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Termite soldiers contribute to social immunity by synthesizing potent oral secretions

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Abstract

The importance of soldiers to termite society defence has long been recognized, but the contribution of soldiers to other societal functions, such as colony immunity, is less well understood. We explore this issue by examining the role of soldiers in protecting nestmates against pathogen infection. Even though they are unable to engage in grooming behaviour, we find that the presence of soldiers of the Darwin termite, *Mastotermes darwiniensis*, significantly improves the survival of nestmates following entomopathogenic infection. We also show that the copious exocrine oral secretions produced by Darwin termite soldiers contain a high concentration of proteins involved in digestion, chemical biosynthesis, and immunity. The oral secretions produced by soldiers are sufficient to protect nestmates against infection, and they have potent inhibitory activity against a broad spectrum of microbes. Our findings support the view that soldiers may play an important role in colony immunity, and broaden our understanding of the possible function of soldiers during the origin of soldier-first societies.

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Introduction

In social insects, the evolution of a reproductively altruistic (ie sterile) caste was a fundamental step, as it facilitated the evolution of advanced division of labour and the emergence of sophisticated caste structures, in turn leading to vast ecosystem-dominating life forms (Oster & Wilson, 1978). The routes to reproductive altruism occurred via the evolution of sterile workers, as in Hymenoptera, or via the evolution of sterile soldiers, as in termites (Tian & Zhou, 2014). In termites, the soldier caste was the first sterile caste to evolve, and represents the only true sterile caste in many termite species (Korb & Hartfelder, 2008; Howard & Thorne, 2010; Tian & Zhou, 2014). Only a small minority of termite species are thought to have entirely lost the soldier caste (Bourguignon *et al*., 2016b).

In general, social insect castes, including termites, harbour multiple morphological, physiological and behavioural adaptations that are linked to specific tasks (Hölldobler & Wilson, 2009; Tian & Zhou, 2014; Engel *et al*., 2016; Kaji *et al*., 2016; Robson & Traniello, 2016). For example, workers (where present), the most abundant individuals in a colony, typically carry out many housekeeping roles such as brood care and foraging. By contrast, soldiers cannot feed themselves and depend on trophallaxis with workers for adequate nutrition. Soldiers make up 5–20% of a typical insect colony and display defensive adaptations (Thorne *et al*., 2003; Sobotnik *et al*., 2010; de Roode & Lefèvre, 2012; Tian & Zhou, 2014; Kaji *et al*., 2016). In termites, soldiers have specialized head and mandibular morphologies and/or chemical secretions that are thought to be synthesized in exocrine glands such as the salivary and the recently described labral gland (Moore, 1968; Prestwich, 1979; Sobotnik *et al*., 2010; Palma-Onetto *et al*., 2018) (Fig. 1A), which can be employed to physically attack or deter intruders. In some termite species, mandibles

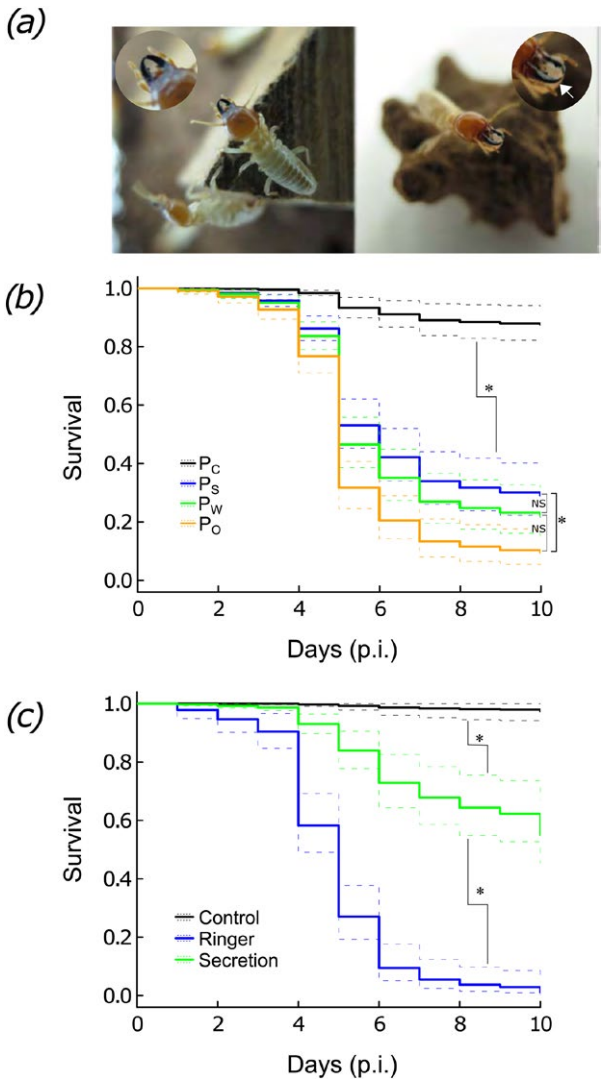

Figure 1. (A) A soldier before (left) and after (right) secretion of oral fluids (white arrow). (B) Survival of infected *Mastotermes darwiniensis* workers (two) in days postinfection (p.i.), after exposure to *Metarhizium anisopliae*. Infected workers are placed in the following treatments: two infected workers with one unmanipulated soldier (soldier treatment 'P_S'), two infected workers with one unmanipulated worker (worker treatment 'P_W') or only two infected workers (pair-only treatment 'P_O'); Survival of uninfected workers from each of the above three categories was combined and is displayed as a single treatment (treatment 'P_C'). (C) Worker pair survival following *Me. anisopliae* infection after topical treatment with Ringers' solution (Ringer) or soldier-derived oral secretion (Secretion); uninfected worker pairs treated with Ringer's solution or oral secretion were combined and are displayed as a single treatment (Control). *, Significant survival differences between treatments following post-hoc pairwise comparison. Dashed lines indicate the 95% confidence intervals of the corresponding fitted survival curve mean.

as defensive structures have been entirely lost and replaced with ejectable chemically active fluids (Moore, 1968; Prestwich, 1979, 1984).

