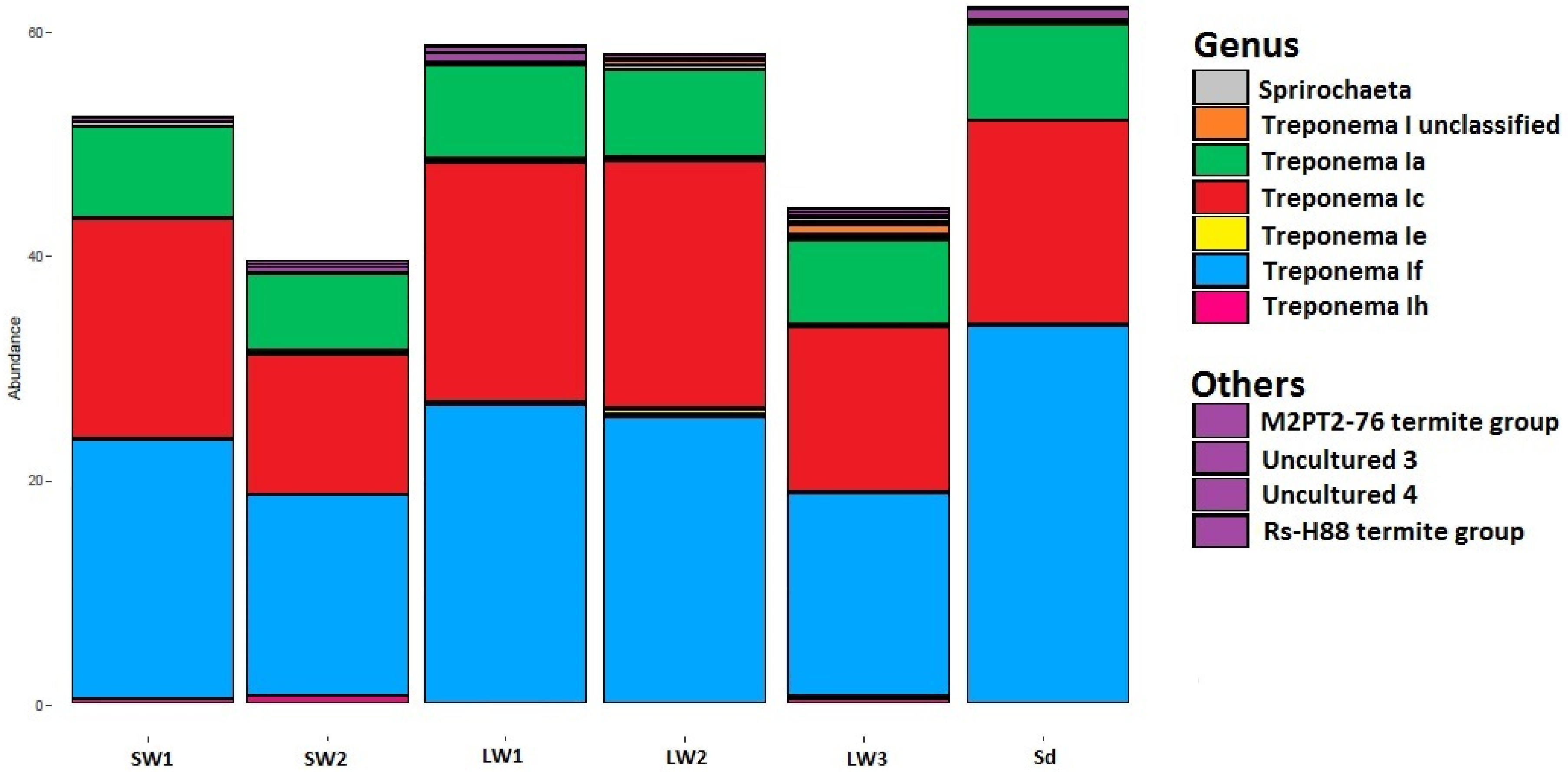

Fig 2. Diversity and abundance of Spirochaetes-related OTUs present in the hindgut of neuter instars of *Nasutitermes ephratae*. Note the decrease in relative abundance from young (SW1, LW1 and LW2) to old (SW2, LW3) instars.

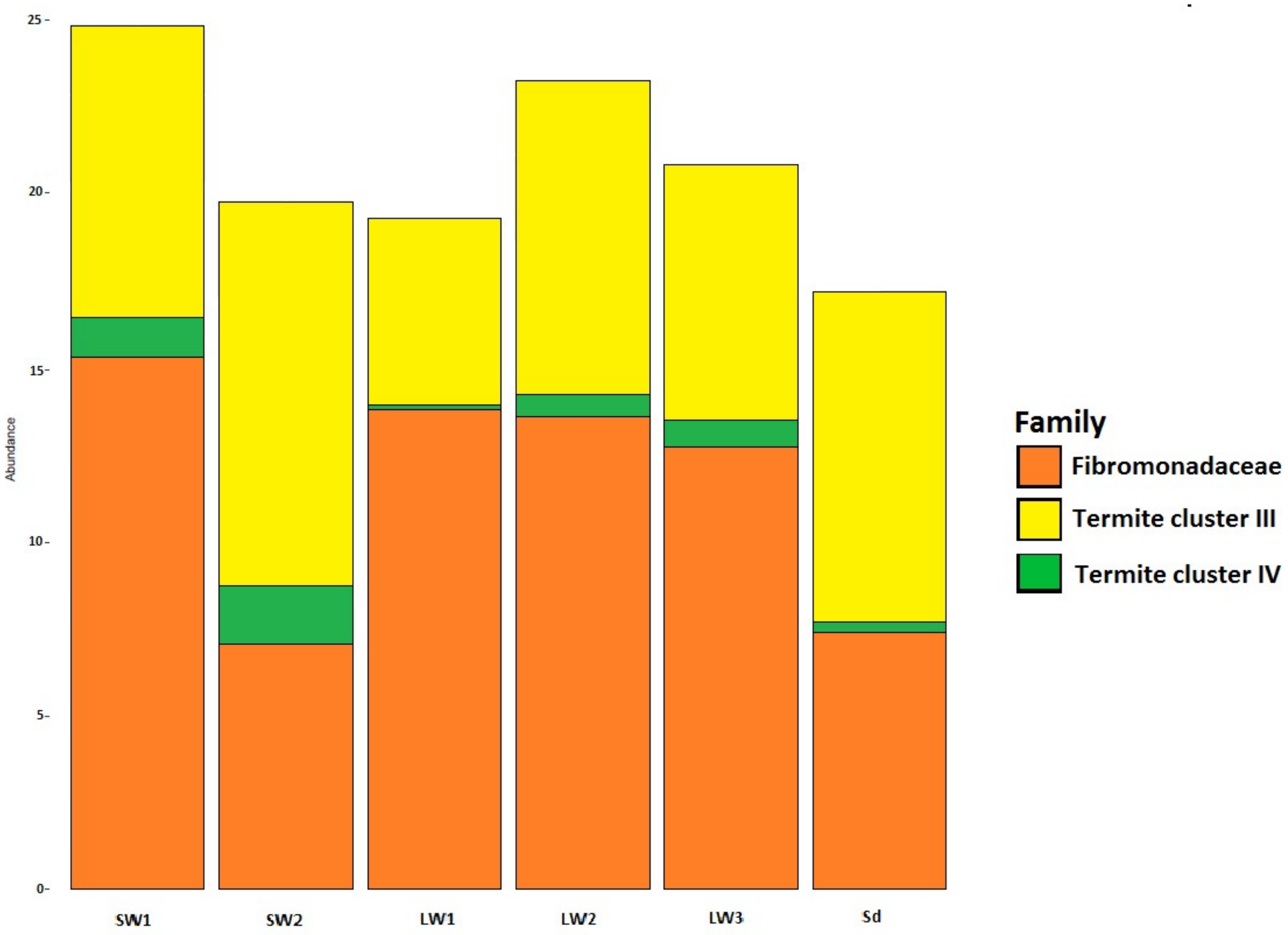

Fig 3. Diversity and abundance of Fibrobacteres-related OTUs associated to hindgut of neuter instars of *Nasutitermes ephratae*. Note the transition of Fribomonadaceae to Chitinivibrionia after that occurs when SW1 moult into SW2.