Such costly synthesized exocrine secretions are one important component of a broad suite of defensive mechanisms found in termite societies (Prestwich, 1979; Sobotnik *et al*., 2010; Sillam-Dusses *et al*., 2012; Kaji *et al*., 2016), which depending on the species consists in large part of terpenoids or acetate-derived chemicals (Moore 1968; Prestwich 1979, 1984). Aside from these abundant semiochemical compounds, there are a number of proteins in soldier secretions that have never been fully identified (Moore 1968). Studies in other insects have shown that excreted proteins can play an important role in digestion (Sillam-Dusses *et al*., 2012; Kambara *et al*., 2014), defence (Negulescu *et al*., 2015; Lawson *et al*., 2017), immunity (Negulescu *et al*., 2015; Palmer *et al*., 2016), and caste regulation (Drapeau *et al*., 2006). Given the importance of externally secreted molecules to eusocial insect immunity (Otti *et al*., 2014), the highly enriched exocrine secretions of termite soldiers could make them ideal reservoirs of externally applied immune molecules.

Some evidence from both termites and other soldier-first societies such as thrips (Turnbull *et al*., 2012) indicates that soldiers can contribute to colony-level immunity (Rosengaus *et al*., 2000; Fuller 2007; Gao & Thompson 2015; Negulescu *et al*., 2015; Mitaka *et al*., 2017). However, most investigations into social immunity have been conducted in workers, where a large number of mechanisms have been shown to protect social insect colonies against pathogen exposure (Cremer *et al*., 2007; Hamilton *et al*., 2011; Stroeymeyt *et al*., 2014; Meunier 2015; Vannette *et al*., 2015; Wang *et al*., 2015). These encompass worker behaviours that reduce parasites by barring, burying or even cannibalizing infected individuals (Cremer *et al*., 2007), or communicating the presence of pathogens to other nestmates (Rosengaus *et al*., 1998, 1999). They also extend to hygienic behaviours such as mutual grooming (Konrad *et al*., 2012; de Roode & Lefèvre 2012); the collection (de Roode & Lefèvre 2012; Vannette *et al*., 2015) or synthesis of antimicrobial compounds (Bulmer *et al*., 2009) that reduce infectiousness and disease susceptibility; and even socially mediated immunization (Hughes *et al*., 2002; Traniello *et al*., 2002; Konrad *et al*., 2012), whereby prophylactic transfer of molecular effectors (Hamilton *et al*., 2011) or low pathogen doses (Hughes *et al*., 2002; Hamilton *et al*., 2011; Konrad *et al*., 2012) lead to protection of susceptible nestmates against infection.

In this study, we employed the Darwin termite, *Mastotermes darwiniensis*, to examine the role of soldiers in group-level immunity. *Ma. darwiniensis* is located basally in the termite phylogeny (Inward *et al*., 2007; Legendre *et al*., 2008), is the only termite species to have retained the bacterial endosymbiont *Blattabacterium* – an obligate intracellular mutualist of cockroaches (Sabree

et al., 2012), and unlike many lower termite species, this species is unusual in possessing true workers (Inward *et al*., 2007; Legendre *et al*., 2008). These factors single out *Ma. darwiniensis* as a significant target of research into termite evolution, ecology and sociality. We conducted survival experiments to measure the impact of soldiers on nestmate survival following lethal exposure to an entomopathogen. We also examined the effect of orally derived soldier secretions on survival when applied directly to nestmates, and characterized the antimicrobial activity and the proteome of the oral secretions.

Results

Effect of soldiers on worker pair survival

Survival across all group types was significantly reduced by fungus treatment. Bonferroni-corrected Tukey post-hoc comparisons of model means are as follows: survival of infected worker pairs kept on their own was significantly lower than the control treatment [pair-only treatment (P_O) vs. control treatment (P_C), hazard ratio = 16.28, $z = 9.50$, $P < 0.001$]; as was infected worker pair survival when cohabited with one uninfected soldier [soldier treatment (P_S) vs. P_C, hazard ratio = 8.88, $z = 7.26$, $P < 0.001$] or one uninfected worker [worker treatment (P_W) vs. P_C, hazard ratio = 11.40, $z = 8.15$, $P < 0.001$]. The survival of infected worker pairs cohabited with one uninfected soldier was significantly greater than infected worker pairs only (P_S vs. P_O, hazard ratio = 0.54, $z = -3.86$, $P < 0.001$), whereas the survival of infected worker pairs cohabited with one uninfected worker was not significantly different from infected worker pairs only (P_W vs. P_O, hazard ratio = 0.70, $z = -2.33$, $P = 0.118$). Survival did not differ between infected worker pairs that were cohabited with either one uninfected soldier or one uninfected worker (P_S vs. P_W, hazard ratio = 0.78, $z = 1.527$, $P = 0.761$; Fig. 1B).

Group behavioural observations and mass of soldier secretions

We observed soldier–worker behavioural interactions five times in a 24-h time-frame following fungus exposure. We found no proctodeal trophallaxis with soldiers acting as donors, and only a very limited number of proctodeal exchanges in total with workers as donors (control: two times; infected: four times). Additionally, stomodeal trophallaxis between workers and soldiers did not differ between infected and control groups ($z = -0.376$, $P = 0.707$). Allogrooming of soldiers by workers was higher in infected vs. control groups, but not significantly ($z = 1.765$, $P = 0.077$). Soldiers in infected groups were significantly more active (involved in recorded behaviours except staying still: 'inactive') than soldiers in control groups ($z = 7.758$, $P < 0.001$). Soldier secretions were collected and weighed after 48h of pairing with fungus-infected workers. The mass of soldier exudate following exposure to fungus-treated workers differed by treatment, but only in colony I (colony I: $t = -2.463$, $P = 0.017$, colony II: $t = 1.301$, $P = 0.200$), indicating a significant effect of colony on soldier secretion use (Fig. 2).

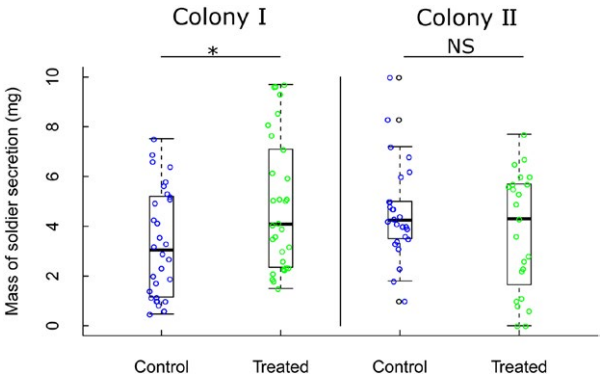

Figure 2. The mass of soldier oral secretion from two colonies (Colony I and Colony II) following cohabitation with *Metarhizium anisopliae*-exposed workers. *, Significant difference between treatments ($P < 0.05$). [Colour figure can be viewed at wileyonlinelibrary.com]

Effect of soldier-derived oral secretions on worker survival

We tested the role of soldier oral exudates in group immunity by applying a droplet of exudate topically to individual workers before exposure to a lethal dose of *Metarhizium anisopliae*. As expected, we found that fungal infection significantly reduced survival of worker pairs compared with controls. Bonferroni-corrected Tukey post-hoc comparisons of model means are as follows: Ringer's solution-treated worker pair survival vs. control (Ringer vs. control, hazard ratio = 268.99, $z = 5.490$, $P < 0.001$); soldier-exudate treated worker pair survival vs. control (secretion vs. control, hazard ratio = 27.39, $z = 3.262$, $P = 0.003$). The application of soldier-derived oral secretions also significantly improved survival of worker pairs compared to Ringer's solution treated worker pairs (secretion vs. Ringer, hazard ratio = 0.10, $z = -9.676$, $P < 0.001$; Fig. 1C).

Antimicrobial activity of soldier-derived oral secretions

We tested the antimicrobial activity of soldier-derived oral secretions on four different bacterial species (two Gram-negative and two Gram-positive bacteria) and the entomopathogenic fungus *Me. anisopliae*. We found clear zones of inhibition around the disc for all exposed bacterial species (*Pseudomonas entomophila*, *Bacillus*

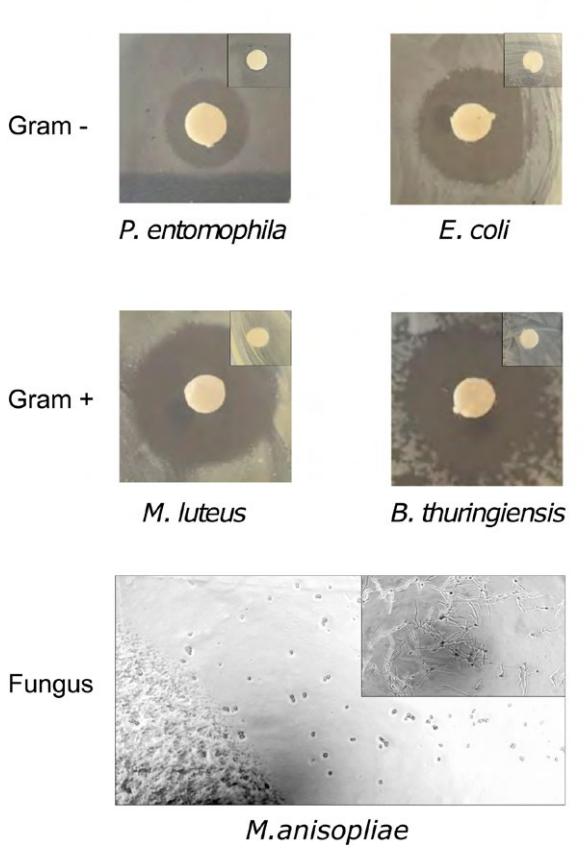

Figure 3. Representative growth inhibition by soldier oral secretions of *Pseudomonas entomophila*, *Escherichia coli*, *Micrococcus luteus* and *Bacillus thuringiensis* following disc diffusion tests and germination inhibition of *Metarhizium anisopliae* conidia following exposure to soldier-derived droplet. The secretion droplet is visible in the lower left-hand corner of the image. The corresponding controls for each antimicrobial assay are shown at the top-right hand corner of each image. [Colour figure can be viewed at wileyonlinelibrary.com]

thuringiensis, *Escherichia coli* and *Micrococcus luteus*) measuring 1.08 ± 0.05, 1.60 ± 0.09, 1.97 ± 0.12, 2.07 ± 0.04 cm, respectively (Fig. 3), with Ringer's solution-treated discs as control, which did not harbour inhibition zones. The secretions also possess high antifungal activity: germination of *Me. anisopliae* conidia was entirely suppressed compared with Ringer's solution (germination rate: 0 vs. 92.80 ± 1.57%).

Protein identification

To prepare the database for protein identification, we *de novo* sequenced the transcriptome of *Ma. darwiniensis*. In total 36 600 000 paired reads were generated from the transcriptome library, 1184 of which were excluded after trimming and quality control, leaving 36 500 000 reads available for subsequent *de novo* transcriptome assembly. The raw reads are available in the Sequence Read Archive in the National Center for Biotechnology (NCBI) database (BioProject PRJNA420017). The *de novo* assembler Trinity produced 185 829 transcripts with an E90N50 of 1399 bp. By assessing transcriptome completeness by Benchmarking Universal Single-Copy Orthologs (BUSCO), we identified 89.7% complete (61.1% single-copy orthologues and 28.6% duplicated orthologues), 8.7% fragmented and 1.6% missing orthologues, indicating high transcriptome completeness. We used Trinotate to annotate our assembly and annotated 37.12% of the transcripts using at least one of these package tool. After prediction by TransDecoder, there were in total 90 565 predicted proteins, including 18.1% complete, 18.9% 5′ partial, 6.5% 3′ partial and 56.5% internal proteins. The predicted proteins with the annotations from Trinotate were subjected to the following tandem mass spectrometry (MS/MS) data analysis.

We excised 20 bands from separated secretion proteins after SDS-PAGE and analysed by liquid chromatography-MS/MS (LC-MS/MS). After searching the transcriptome database, 139 proteins (Supporting Information Table S1) were identified, of which 135 were annotated using the Trinotate annotations from the transcriptome. The significance values of these search results are presented as e-values in Table 1 and Supporting Information Table S1 along with the corresponding protein names and Uniprot IDs. Identified proteins consisted of 56.8% complete, 32.4% 5′ partial, 5% 3′ partial and 9.4% internal proteins. Detailed information concerning all peptide matches are given in Supporting Information Table S1.