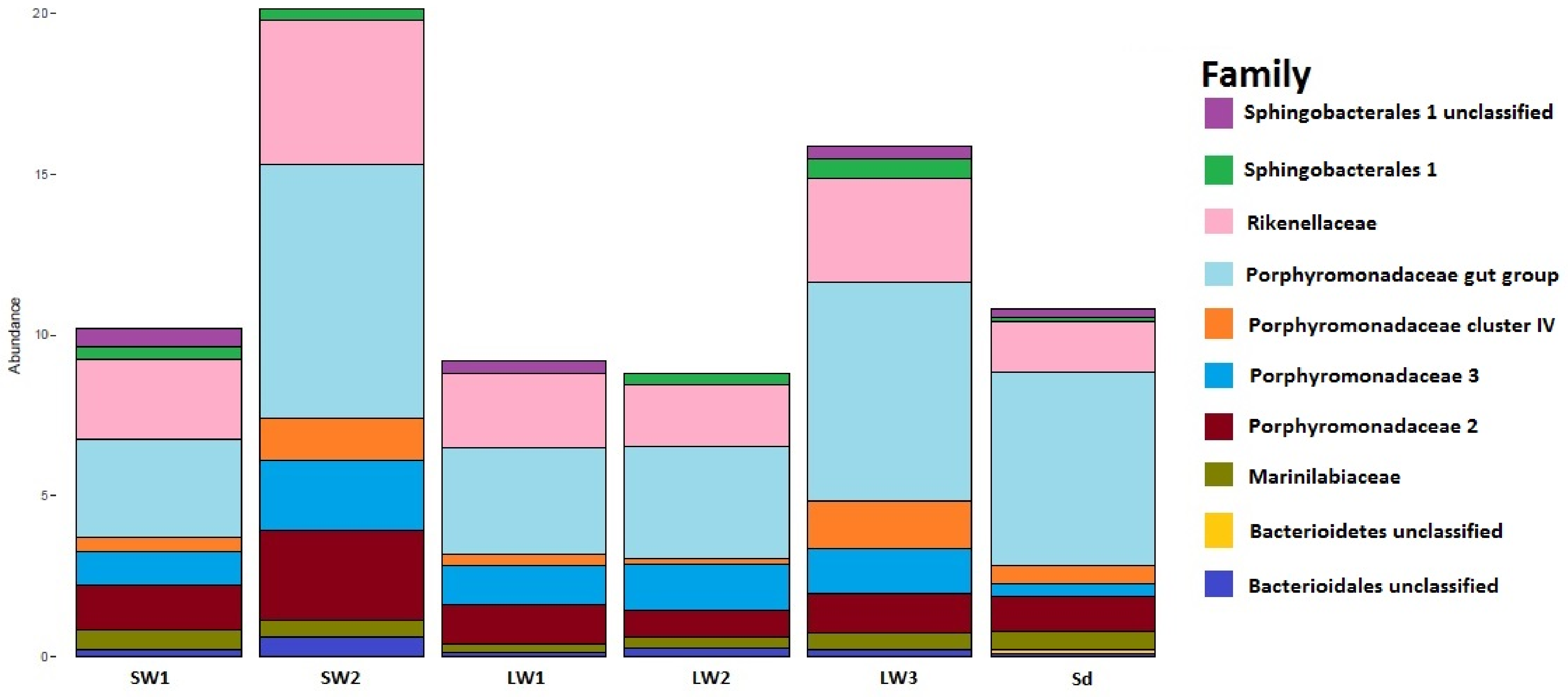

Fig 4. Diversity and abundance of Baterioidetes-related OTUs associated to hindgut of neuter instars of *Nasutitermes ephratae*. Note the increase in the abundance of Porphyromonadaceae-Gut-group, Porphyromonadaceae Cluster V and Rikenellaceae in older instars.

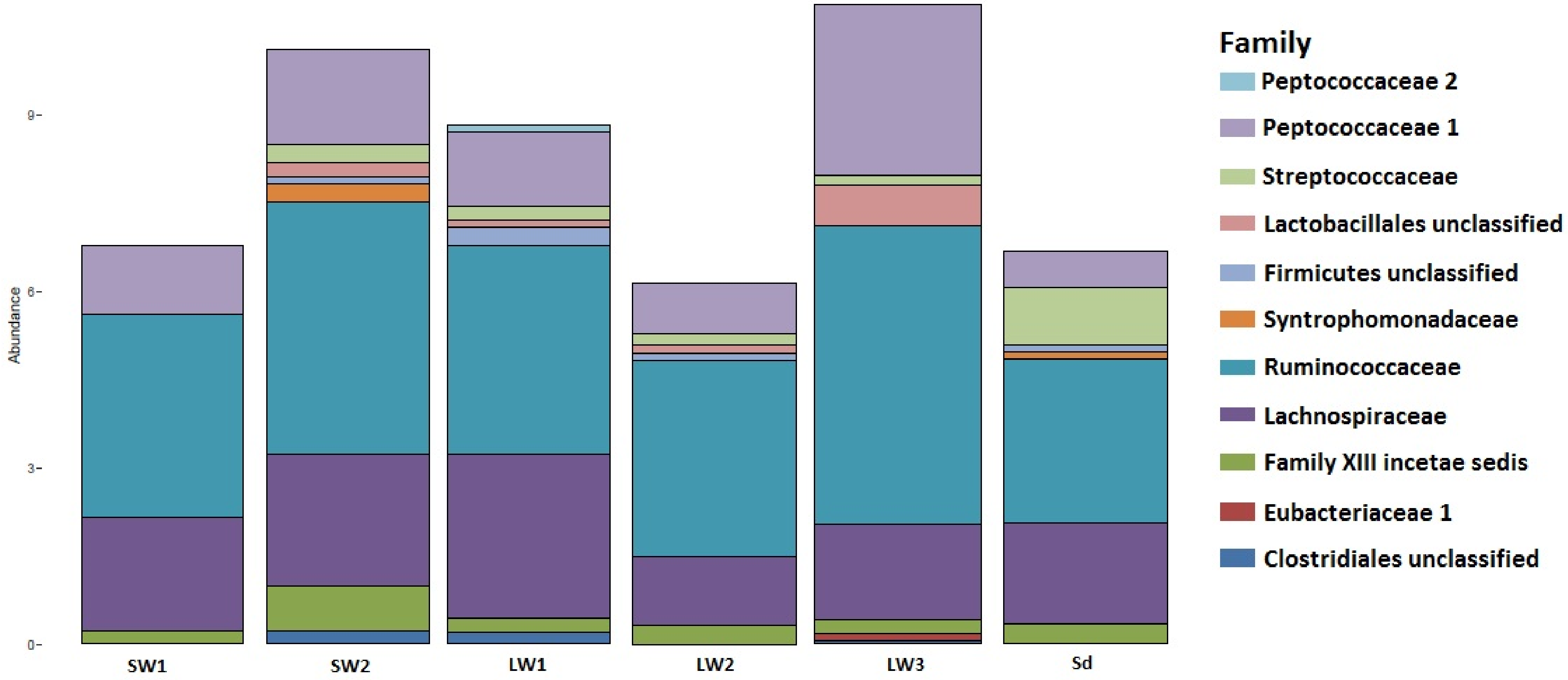

Fig 5. Abundance of Firmicutes gut symbionts present in apterous instars of *Nasutitermes ephratae*. The enhance in abundance for Ruminococcaceae and Peptococcaceae 1 in later instars was the most remarkable characteristic related to this phylum.

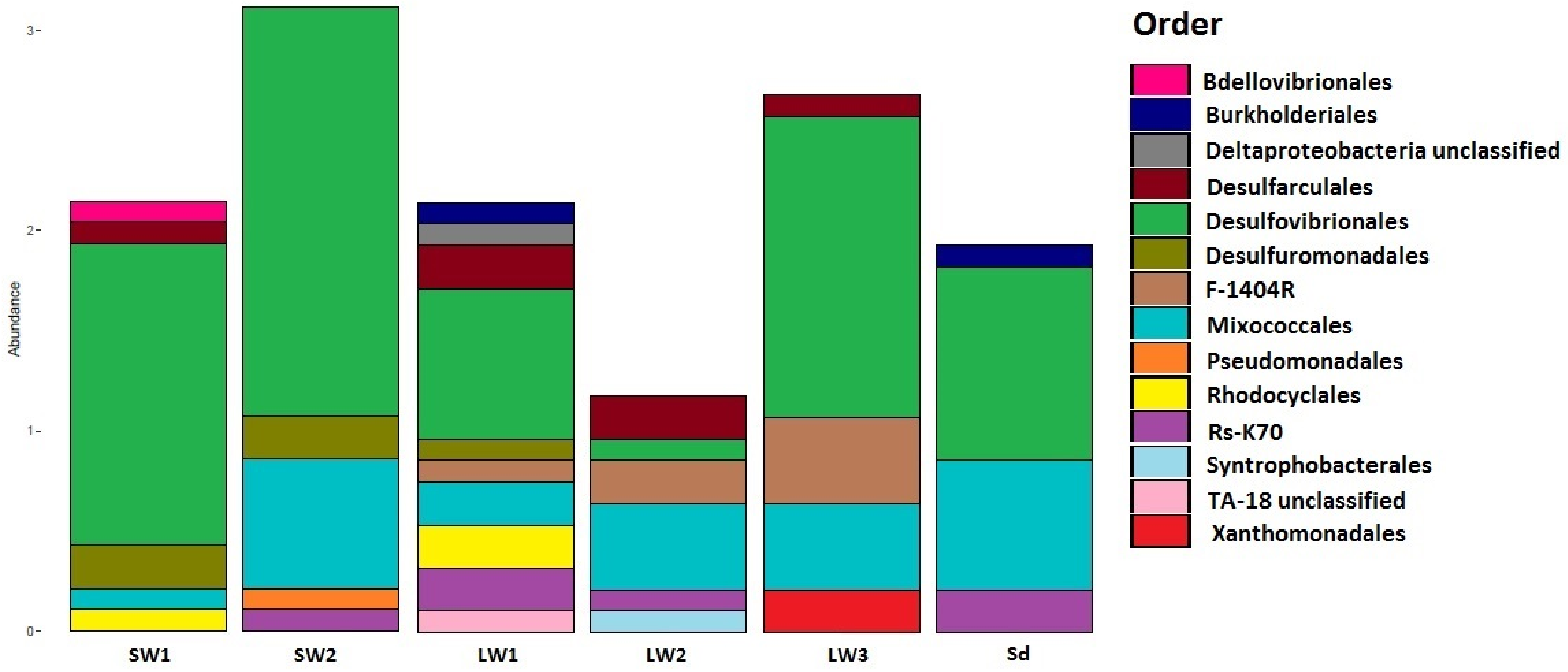

Fig 6. Abundance and species richness of Proteobacteria intestinal microbes associated to apterous instars of *Nasutitermes ephratae*. Defulsovibrionales OTUs were the most abundant in all lineages, except in LW2. Several Proteobacteria OTUs seems to be instar-specific and could not be observed outside their particular lineage.