The identified proteins are mainly related to metabolism, protein regulation, stress response, muscles or other functions. Regarding metabolism, we identified glucose-degradation enzymes (maltase 2, glucosylceramidases, trehalase and alpha-amylase, beta-mannosidase, 5′-nucleotidase), lipid metabolism related proteins (such as family with sequence similarity 151 member B (FAM151B), esterase FE4, lipase 3 and apolipoproteins), and some Adenosine triphosphate (ATP)-related proteins (ATP synthase, succinyl-Coenzyme A ligase). For protein regulation, proteases (cathepsin B, cathepsin L, serine/threonine-protein kinase, Lysosomal Pro-Xaa carboxypeptidase, Ras-like protein 3), protease inhibitors (leukocyte elastase inhibitor C, serpin, serpin-like proteins), some histones and a polyubiquitin were also identified in secretions. Furthermore, we identified peroxidase, superoxidase dismutase, heat-shock proteins and glutathione S-transferase, which are all related to stress response. In addition, some muscle-related proteins were found in secretions, such as myophilin, tropomyosin, tubulin beta chain, actin, and synaptic vesicle membrane protein. We also identified two insect-specific proteins in the soldier secretions: protein yellow and haemolymph juvenile hormone binding protein. Interestingly,

Table 1. Top 30 putative proteins identified from *Mastotermes darwiniensis* soldier secretions

Description	Score	M. (kDa)	No. pept.	SC (%)	emPAI	Prot. ID	E-value
Digestion							
Maltase 2	72145	66 619	16	0.86	112.74	O16099	0
Glucosylceramidase[1]	33167	60 438	40	0.67	17.58	Q70KH2	1.66e-132
Glucosylceramidase[2]	3024	60 426	23	0.44	5.54	P17439	1.44e-130
Trehalase	5351	65 790	20	0.4	3.63	Q8MMG9	0
Alpha-amylase 1	4687	56 544	16	0.42	3.10	Q23835	0
Immunity							
Glucose dehydrogenase[1] [FAD-quinone]	26018	69 640	36	0.6	23.58	P18172	0
Leukocyte elastase inhibitor C	4768	45 561	12	0.43	8.12	Q5SV42	2.99e-68
Serpin B6[1]	3349	45 128	14	0.38	3.02	Q4R3G2	1.06e-53
Glucose dehydrogenase[2] [FAD-quinone]	3828	68 117	15	0.35	2.89	P18172	2.99e-157
Peptidyl-prolyl cis-trans isomerase FKBP14	408	28 617	7	0.32	2.21	Q5R941	1.85e-53
Lysosomal aspartic protease	1412	41 553	12	0.34	2.36	Q03168	0
Peroxidase	4602	76 890	20	0.38	2.33	Q01603	0
Glutathione S-transferase 1-1	154	24 465	7	0.32	2.30	P30108	1.96e-109
Other							
Apolipoprotein[1,a]	4054	24 153	18	0.63	21.07	PF01442.15	0.0027
Protein yellow	8199	50 819	29	0.66	20.19	Q9BI18	3.54e-76
L-ascorbate oxidase	33498	72 917	28	0.66	13.20	P14133	9.19e-56
Apolipoprotein[2,a]	2003	30 695	17	0.46	9.05	PF01442.15	7.40e-08
Apolipoprotein[3,a]	17939	25 920	14	0.39	8.44	PF01442.15	0.066
Fasciclin-2	4601	85 753	39	0.48	8.09	P22648	0
Venom allergen 3[1]	843	21 331	7	0.36	6.01	P35779	4.05e-46
Venom allergen 3[2]	1376	27 403	9	0.51	5.20	P35778	7.28e-61
Regucalcin	6257	37 834	14	0.21	4.86	Q2PFX5	9.25e-66
Multiple inositol polyphosphate phosphatase 1	3276	52 017	9	0.3	4.01	Q5R890	1.25e-38
Lazarillo protein	513	21 326	8	0.52	3.75	P49291	1.81e-06
Polyubiquitin	637	11 469	4	0.46	3.17	P23398	1.69e-68
Actin- clone 403	2040	41 827	3	0.35	3.06	P18603	0
Cathepsin L	751	38 004	10	0.33	2.75	Q26636	2.07e-168
Haemolymph juvenile hormone binding protein	241	9270	2	0.27	2.68	PF06585.8	3.2e-17
Glutaminyl-peptide cyclotransferase	643	39 910	10	0.38	2.52	Q16769	6.56e-101
14-3-3 protein zeta	889	28 099	6	0.28	2.28	Q2F637	9.4e-179

Score, mass score; M (kDa), predicted protein mass; No. pept., number of significant unique peptide sequences; emPAI, exponentially modified protein abundance index; Prot. ID, homology search protein ID of predicted proteins based on transcriptome data; E-value, blastp search e-value; FAD, flavin adenine dinucleotide; FKBP14, FK506 binding protein 14.

[1,2,3] represent different isoforms of the same protein; [a] represents the annotation of protein from pfam.

Identified proteins were sorted by emPAI and the listed 30 proteins were categorized into digestion, immunity and others.

there are also oxidoreductases [glucose dehydrogenase [flavin adenine dinucleotide (FAD)-quinone], 15-hydroxyprostaglandin dehydrogenase [NAD(+)], short-chain dehydrogenase/reductase family member 11], isomerases (FK506 binding protein 14, protein disulphide-isomerase), transferases (glutaminyl-peptide cyclotransferase, protein O-linked-mannose beta-1,2-N-acetylglucosaminyltransferase) and also a laccase (laccase 2). Some venom allergens, like venom allergen 3, histones, ribosomal proteins and elongation factors were also found.

Proteins relating directly to insect immunity included beta-1,3-glucan binding protein 2 (also known as Gram-negative binding protein 2, GNBP2), the antimicrobial activity of which has been studied in detail in termites (Bulmer *et al*., 2009, 2012); a protein Niemann-Pick type C homologue, which is suggested to be involved in immune deficiency pathways in *Drosophila melanogaster* as well as sterol homeostasis and ecdysteroid biosynthesis (Huang *et al*., 2007; Shi *et al*., 2012); lysosomal aspartic protease (also known as cathepsin D), which is potentially involved in antimicrobial activity in the carpenter ant *Camponotus pennsylvanicus* (Hamilton *et al*., 2011); pathogenesis-related protein 5, which has been shown to harbour antimicrobial activity in some insects (Drapeau *et al*., 2006; Altincicek *et al*., 2008); and phospholipase A2, which has been suggested to possess antimicrobial activity in the tobacco hornworm, *Manduca sexta* (Tunaz *et al*., 2003; Tunaz & Stanley, 2004) (Supporting Information Table S1). Some proteins with other putative pleiotropic functions may also play a role in termite immunity, as has been shown in other insects. These include proteases (Colbert *et al*., 2009), protease inhibitors (Cerenius *et al*., 2008), stress response proteins (Napping, 1995; de Morais Guedes *et al*., 2005; Colinet *et al*., 2011; Wojda, 2016) and oxidoreductases like glucose dehydrogenase [FAD-quinone] in the larvae of the moth *M. sexta* (Cox-Foster & Stehr, 1994). The top 30 proteins, as sorted by their relative abundance and categorized by function [based on exponentially modified protein abundance index (emPAI) value], are listed in Table 1.

Discussion

Owing to their specialized head and mouth morphology, termite soldiers cannot feed by themselves or engage in allogrooming. Soldiers could therefore be viewed as a potentially costly component of colony integrity during pathogen exposure. However, our findings show that termite soldiers can contribute to external immunity by confering equal or better group-level survival benefits as compared to workers. We find that the presence of Darwin termite soldiers protects nestmates against cuticular pathogen infection, and that nestmate survival is significantly improved when soldier-derived oral secretions are applied directly to the cuticle of workers following pathogen exposure. The secretions showed strong antibacterial as well as antifungal activity, although we found that secretion quantity and trophallactic exchanges between soldiers and workers did not consistently differ by treatment in the two colonies tested following interaction with pathogen-exposed worker nestmates, pointing towards a stronger role for prophylaxis over therapy for termite soldiers. We identified 139 protein components of soldier secretions, including proteins involved in metabolism and chemical biosynthesis, as well as proteins associated with antimicrobial activity and immunity.

Termite soldier secretions are known to contain organic compounds that have broad cytotoxic activity such as quinones and terpenoids, which are synthesized in the labial and/or frontal glands (Prestwich, 1979; Sobotnik *et al*., 2010; Sillam-Dusses *et al*., 2012). In *Ma. darwiniensis* soldiers, the identified chemical compounds are thought to be mixed in the labial glands (Sillam-Dusses *et al*., 2012) with *p*-benzoquinone being the dominant component found in secreted fluids (Moore, 1968) (Table 2). The process of quinone production had been hypothesized in *Eleodes longicollis* and *Tribolium castaneum* (Happ, 1968), and also as a part of luciferin biosynthesis in the firefly *Luciola lateralis* (Oba *et al*., 2013) but the pathways responsible for the production of these chemicals has remained largely unclear. In our study, we identified glucosylceramidase, oxidoreductases (in particular, glucose dehydrogenase [FAD-quinone]) and laccase 2, which are likely to be involved in benzoquinone biosynthesis

Table 2. Chemical compounds from *Mastotermes darwiniensis* soldier secretions.

Chemical name	Origin
p-benzoquinone derivative toluquinone	Secretion (Moore, 1968)
p-benzoquinone hydroquinone 2-methoxyhydroquinone methyl glucopyranoside *p*-arbutin	Labial gland extract (Sillam-Dusses *et al.*, 2012)

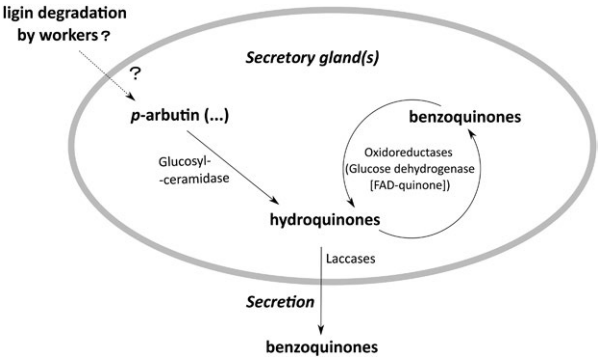

Figure 4. Proposed *Mastotermes darwiniensis* soldier secretion chemical biosynthesis pathway. →, possible metabolic path; ?, unknown enzymes or origin of source metabolites. Owing to the cytotoxicity of benzoquinones, we propose that *Ma. darwiniensis* employs a protection system mediated by oxidoreductases [eg glucose dehydrogenase (flavin adenine dinucleotide quinone)] to convert oxidized benzoquinones back to hydroquinones at the site of synthesis.

in soldier secretions. In Fig. 4, we propose a pathway for benzoquinone biosynthesis in *Ma. darwiniensis*. Glucosylceramidase, which belongs to the glucoside hydrolase family, can digest glucan hydrolyse O- and S-glycosyl compounds. In *Ma. darwiniensis* soldiers, we hypothesize that glucosyceramidase hydrolyses *p*-arbutin to glucose and hydroquinone, which have also been reported in labial glands (Table 2). After being secreted and exposed to oxygen, hydroquinone is oxidized by oxidases (such as laccase 2) into benzoquinone, which is the dominant chemical compound found in *Ma. darwiniensis* soldier secretions. As benzoquinones are highly cytotoxic, oxidoreductases such as glucose dehydrogenase [FAD-quinone] may be involved in preventing the hydroquinone from oxidizing prior to secretion. Such a protection mechanism has also been found in old workers of *Neocapritermes taracua* (Bourguignon *et al*., 2016a). Our identification of isomerases and transferases, characterized here in termites for the first time, indicate that quinones are probably biosynthesized *de novo* by soldiers, as is the case with terpene biosynthesis in nasute termite soldiers (Prestwich *et al*., 1981), possibly from lignin degradation by workers (Geib *et al*., 2008). These secreted chemical compounds could have multiple functions in a colony, including defence, phagostimulation, caste regulation and immunity (Prestwich, 1984; Rosengaus *et al*., 2000; Reinhard *et al*., 2002; Tarver *et al*., 2009; Sobotnik *et al*., 2010; Mitaka *et al*., 2017). Such chemicals offer clues relating to the potential function of soldiers in colonies, and imply that direct behavioural contacts with other colony members may be less relevant in soldiers than workers (Rosengaus *et al*., 2000; Mitaka *et al*., 2017), a hypothesis that is also supported by our behavioural

assay, where limited interactions between soldiers and other nestmates were observed.