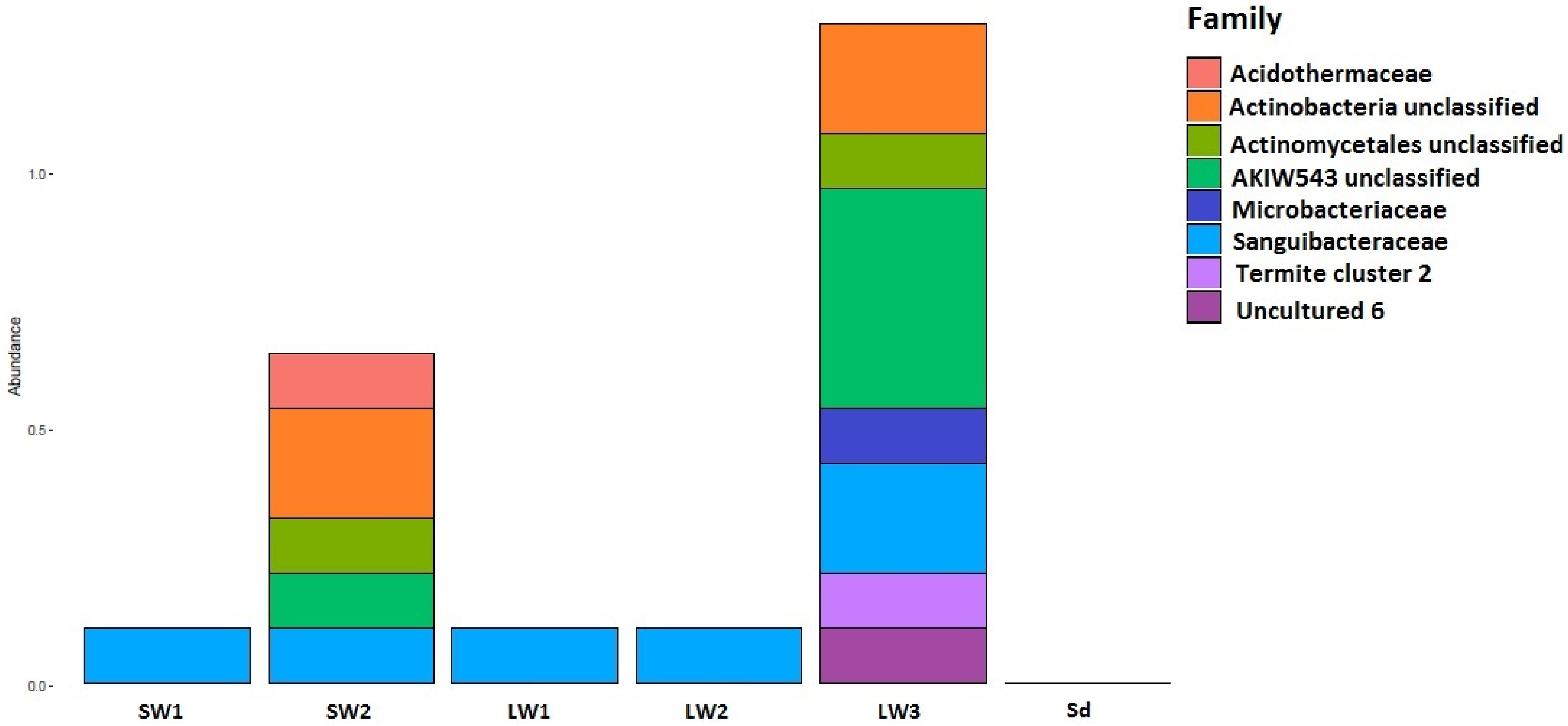

Fig 7. Actinobacteria-like OUT richness found to be associated to neuter lineages of *Nasutitermes ephratae*. Note the intriguingly rise in OUT diversity in older worker instars SW2 and LW3; and the complete absence of Actinobacteria in soldier caste.

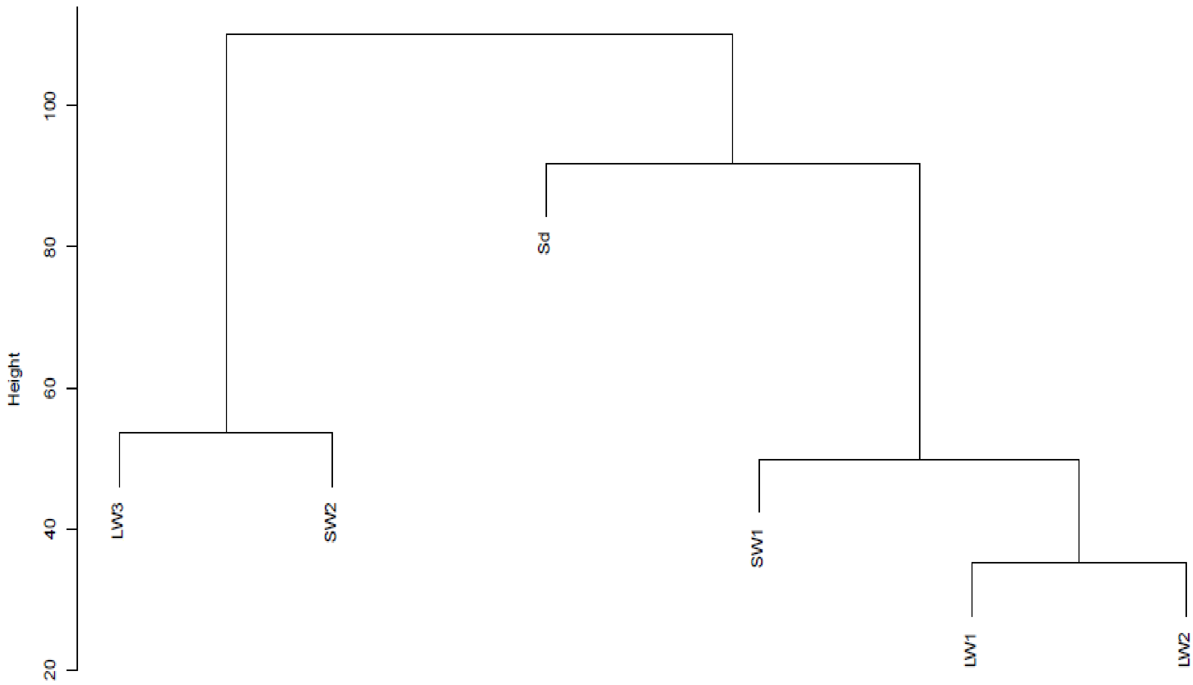

Fig 8. UPGMA cluster dendrogram grouping *Nasutitermes ephratae* apterous instars based on species richness and abundance of associated intestinal microbiota [*dist(t(OTU2), method = "euclidean"); hclust (*, "average")*]. Worker instars clustered by age rather than size.

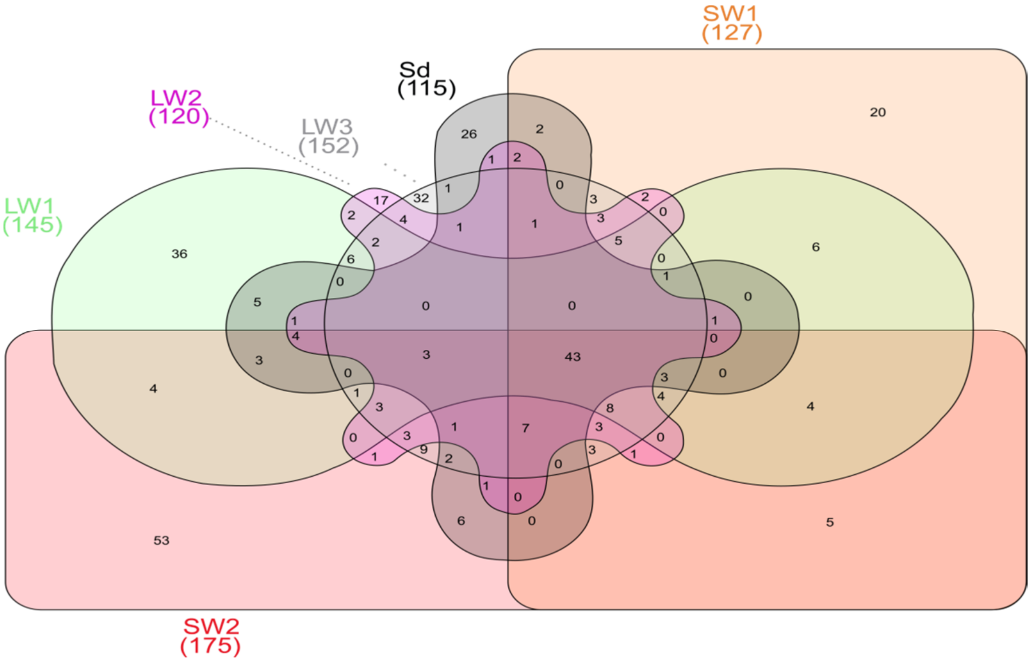

Fig 9. Venn diagram of bacterial OTUs (at 97% identity) common to the Illumina MiSeq libraries for worker instars and soldier of *N. ephratae*. Numbers within parentheses represent the total OTUs observed per instar. Only 43 OTUs from 355 were common to all neuter lineages.