We identified 28 and 21 proteins (out of 139) with similarities to oral secretions from ants and a honey bee, respectively (LeBoeuf *et al*., 2016). In both ants and termites, the most dominant proteins were involved in metabolism, including maltase, amylase and trehalase. This is reasonable as these insects require a repertoire of enzymes to assist sugar digestion, and salivary glands are the main origin of these proteins. In *Ma. darwiniensis*, we found several proteins relating to insect immunity, which was also the case in ants. These included proteins with putatively similar functions, such as glucose dehydrogenase (Cox-Foster & Stehr, 1994), some serpins (Cerenius *et al*., 2008), an NPC2 homologue (Shi *et al*., 2012) and several proteases. However, we also found that many immune-related proteins were unique to *Ma. darwiniensis*, including peroxidase, peroxiredoxin and GNBP2 (Bulmer *et al*., 2009; Bulmer *et al*., 2012). The identification of these immune-related proteins in soldier secretions further strengthens the view that externally secreted molecules derived from soldiers play a role in colony-level immunity.

In addition, we detected a significant amount of protein yellow, which in *Drosophila* has a dual function in pigmentation and behaviour (Wittkopp *et al*., 2002; Drapeau *et al*., 2003; Tian *et al*., 2004). Although its function in other insects is barely known, the closely related major royal jelly protein genes, which originated following the rapid radiation of a *yellow* gene precursor (Drapeau *et al*., 2006) regulate caste and fertility in the honey bee (Kamakura, 2011; although see Buttstedt *et al*., 2016). Interestingly, we also identified haemolymph juvenile hormone binding protein (JHBP). JHBP binds juvenile hormone (JH), which in turn is thought to regulate polyphenism and caste development in termites (Korb, 2015; Korb & Belles, 2017). Interestingly, JH may also be involved in immunity as it has been suggested to suppress the expression of antimicrobial peptides in *D. melanogaster* (Flatt *et al*., 2008). The presence of JHBP and protein yellow indicates that caste regulation in *Ma. darwiniensis* may be mediated by the regulation of JH by soldiers, which in turn also suggests that soldier oral secretions have multiple functions in colonies.

The functions of individual proteins found in soldier oral secretions require further investigation. A possible role for pleiotropy in many such proteins, such as enzymes involved in quinone biosynthesis should be considered, as these may also be involved in other key processes such as cuticle sclerotization (Hopkins & Kramer, 1992). An additional question concerns the origin of secreted proteins, which by and large are expected to be synthesized from the termite host itself (Hojo *et al*., 2005) by head and/or abdominal glands, which may then spread via trophallaxis between worker and soldier castes within colonies. However, the microbiota itself may also represent an important source of enzymes that can be co-opted by the host (Warnecke *et al*., 2007; Rosengaus *et al*., 2014; Brune & Dietrich, 2015; Morella & Koskella, 2017).

We showed that *Ma. darwiniensis* soldier oral secretions harbour broad constitutive antimicrobial activity by inhibiting the growth of diverse bacteria, including two pathogens and an entomopathogenic fungus. As the secretion is made up of both proteins and chemicals, this activity could be mediated by immune-related proteins such as GNBP2, as well as the actions of cytotoxic quinones, which we predict are synthesized by glucosylceramidase amongst other enzymes. It remains unclear to what extent the proteins detected in soldier oral secretions are directly or indirectly involved in antimicrobial activity. For example, GNBP2 is known to have direct antimicrobial properties (Bulmer *et al*., 2009). However, evidence also shows that insect- (including termite) secreted chemicals can possess antimicrobial activity (Rosengaus *et al*., 2000; Otti *et al*., 2014; Mitaka *et al*., 2017). Chemically mediated microbial inhibition, perhaps via benzoquinone, could therefore play a significant role in the inhibition we observed in *Ma. darwiniensis*. Proteins may be indirectly involved here, owing to their putative involvement in the chemical biosynthesis of benzoquinone (Fig. 4). However, as noted above, it remains possible that such chemicals could be synthesized via unknown non host-derived metabolic pathways (eg microbiota-derived). Our data also hint at a potential therapeutic role for soldiers because the mass of secretions increased following cohabitation with pathogen-exposed workers. However, this effect was not consistently observed across colonies and therefore remains equivocal. This different response between colonies could indicate fixed- or condition-dependent colony differences in pathogen sensitivity. Although *Ma. darwiniensis* reproductives, and colonies, are known to be very long-lived (Keller, 1998), we cannot exclude the possibility that the relatively long period of time that these colonies have been kept under laboratory conditions may have also contributed to this variation.

Ma. darwiniensis is the only extant representative of the most basally located termite lineage, Mastotermitidae (Eggleton, 2001). Previous studies exploring the antimicrobial activity of soldier-derived secretions focused on species that are distantly related to *Ma. darwiniensis* (Rosengaus *et al*., 2000; Fuller, 2007; Mitaka *et al*., 2017). The occurrence of antimicrobial activity in diverse termite lineages therefore raises the possibility that an immune role for soldiers is a conserved ancestral termite trait. This is supported by the detection of GNBP2

in *Ma. darwiniensis* oral secretions, which resulted from a gene duplication that preceded the divergence of Cryptocercidae and the termite crown group (Bulmer *et al*., 2012). GNBP2 is evolutionarily conserved across Isoptera and has been shown to be a key regulator of social immunity in termites (Bulmer & Crozier, 2004; Bulmer *et al*., 2009). If this hypothesis is correct, it would be consistent with other soldier-first insect societies, such as the thrips, where a dual defensive/immune role for soldiers, via the synthesis of potent antifungal compounds, has been shown (Turnbull *et al*., 2012). Taken together, these findings raise the interesting hypothesis that soldier involvement in colony-level immunity may be required for the evolution of soldier-first societies (Tian & Zhou, 2014). Overall, we hope our findings will stimulate further research on the role of soldiers in colony immunity, and on the relationship between colony immunity and the evolutionary origins of eusociality in general.