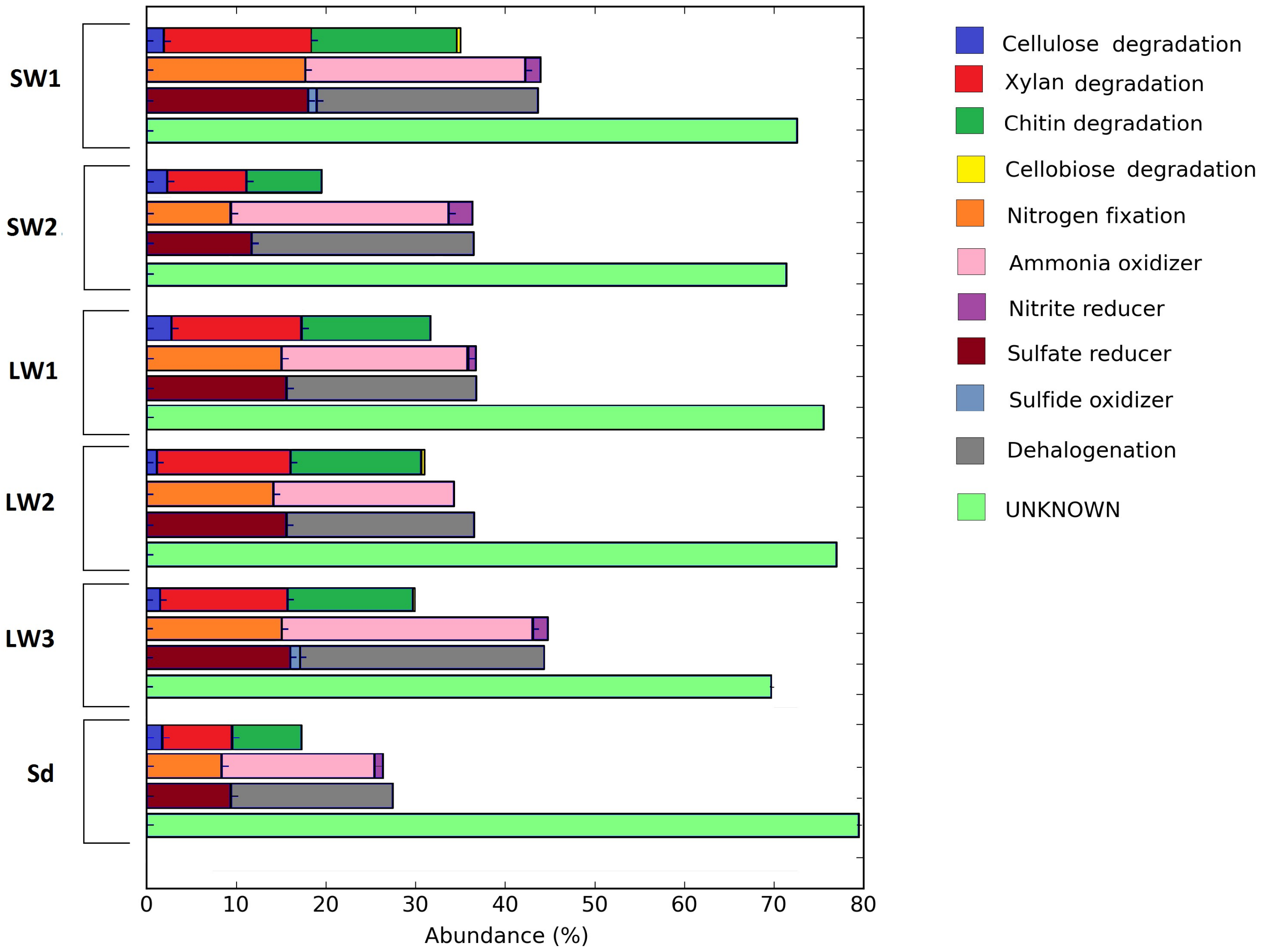

Fig 10. Barplot of the abundance of taxonomy-to-phenotype mapping among neuter lineages of *N. ephratae*. Only traits with an abundance ≤ 1% in at least one instar were kept, except for “cellobiose degradation”.

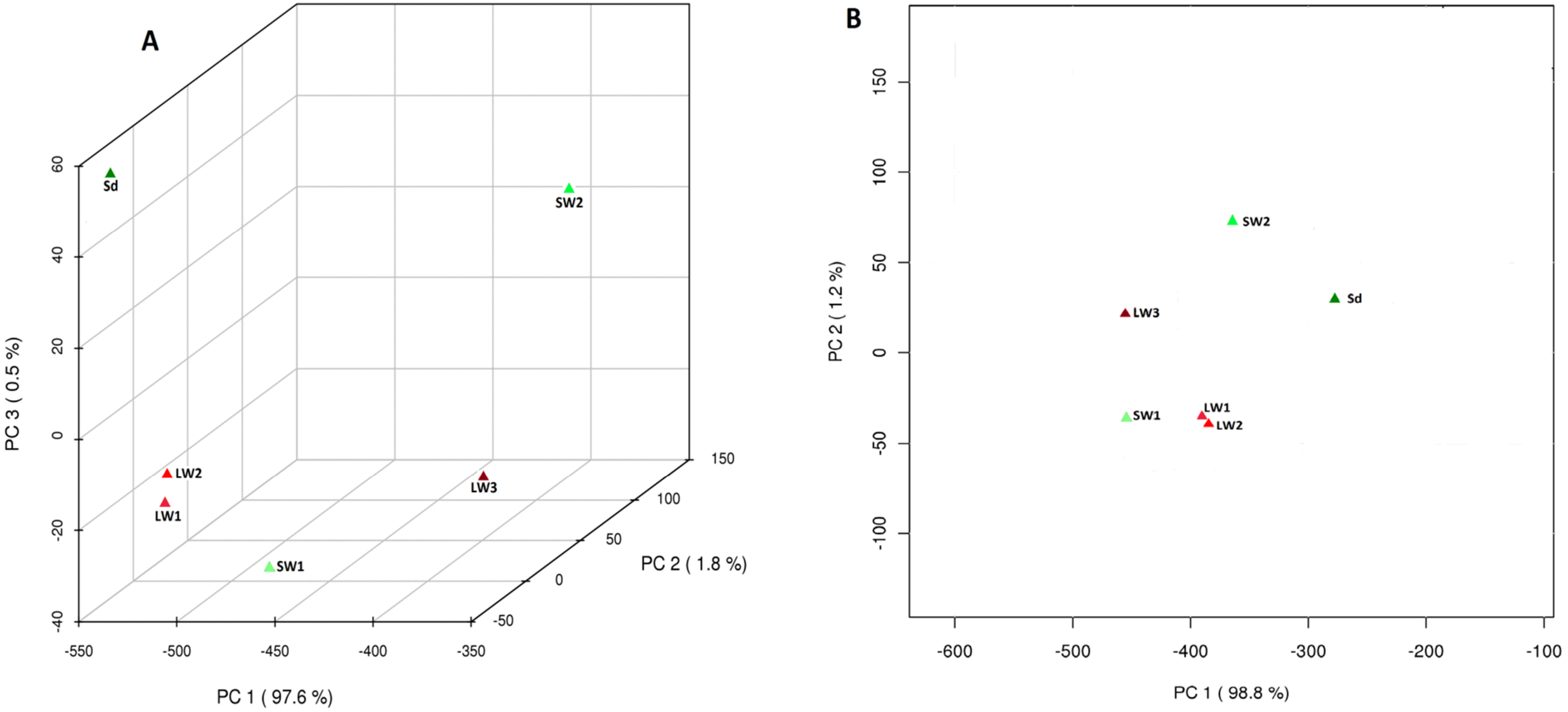

Fig 11. Principal component analysis, evaluating changes in the abundance and species richness of intestinal microbial symbionts among instars of *Nasutitermes ephratae*. A: Phylogeny-based 3D plot at phylum level. B: Microbial metabolism-based 2D plot. In both plots, younger workers were more closely related among them, than with their older respective counterparts regarding gut bacterial community structure.

CAPÍTULO IV

POLYCALISM AND NEST PLASTICITY IN *Nasutitermes ephratae* HOLMGREN (BLATTARIA: TERMITIDAE)

*Artigo a ser submetido à Revista Sociobiology.

RESEARCH ARTICLE - TERMITES

Polycalism and Nest Plasticity in *Nasutitermes ephratae* Holmgren (Blattaria: Termitidae)

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Running Title: Nesting Plasticity in *Nasutitermes ephratae*

ABSTRACT - We described for the first time a policalic nest of *Nasutitermes ephratae* and report the occurrence of an epigeal domed nest for this species in the Cerrado region in Central Brazil, alternatively to arboreal nest commonly found in humid forests. The policalic arboreal nest was formed by two calies constructed in a same support stalk (CAP: > 10 cm) and interconnected by a foraging tunnel. Royal chamber was in the largest calie and only one royal couple was found. Eggs and nymphs were present also only in the major calie, mainly surrounding the royal chamber. In the minor calie, only larvae 2, workers and soldiers were found, suggesting this satellite-nest works as a shelter for neuter castes. The occurrence of *N. ephratae* in the Cerrado region was confirmed by morphological identification and sequencing three mitochondrial genes (COII, rRNA 12S and rRNA 16S). Its epigeal domed nest showed a particular architecture without a recognizable royal chamber, indicating plasticity in nesting behavior for that termite species.

Keywords: termite, Nasutitermitinae, policalism, nest architecture.

Introduction

The termite *Nasutitermes ephratae* Holmgren is a Neotropical Nasutitermitinae species widely distributed throughout Central America and in humid forests of South America (Constantino, 1998). It is a xylophage and arboreal species (Vasconcellos et al, 2005), that usually makes its carton nests on branches or tree trunks, which have a quite distinct architecture (Thorne, 1980).

In *N. ephratae* nests (**Fig.1**), the royal chamber is near the center of the nest and it is surrounded by a capsule of hard, dense carton. This cell is suspended in a matrix of thin carton composed of relatively large galleries. The thin carton nest interior is encased in an outer band of very hard, dense carton containing only small galleries (Thorne, 1980). The external layer is a carton envelope with tiny perforations over the entire surface that possibly functions in air exchange and thermoregulation within the colony (Noirot, 1970).

For some species of genus *Nasutitermes*, polycalic nests seem to be a common phenomenon, having been observed in *N. polygynus* Roisin & Pasteels and *N. corniger* Motschulsky (Roisin & Pasteels, 1896A; Vasconcellos & Bandeira, 2006). *N. ephratae*, however, is a predominantly monocalic species. Muradian et al (1998), cite in their study that *N. ephratae* nests have normally only one central structure, although exceptionally they can have more than one. This is possibly the unique reference about polycalism in *N. ephratae*, but such colony organization was never described before for this species.

Nest plasticity for *N.* cf. *ephratae* was suggested by López-Hernández et al (1989), who described low domed mounds for this termite species in savanna enclaves in Orinoco Llanos (Venezuela and Colombia), located in the Amazon Region. This study, however, was focused on measured phosphate accumulation in termite mounds and the correct taxonomic identification of the termite species remains unclear, due to the absence of termite samples collected from that type of nests.

In this study, we describe for the first time the organization of a polycalic nest and confirm the occurrence of mound nest of *N. ephratae* in the Cerrado *stricto sensu* of Central Brazil.

Material and Methods

Collection of Polycalic Nest

A policalic nest of *N. ephratae* (Ne - ARF) was found in September/2013 on Mata do Buraquinho Forestry Complex (7°08'18.9"S; 34°50'34.7"W), an urban fragment of Atlantic Rain Forest located in João Pessoa – PB, Brazil. Nest volumes were calculated using the formula for an ellipsoid: $\mathbf{V = 4/3\ \pi\ hDd}$, where $\mathbf{h}$ = ½ nest height, $\mathbf{D}$ = ½ widest diameter, and $\mathbf{d}$ = ½ narrowest diameter. These measurements were taken in three opportunities (September/2013, September/2014 and September/2015) to estimate the growth of the colony over two years.

The colony were collected in totality in September/2015, putted in plastic bags and brought to the laboratory. The calies were carefully dissected to allow the description of nest architecture. Then the nest was broken into pieces and termites were separated from nest debris by successive water flotation. Collected individuals were fixed in FAA solution and subsequently stored in 70% ethanol. Three subsamples with 5 g each from both calies were taken and the termites were separated according their respective caste and counted to estimate the proportion among instars.

Collection of Mound Nest

A mound nest of a Nasutitermitinae species (*Nasutitermes* cf. *eprhatae* - CER) was collected on September/2015 in an urban Cerrado fragment located in Brasilia – DF, Brazil (15°46'34.5"S; 47°52'04.2"W). Only the nest dome above soil surface was collected, leaving intact the subterranean part. The nest was kept in plastic box and transported to the lab for dissection. A subsample of 50 termites, including soldiers and workers, was preserved in both 70% ethanol and RNALater solution for further analysis.

Morphological and Genetic Identification

Samples of *N. ephratae* were collected from both polycalic and mound nests, as described above. A group of three other species of Nasutitermitinae was used as complementary sequences: specimens of *Nasutitermes gaigei* Emerson and *Subulitermes microsoma* were also collected in Mata do Buraquinho Forestry Complex (João Pessoa –PB). Another Nasutitermitinae (*Diversitermes* sp. Holmgen) was collected in Brasília – DF, near to the domed nest of *N.* cf. *ephratae*.

Termites were identified based on the morphology according to Ensaf et al (2003) and comparisons with samples deposited on the Isoptera Collection – UFPB. For genetic identification, DNA was isolated from 15-20 workers using NucleoSpin® Soil (Macherey-Nagels) according the manufacturer instructions. Three mitochondrial genes – COII, 16S RRNA and 12S rRNA - were amplified by polymerase chain reaction using the primers set listed on

Table 1. Amplicons were Sanger sequenced commercially (GATC Biotech, Konstanz, Germany). An additional sequence from mitochondrial genome of *Nasutitermes corniger* (GenBank Accession: KP091691) was used as reference for alignment. Concatenated sequences were aligned using MUSCLE (Edgar, 2004) implemented into MEGA v. 6.06 (Tamura et al, 2013) and manually curated for adjustments. Maximum-likelihood trees (substitution type: DNA; Model: GTR (G+I); 1000 bootstraps) were calculated for concatenated sequences of all three mitochondrial genes.

Results

Polycalic Nest

This is the first time the architecture organization of a nest of *N. ephratae* is described. The polycalic colony of *N. ephratae* was formed by two calies, far apart 7.5 cm each other and connected by a foraging tunnel (**Fig. 2**). The calies were attached to one same support tree trunk which had a circumference < 10 cm. The calies had a similar volume, having the major calie 9.8 L and the minor 9.5 L. No significant increase in the nest volume was verified after two years of observation.

Regarding to the nest architecture (**Fig. 3**), only one royal cell was identified and it was located in the large calie. Also only one royal couple was registered. The queen chamber was situated on the dense outer band instead on the center of the nest, as described by Thorne (1980). Eggs and nymphs (3^{rd} to 5^{th} instars) were found only in the major calie, surrounding the queen cell. On the minor calie, only immature and neuter castes were found. We have counted circa two times more larvae 2 (2.1:1), soldiers (1.9:1) and large workers (2.2:1) inside minor calie than the major one. Regarding larvae 1, this instar was more common inside major calie (2.3:1). The proportion among small workers (SW) seems to be equivalent in both calies (1.2:1). No presoldiers were recorded in the selected subsamples.

Mound Nest

The aerial portion of the mound nest collected at Cerrado biome had a peculiar architecture, with a dome composed mainly by soft carton with large tunnels, surrounded by an outer band of dense carton and a thin external carton envelope. The outer band was very thin compared to that observed in Atlantic Rain Forest arboreal nests of *N. ephratae*. No royal chamber was present in the upper part collected (**Fig. 4**).

Identification of N. ephratae *from Cerrado*

The specimens collected in Brasília were attribute to the *N. ephratae* due to the following characters: head dark reddish brown and with an elevation at base of nasus; only 3-4 bristles at base of nasus and two on vertex; abdominal tergites without microscopic hair; presence of 12-13 antennomeres; third and fifth antennomeres longer than fourth. This set of characteristics differentiate this specimen from *Nasutitermes coxipoensis*, a common mound nest builder in Cerrado biome.

To confirm the affiliation of this Nasutitermitinae species as *N. ephratae*, three mitochondrial genes were sequenced. For all three genes, *N. ephratae* ARF and *N*. cf. e*phratae* -

CER showed the higher Max scores (704 to 1158), Querry cover (99%) and Identity (98% to 99%) than when compared with the other sequences. COII sequence of *N*. cf. e*prhatae* CER also showed a high coverage (99%). The concatenate phylogenetic tree has confirmed the affiliation between both Cerrado/Atlantic Forest *N. ephratae* species (**Fig. 5**).

Discussion

For *Nasutitermes ephratae*, polycalism seemingly is not a frequent phenomenon and in this specific case related here, the construction of two closed calies was made possibly to distribute the nest weight along the tree trunk. We could not verify nest growing after two years of observation, indicating this limitation on nest size can be due to the tiny circumference and low resistance of the support stalk.

The construction of nest with two or more calies near each other may facilitate gas exchange promoting an increase of surface when compared to monocalic nests, as postulated by Holt & Easey (1985). This does not seem to be, however, the case of the polycalic colony under analysis. In arboreal termite species, the separation of the colony in two or more closer calies seems to be a strategy to prevent detachment from support trees because of its own weight, as happens to large nest of *N. princeps* Desneaux in coconut plantation (Roisin & Pasteels, 1896 B).