Experimental procedures

Termites and pathogens

Workers (approximate head width: 2.15–2.45 mm) and soldiers (approximate head width: 2.70–2.95 mm) were extracted from two colonies of *Ma. darwiniensis*, collected from Townsville (Australia) in 1972 and kept at 28 °C and 70% humidity in the Federal Institute of Materials Research and Testing (BAM), Berlin. Termite colonies were fed regularly with predecayed birch wood. Two Gram-negative bacteria (*P. entomophila*, DSM 28517^T and *E. coli* K12), two Gram-positive bacteria (*Mi. luteus*, DSM 20030^T and *B. thuringiensis*, DSM 2046^T) and one fungus (*Me. anisopliae*, DSM 1490^T) were stored in the BAM and cultivated for use in subsequent experiments.

Me. anisopliae *conidia preparation and termite infection*

Me. anisopliae conidia were kept at −70 °C and grown on malt extract agar at 24 °C for 3–4 weeks prior to each experiment. Conidia were harvested by suspending them in 0.1% Tween 80 (Merck, Rahway, NJ, USA) and storing at 4 °C for a maximum of 2 weeks. The viability of conidia was confirmed directly before each experiment by quantifying the germination of conidia as described previously (Rosengaus *et al*., 1998), with germination rates exceeding 90% in all experiments. Termites were exposed to conidia in solution by allowing them to walk freely for 1h in groups of 10 in Petri dishes (diameter: 90 mm) lined with filter paper (Whatman No. 5, Merck KGaA, Darmstadt, Germany) moistened with 1 ml defined conidia concentration. After exposure, termites were transferred randomly into new sterile Petri dishes to establish subgroups for subsequent experiments. Subgroups were kept in Petri dishes lined with filter paper (Whatman No. 1) moistened with 1 ml water. All Petri dishes were subsequently put in plastic boxes lined with moistened paper towels and maintained at 27 °C and 65% relative humidity in complete darkness.

Survival of termite workers following infection with Me. anisopliae

Workers were treated with 1.0×10^7 conidia/ml of *Me. anisopliae* suspended in 0.1% Tween 80 or an equivalent 0.1% Tween 80 control solution. To minimize the synergistic effects of worker group size on factors such as grooming frequency, we measured the survival of (two) infected termite workers placed in the following treatments: two infected workers with one unmanipulated soldier (soldier treatment 'P_S'), two infected workers with one unmanipulated worker (worker treatment 'P_W') or only two infected workers (pair-only treatment 'P_O'). For the fungus-exposed treatments, each category was replicated in colonies I ($N = 25$) and II ($N = 30$). Equivalent uninfected control subgroups consisting of all three categories were also established ($N = 10$ for both colonies I and II), which were combined as a single treatment in subsequent survival analyses (treatment 'P_C'). All subgroups were kept in Petri dishes (diameter: 55 mm) with moistened filter paper (Whatman No. 1) and the mortality of the predetermined termite worker pairs from each subgroup was recorded for 10 days following treatment.

Soldier–worker interactions and oral secretion quantification

Groups of five workers were infected with either 5.0×10^6 conidia/ml of *Me. anisopliae* suspended in 0.1% Tween 80 ($N = 10$ groups from each colony) or an equivalent 0.1% Tween 80 control solution ($N = 10$ groups from each colony). Each group was kept in a Petri dish (diameter: 90mm) lined with moistened filter paper (Whatman No. 1) in the presence of an unexposed soldier. We conducted 30 behavioural observations over the course of 1h at each of five time-points following establishment of groups (0.5, 2.5, 4.5, 6.5, 24h). Only behaviours between soldiers and workers were considered, and the following types of behavioural states were counted during each observation: allogrooming (worker to soldier), stomodeal or proctodeal trophallaxis (soldier to worker, or worker to soldier), moving and inactivity (staying still). Soldiers engaging in the first four behavioural states were also described as 'active'. Soldier oral secretions were extracted at 48h and weighed. Secretions were collected by removing the total exudate produced from the

mouth of each living soldier with a pipette after triggering secretion by blowing air.

Survival of infected workers following exposure to soldier-derived oral secretions

Workers were treated with 1.0×10^7 conidia/ml of *Me. anisopliae* suspended in 0.1% Tween 80 ($N = 80$ for each colony) or an equivalent 0.1% Tween 80 control solution ($N = 20$ for each colony). Workers from each treatment were divided randomly into 50 pairs ($N = 40$ *Me. anisopliae* exposed pairs, $N = 10$ control pairs) and kept in Petri dishes (diameter: 55 mm) lined with moistened filter paper (Whatman No. 1). Worker pairs were further subdivided into two groups wherein pairs were treated with 0.3 µl soldier-derived oral secretion or Ringer's solution. Oral secretions or Ringer's solution were applied topically to the abdomens of each infected worker pair, with a different soldier being used for each worker pair. After application, the mortality of workers was recorded for 10 days.

Antimicrobial activity of soldier-derived oral secretions

Soldier oral secretions were collected as described above, before being dissolved in Ringer's solution and preserved at –20 °C. Antimicrobial assays against four bacteria and one fungus were used to detect the antimicrobial properties of soldier oral secretions. A diffusion disc method and a germination rate inhibition assay were employed for the bacterial and fungal species, respectively. Briefly, for the antibacterial assay, test agar plates (diameter: 90 mm) appropriate for each species were prepared following Deutsche Sammlung von Mikroorganismen und Zellkulturen guidelines and inoculated with 100 µl cultured bacteria (1.0×10^7 colony-forming units/ml). Sterile paper discs (diameter: 5 mm) containing 5 µl soldier oral secretion solution, or the equivalent Ringer's solution, were placed in replicate onto the agar plates. The plates were kept for 24 h at 28 °C for *P. entomophila*, 30 °C for *Mi. luteus* and *B. thuringiensis*, and 37 °C for *E. coli* and the mean minimum and maximum diameter of each clearance zone was recorded. For the fungal inhibition assay, we modified the method from Rosengaus *et al*. (2000). Briefly, 1 µl soldier exudate preparation, or an equivalent Ringer's control solution was dropped directly onto potato dextrose agar plates inoculated with 100 µl 0.1% Tween 80 containing 1.0×10^7 *Me. anisopliae* conidia/ml. The germination of conidia was used as a measure of conidia viability, as determined by recording the proportion of visible germ tubes within an area of a circle (diameter: 1 cm) around the experimental droplet. Secretions from five soldiers from the same colony were mixed ($N = 3$ for each colony) and used for each microbial species.