Further observation on additional *N. ephatae* colonies in the same area of Mata do Buraquinho showed only monocalic nests constructed around tree stalks that apparently grow in size and volume as long the tree trunk enlarges in circumference. Most parte of these nests were attached to trees with circumference > 15 cm. The observation of some dead large monocalic nests – still attached to thin stalks – fallen on the ground, corroborates the idea of exceptional polycalism conditioned by the characteristics of the support tree.

Regarding nest inner architecture, royal chamber was attached to the hard carton outer band. The density of the carton material of the royal chamber and the outer band seems to be similar, leading us to postulate that multiple queen chambers are constructed and remodeled as long the colony grows, being the first one close or even attached to the outer band. As mentioned above, when the support tree grows in circumference, its apparently stimulates growth in the nest, leading the termites to remodel the inner nest architecture and build/rebuild several royal chambers. In the case of the polycalic nest, as the tree trunk did not grow substantially in circumference, there was no stimulus for major calie be remodeling, which may be the cause for the first and sole royal chamber remained intact attached to the outer band.

For this *N. ephratae* polycalic colony, the minor calie works as satellite to house surplus workers and soldiers not required at some moment to maintain the large calie. On the other hand, the large nest structure may function as a nursery and as an egg and nymphs shelter. A similar organization - reproductives present at most in one calie - was described for polycalic nest of *N. polyginus* that were built close by each other on the same tree (Roisin & Pasteels, 1986 C).

Nesting Plasticity in N. ephratae

Epigeal nests of *Nasutitermes* spp. in savanna-like vegetation was previously describe by San José at al. (1989) in Orinoco Llanos - Venezuela. Those colonies were found in well-drained, coarse-textured soils and randomly distributed throughout the region. The mounds were built using sand particles and fecal material as cement, and an accumulation of herbaceous material collected surrounding. This nest description is similar to that made by Laffont et al (2012) for *Nasutitermes coxiopensis* Holmgren, a well-known mound nest builder from Cerrado region.

When we compare the architecture of epigeal nest of *N. coxipoensis* and this unique domed nest of *N. ephratae* described in this paper, we can highlight some remarkable differences. Regarding nest surface of *N. coxipoensis*, Laffont et al (2012) described it as irregular thin layer of fragile and easily removable sandy consistency, with granular-like protuberances. This layer was immediately followed by an internal soft cardboard-like material, with an approximately homogeneous structure, without remarkable differences between the peripheral and central regions of the nest. The building material included partially digested plant debris and sand particles coated with excreta of the workers. No hard carton outer band or royal cell was observed.

For the epigeal nest of *N. ephratae*, the surface was coverage by a thin carbon carton envelope with several perforations, followed by a sharp but more resistant carton outer band. The interior of the epigeal part of the nest consisted in soft and relatively moist carton material, pointing a remarkable transition between the center and the periphery of the nest. No evident soil particles were observed, but carton material only, similar to nest of *N. ephratae* from Atlantic Rain Forest, indicating that only lignocellulolytic debris and feces were used as building materials. Similar to *N. coxipoensis* nests, no royal cell was found, suggesting the chamber is absent in domed nest or located underground, possibly due to the less protection promoted by the tiny outer band. The mound nest of *N. ephratae* showed a soft inner structure but with a relatively harder surface, requiring specific tools – like a hatchet - to be opened.

Nest plasticity was reported for several termite species, been related to a number of environmental factors, as thermoregulation, gas exchange, response to the soil type, or a result of intrinsic behaviors of the species (Roisin & Korb, 2011). Nest plasticity as a response for flood pulse was documented to *Amitermes laurensis* e *A. vitiosus* in Australia (Gay & Calaby, 1970) and to *Cornitermes silvestrii* Emerson in Pantanal Region, Brazil (Plaza et al, 2014).

The transition between epigeal domed and arboreal nests was described to *Microcerotermes crassus* Snyder in Thailand (Inoue et al, 2005). Among subfamily Nasutitermitinae, this behavior is known to *Constrictotermes cyphergaster* Silvestri who build domed nest on rocks or arboreal nests surrounding tree trunks in Caatinga biome, Brazil (Vasconcellos et al, 2007).

We postulate this nesting plasticity behavior in *N. ephratae* can be due to the absence or reduced number of support trees in the area where the nest was collected plus the competition with arboreal nest builder commonly found in Cerrado, as *C. cyphergaster*. Additionally, as *N. ephratae* and *N. coxipoensis* are sister groups (Roy et al. 2014), this phylogenetic proximity can help to explain the nesting plasticity observed in *N. ephratae*, being both able to build mound nests.

The description of this domed nest of *N. ephratae* is, possibly, the first record of this species for Cerrado *stricto sensu* in Brazil, where the species build domed nest on soil instead spherical arboreal nests. The occurrence of *N. ephratae* in savanna-like biome was reported by Ensaf et al (2003) in coastal savanna and in inselberg-rocky outcrops savanna type in French Guiana and suggested additional studies to verify if this species can be considered as an ubiquitous or a specialized-habitat species of open savannas.

Despites a sole nest of *N. ephratae* was found in Cerrado region, is not possibly to affirm that species can naturally and commonly occur in this biome. The nest was found in a very anthropic fragment of Cerrado and its presence can be restricted to that area and be a result of human transportation from Tropical Rain Forests. More studies are required before propose new delimitations for the natural occurrence of *N. ephratae*. Additional studies, as *ex-situ* rearing experiments with *N. ephratae* can help to document the phenomenon of nesting plasticity in this termite species.

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Table 1. List of primers used to amplify mitochondrial genes from termites.

Primer	Direction	Gene	Sequence (5' to 3')	Length
16SBr	Forward	mt-16S rRNA	CCG GTC TGA ACT CAG ATC ACG T	22
16SAr	Reverse		CGC CTG TTT ATC AAA AAC AT	20
C2-J-3096f	Forward	mt-COII	AGA GCA TCA CCA ATC ATA GAA CA	23
TK-N-3807r	Reverse		GTT TAA GAG ACC ATT ACT TA	20
12S-f	Forward	mt-12S rRNA	TAC TAT GTT ACG ACT TAT	18
12S-r	Reverse		AAA CTA GGA TTA GAT ACC C	19

Table 2 – Blast results of 12S rRNA, 16S rRNA and COII mitochondrial genes sequences of *Nasutitermes* cf. *ephratae* found I Brazilian Cerrado against several Nasutitermitinae. Note the high coverage and similarity with *N. ephratae* from Atlantic Rain Forest.

Gene	Species	Max Score	Total Score	Query cover	E-value	Identity	Accession
12S rRNA	*Nasutitermes ephratae*	704	704	99%	0.0	99%	_____
	Nasutitermes gaigei	558	558	84%	$8e^{-164}$	96%	_____
	Diversitermes sp.	601	601	98%	$2e^{-176}$	94%	_____
	Subulitermes microsoma	630	630	99%	0.0	95%	_____
	Nasutitermes corniger	671	671	95%	0.0	98%	KP091691
16S rRNA	*Nasutitermes ephratae*	905	905	99%	0.0	99%	_____
	Nasutitermes gaige	821	821	99%	0.0	97%	_____
	Diversitermes sp.	719	719	100%	0.0	93%	_____
	Subulitermes microsoma	715	715	98%	0.0	93%	_____
	Nasutitermes corniger	808	808	99%	0.0	99%	KP091691
COII	*Nasutitermes ephratae*	1158	1158	99%	0.0	98%	_____
	Nasutitermes gaigei	887	887	96%	0.0	92%	_____
	Diversitermes sp.	778	778	99%	0.0	88%	_____
	Subulitermes microsoma	693	693	99%	0.0	86%	_____
	Nasutitermes corniger	1092	1092	100%	0.0	96%	KP091691

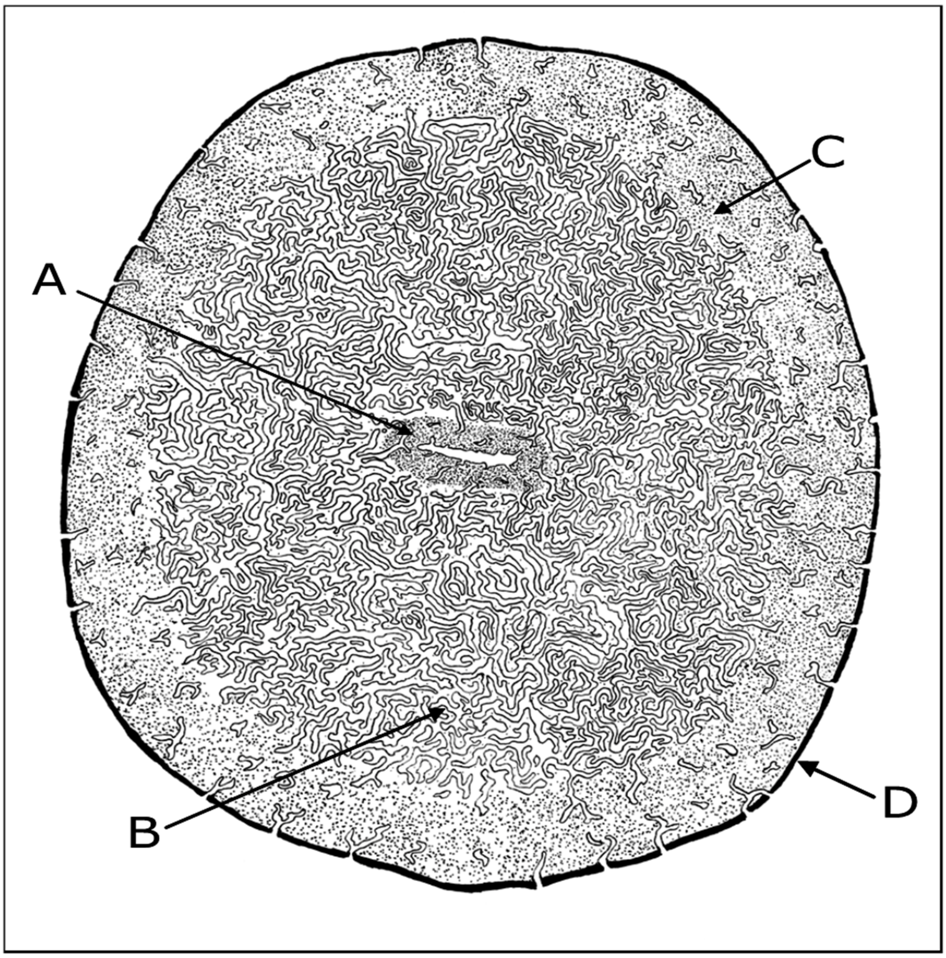

Fig. 1 – Architecture of a monocalic nest of *Nasutitermes ephratae*. A) Royal chamber; B) Thin carton matrix; C) Outer band of dense carton; D) External carton envelope. Based on Thorne (1980).

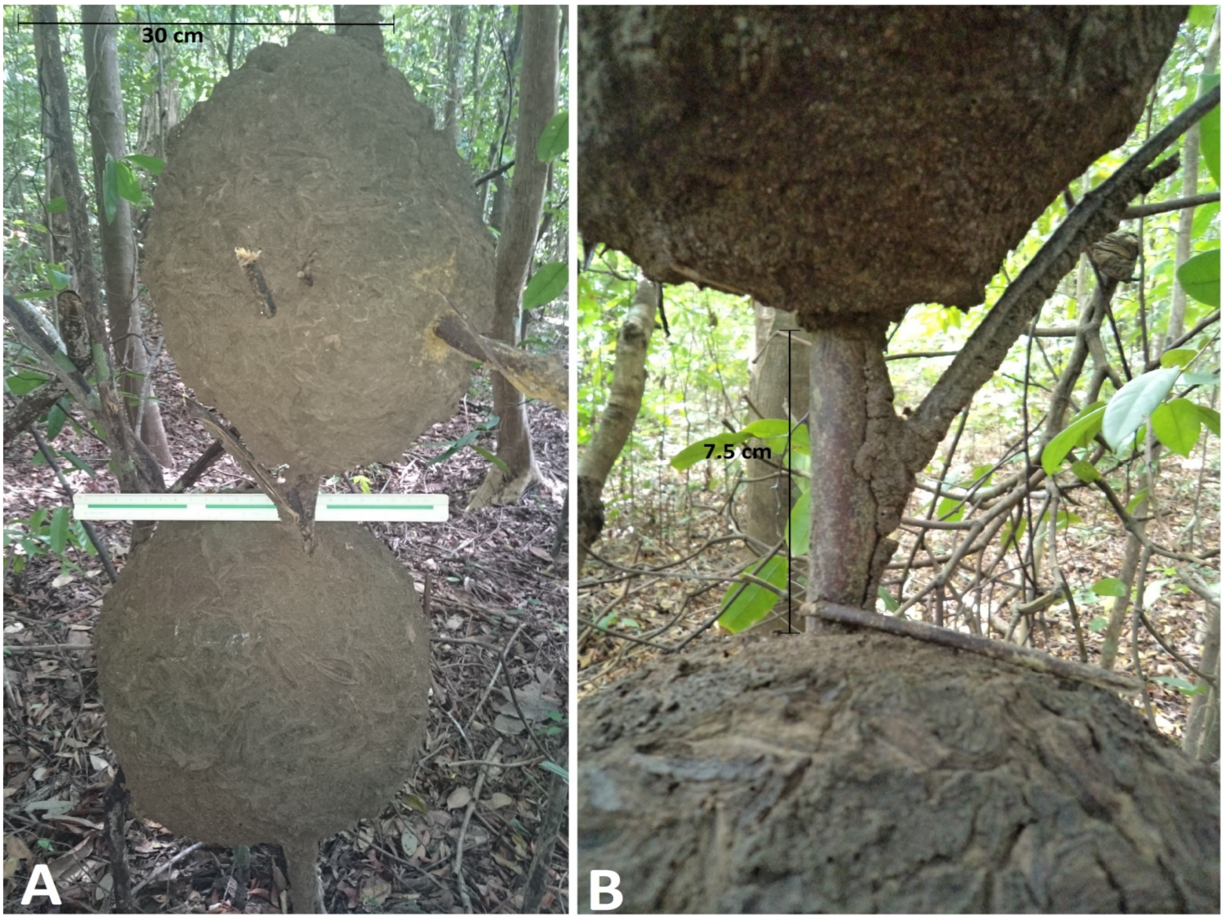

Fig. 2 – Policalic nest of *Nasutitermes ephratae*. **A**) View of both calies attached to a same tree trunck. **B**) Detail of the foraging tunnel connecting the calies.

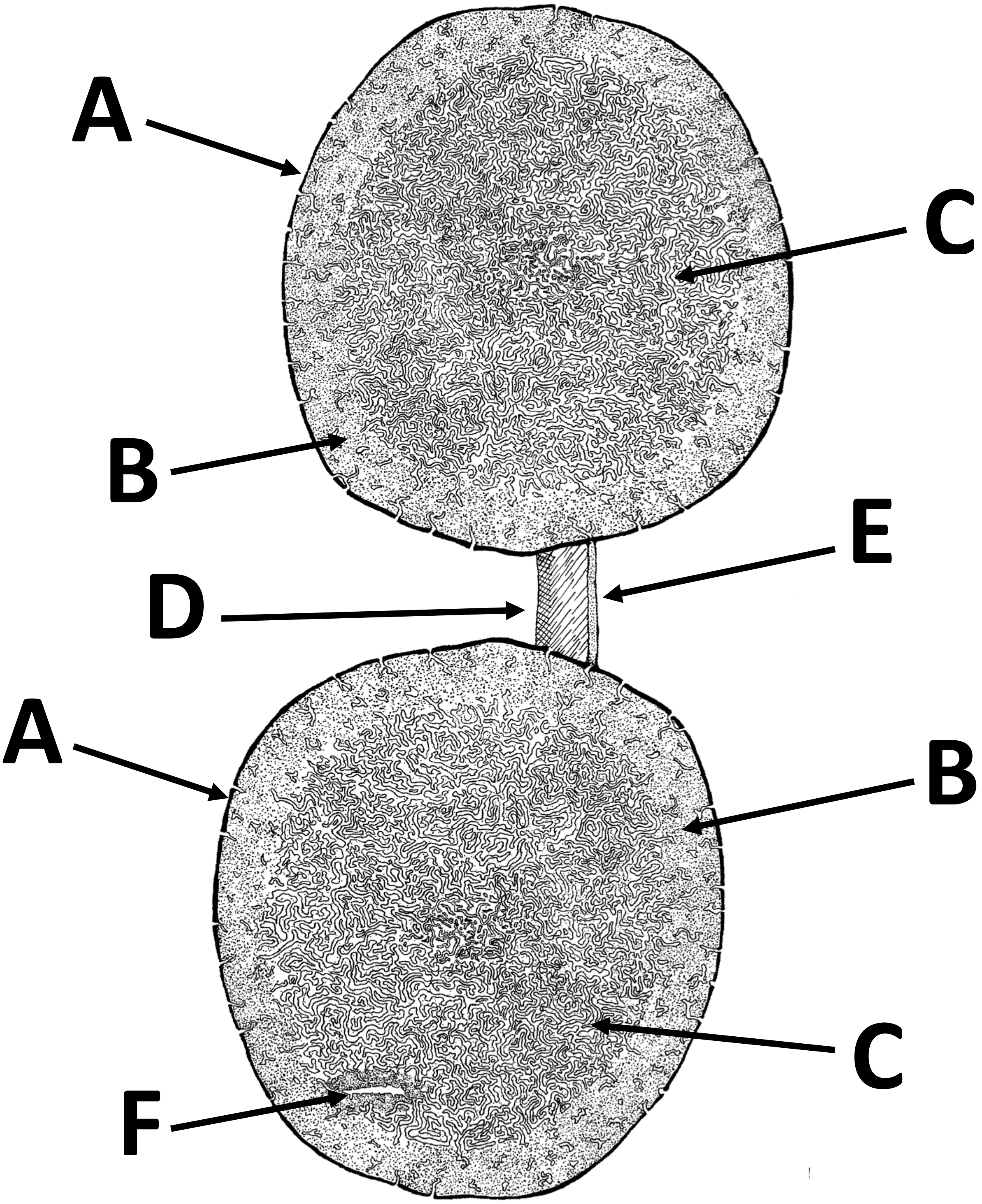

Fig. 3 – Architecture of the polycalic nest of *Nasutitermes ephratae*. A) External carton envelope; B) Outer band of dense carton; C) Thin carton matrix; D) Support tree trunk; E) Connective foraging tunnel; F) Royal chamber.