Proteomic database preparation by de novo *transcriptome sequencing of* Ma. darwiniensis

Whole bodies of four larvae, four workers and four soldiers were pricked through the cuticle with a cocktail of heat-killed microbes (*P. entomophila*, *B. thuringiensis*, *Saccharomyces cerevisiae*) and pooled with an equal number of unmanipulated individuals for total RNA extraction, described briefly as follows. Whole bodies of individual termites were suspended in pre-cooled Trizol (Thermo Fisher Scientific, Waltham, MA, USA) and homogenized using a homogenizer (FastPrep™-24, MP Biomedicals, Santa Ana, CA, USA) with a 5-mm stainless steel bead (Qiagen, Valencia, CA, USA). Recovery of RNA was achieved following the manufacturer's instructions, using chloroform extraction and isopropanol precipitation, and redissolving in RNA storage solution (Ambion, Carlsbad, CA, USA). Subsequently, samples were incubated with 2 units of TurboDNase (Ambion) for 30 min at 37 °C and RNA was purified using an RNAeasy Mini kit (Qiagen) according to the manufacturer's instructions. Quantity and quality of RNA were determined by QUBIT and BIOANALYZER 2100. Equal amounts of total RNA from each individual extraction were pooled together. Subsequently, mRNA library was enriched and was prepared following the NEXTflexTM Rapid Directional mRNA-seq Kit protocols (Bioo Scientific, Austin, TX, USA). The prepared library was sequenced on an Illumina NextSeq500/550 platform (Illumina, San Diego, CA, USA) at the Berlin Center for Genomics in Biodiversity Research (BeGenDiv).

The raw data were trimmed and filtered to remove barcodes, adapters, short reads (< 25 bp) and reads containing low-quality bases using TRIMMOMATIC, as incorporated in TRINITY (v. 2.2.0) (Grabherr *et al*., 2011). Paired-end reads were assembled using TRINITY with default parameters (Kmer size: 25) on a local server. The assembly quality was assessed by BUSCO v. 2 with the Arthropod BUSCO set from OrthoDB (version 9; Simão *et al*., 2015). The transcriptome was annotated by following the guidelines of TRINOTATE (https://trinotate.github.io/). Briefly, assembly and peptide sequences predicted from the assembly by TRANSDECODER (version 3.0.1) (http://transdecoder.github.io/) were used to query SWISSPROT with BLAST (Altschul *et al*., 1990). Protein domains, signal peptides and transmembrane domains were determined by HMMER (v. 3.1b2) against the pfam database (Finn *et al*., 2011), SIGNALP 4.0 (Petersen *et al*., 2011) and TMHMM 2.0 (Krogh *et al*., 2001), respectively. Homology searches, predictions and domain identifications were performed locally and subsequently integrated into the SQLite database with SQLite

version 3.11.0 (https://www.sqlite.org/index.html) at an e-value threshold of $1 \times e^{-03}$.

Protein identification of soldier-derived oral secretions

Soldiers were triggered to secrete an oral droplet by blowing air. The ejected drops of 20 soldiers were collected in 500 µl sterile double-distilled H_2O using a 10 µl pipette and kept on ice until further analysis (maximum 3h). For SDS-PAGE, 25 µl of this secretion was mixed with SDS sample buffer and boiled at 95 °C for 5 min. Subsequently, the sample was loaded onto gels and separated. After separation, gels were stained with Coomassie Blue (Roti©-Blue, Carl Roth, Karsruhe, Germany) for 6h and destained with water. The gel was cut and proteins were digested with trypsin followed by peptide extraction. The purified peptides were analysed by LC-MS/MS (Thermo Scientific). The details of SDS-PAGE and LC-MS/MS are described further in Supporting Information File S1.

Data processing and identification of proteins was performed using the Mascot Server (version 2.5.0, Matrix Science, London, UK) and Mascot Distiller software (version 2.6.1, Matrix Science). Processed spectra were used to search against the peptide database generated by our *Ma. darwiniensis* transcriptome, which explicitly removed all nontermite proteins, eg derived from protists and bacteria. A maximum of two missed cleavages was allowed and the mass tolerance of precursor and sequence ions was set to 10 ppm and 0.35 Da, respectively. Methionine oxidation, acetylation (protein N-terminus), and propionamide modification of cysteines were used as variable modifications. Only peptides with Mascot scores greater than the homology threshold were considered and a significance threshold of 0.05 was used based on decoy database searches. In addition, a peptide ion score cut-off of 20 was applied and only proteins with at least two significant unique sequences are reported in the output tables. After peptides from the LC-MS/MS analysis were matched to our transcriptome-derived predicted protein database, we ascertained the putative functions of proteins by querying the annotated functional database associated with our transcriptome-derived predicted-protein database. The functional annotation of the majority of these proteins was derived from SwissProt queries using BLAST (blastp) and proteins that could not be identified using blastp were assigned using hmmer search results from the annotated functional database. Proteins that could not be annotated with Trinotate suit tools (blastp, HMMER) were assigned 'unknown' function. Finally, identified protein sequences were queried (blastp) against the NCBI database to further corroborate protein identities. Candidate proteins that yielded different search results to the annotation approach are reported where relevant. Proteins are ranked by their emPAI value, which allows for approximate, label-free, relative quantitation of the proteins as described previously (Ishihama *et al*., 2005).

Statistical analyses

All analyses were performed in R v. 3.2.3 (Team, 2016). Termite survival data were analysed by Cox proportional hazard models (R packages 'coxme' and 'coxph'), with models containing subgroup replicate and colonies as random effects and treatment as a fixed effect. Survival curves were depicted graphically excluding random effect terms, as these could not be incorporated in curve fitting ('Survfit' function of a 'coxph' object). The R package 'multcomp' was employed for comparing significant differences between treatment means, using Bonferroni correction to account for multiple testing. The reported test results are as follows: hazard ratio, *z*-value from Wald test and *P*-value. Behavioural data were analysed by generalized linear mixed models (R package 'glmm'), with models containing time, colony and individual as random effects and treatment as a fixed effect. Student's *t*-tests were employed to compare secretion masses. Data are presented as means ± SE.

Data accessibility

For raw data and statistics, see the electronic Supporting Information. Proteomic data are available via ProteomeXchange with identifier PXD009195.

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Supporting Information

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

File S1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis and liquid chromatography-tandem mass spectrometry.

Table S1. Full list of putative proteins identified from *Mastotermes darwiniensis* soldier secretions.