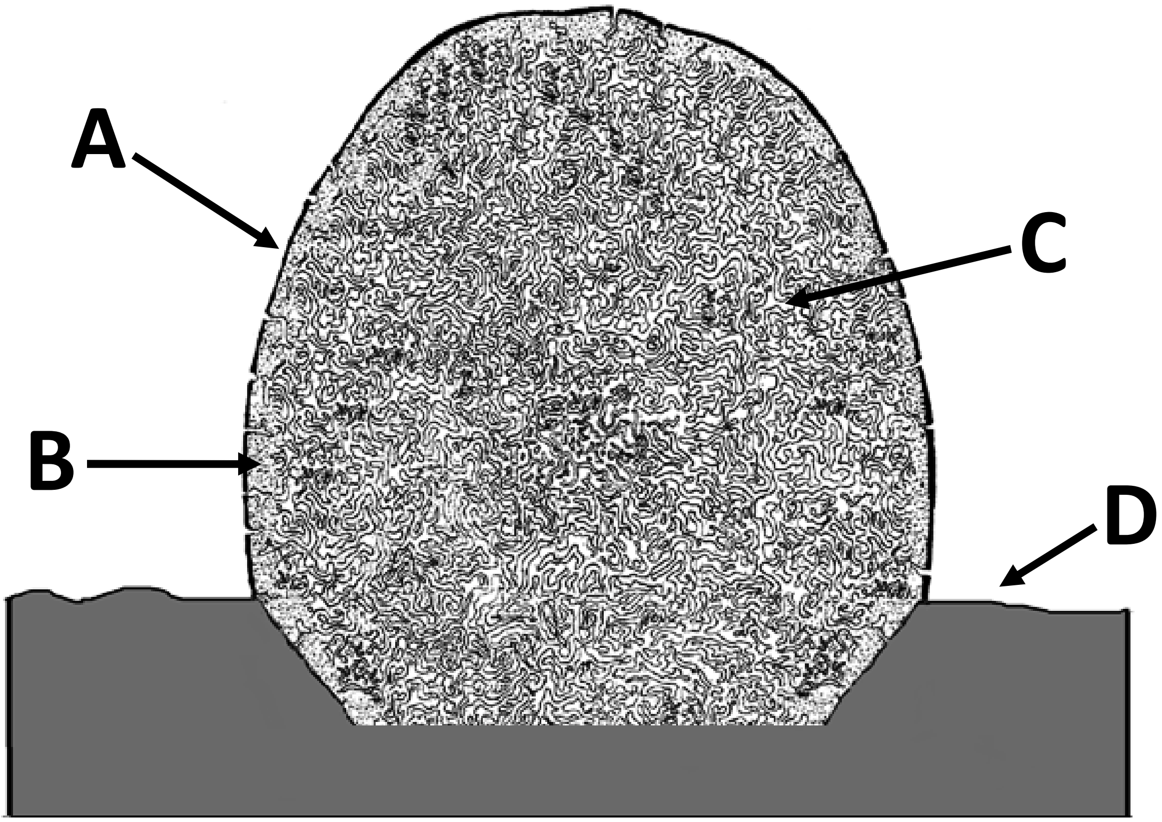

Fig. 4 – Architecture of the mound nest of *Nasutitermes ephratae* found in Cerrado Region. **A**) External carton envelope; **B**) Sharp outer band of dense carton; **C**) Soft carton matrix; **D**) Soil surface. No royal chamber was found. As only the epigeal part of the nest was collected, we could not describe the structure of its subterranean portion, and then it was excluded.

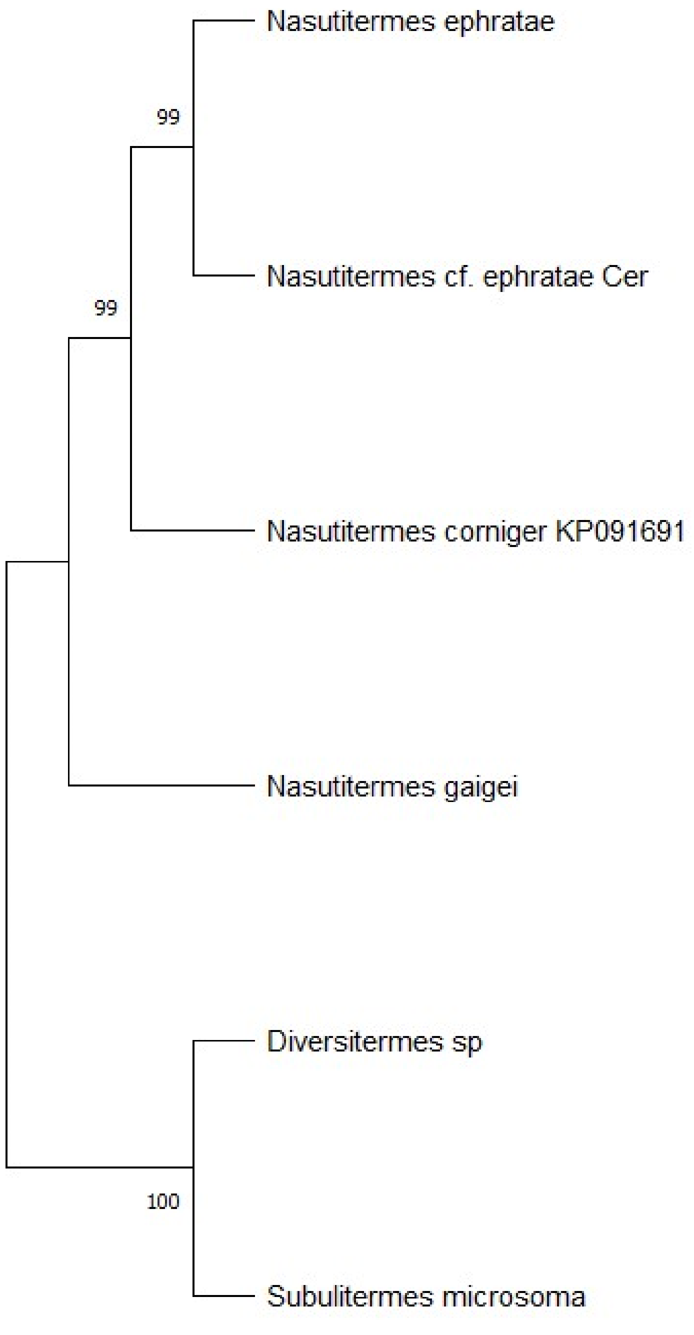

Fig. 5 – Maximum-Likelihood tree showing the phylogenetic affiliation between both species of *Nasutitermes ephratae* from Cerrado and Atlantic Rain Forest with bootstrap values of 99%.

CONSIDERAÇÕES FINAIS

Nasutitermes ephratae é uma espécie de térmita que apresenta um desenvolvimento ontogenético composto por dois ínstares larvais que se bifurcam nas linhagens ninfais (aladas) e neutras (operários e soldados). A linhagem ninfal é formada por cinco ínstares que culminam na formação dos imagos totalmente maduros. A linhagem operária se apresenta bastante polimórfica, havendo um primeiro polimosrfismo por tamanho de origem sexual; e um segundo polimorfismo por idade, com o incremento da pigmentação da cápsula cefálica a medida que os ínstares vão sofrendo novas mudas. Os soldados são monomórficos e se originam a partir de operários pequenos de 1º ínstars. Essa ontogenia ésimilar àquela previamente descrita para outras espécies do gênero *Nasutitermes*, com exceção dos soldados monomórficos.

Esses polimorfismos sexual e por idade interferem no papel fisiológico das castas neutras com relação à degradação da celulose. Mediante anális eda microbiote intestinal de cada ínstar, foi possível observar uma transição de bactérias degradadoras de madeira (Spirochaetes, Fibrobacteres) para bactérias degradadoras de húmus (Bacteriodetes, Firmicues, Proteobacteria) a medida que os operários sofrem novas mudas e ficam mais velhos, evidenciando a ocorrência de polietismo por idade relativo ao processamento da lignocelulose.

Este polietismo fisiológico identificado pro meio da variação da microbiota intestinal sugere que estudos que busquem identificar as espécies microbianas associadas a térmitas ontogeneticamente polimórficos, devem se preocupar em realizar amostragens em todos os diferentes ínstares a fim de aprimorar a cobertura e reduzir as chances de não-identificação de mcrorganismos relevantes para a espécie hospedeira.

Esta plasticidade em *N. ephratae* não se restringe à sua ontogenia e divisão de trabalho, mas se estende também à sua arquitetura de ninhos. Neste estudo descreveu-se pela primeira vez a organização de um ninho policálico para a espécie N. ephratae e postulou-se as razões para tal ocorrência como sendo diretamente relacionada à reduzida circunferência dos troncos de suporte dos ninhos. Identificou-se também um ninho desta espécie construído no solo e com características de montículo, sugerindo que a espécie, na ausência de troncos de suporte, pode iniciar e firmar suas colônias em solo, sem fazer a transição para um estilo de vida arborícola.