ABSTRACT

FUNARO, COLIN FRANCIS. Royal Recognition Behaviors and Pheromones in the Subterranean Termite, *Reticulitermes flavipes*. (Under the direction of Dr. Edward Vargo and Dr. Coby Schal).

Social insects must communicate to effectively cooperate and thrive in large and complex colonies. Termites depend on chemical communication in the form of pheromones to mediate nearly all colony activities, including feeding, building, defense, and reproduction. Queens and kings are the sole reproductive castes within the termite colony and must communicate their status to prevent unwanted reproduction and solicit their attendant workers for care. Identifying the pheromones responsible for encoding these messages and their effects on the colony is an important aspect of understanding termite biology and behavior. Queen pheromones have been identified in multiple social hymenopterans, but queen and king pheromones in termites have received little attention and no royal recognition pheromones have been identified. Moreover, the behaviors associated with royal recognition have not been well studied in termites. In the following studies, we investigate cuticular chemicals of the eastern subterranean termite, *Reticulitermes flavipes,* identify royal-specific cuticular hydrocarbons, develop a behavioral assay of royal recognition behaviors, and we describe the first queen and king recognition pheromone in termites.

In the first study, cuticular extracts from *R. flavipes* collected in North Carolina were analyzed across castes and several colonies. Using gas-chromatography-mass spectrometry (GC-MS), we analyzed hexane extracts of the cuticular surface of queens, kings, and workers to identify compounds that differentiate these castes, and to specify candidate royal pheromones. We identified 21 of the most prominent cuticular hydrocarbons and successfully

distinguished castes and colonies with principal component analysis. Closer inspection of the suite of compounds revealed several previously un-reported and highly royal-enriched long-chain alkanes and a royal-specific alkane, heneicosane, as a candidate queen and king pheromone.

In the second study, we described behaviors that queens and kings elicit from workers and soldiers within the termite colony in an effort to develop a behavioral assay that would guide the isolation and identification of royal pheromones. Royal recognition has not been previously documented in termites, mostly because they do not clearly aggregate around queens or kings (i.e., form a retinue), a common response in social hymenopterans. We designed a behavioral assay to identify behaviors elicited by queens and kings in *R. flavipes*. We found that elevated antennation and rapid lateral shaking behaviors were elicited by queens and kings and that termites reacted in a similar fashion to non-reproductive termites or glass dummies coated with cuticular extracts from queens and kings. We conclude that cuticular compounds are responsible for eliciting royal recognition behaviors.

In the third study, we used the royal recognition bioassay to test whether heneicosane, the queen- and king-specific hydrocarbon, acts as a pheromone eliciting recognition behaviors. Additionally, we explored how termite cuticular compounds affect termite behavioral responses to heneicosane. We found that, when applied to glass dummies, heneicosane elicited a behavioral response in workers identical to that elicited by live termite queens, including increased antennation and vibratory shaking. Additionally, worker cuticular extracts enhanced the effects of heneicosane, indicating that chemical context is integral to termite royal recognition. We conclude that heneicosane is the first royal recognition pheromone in termites and the first king pheromone discovered in social insects.

Royal Recognition Behaviors and Pheromones in the Subterranean Termite, *Reticulitermes flavipes*.

by
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DEDICATION

This dissertation is dedicated to my mother and father, who always encouraged my passions and put up with my shenanigans and to my uncle Pete, for teaching me about science, lively discussion, and fine Irish poetry.

BIOGRAPHY

Colin Francis Funaro was born and raised in South Jersey out of the parking lots of suburbia and the tomato cages of his family's backyard. He graduated with a Bachelor's of Arts degree from the University of Richmond in Biology, with a studio art minor. He then worked as a laboratory technician with Dr. Jake Russell at Drexel University for two years before accepting a graduate assistantship at North Carolina State University.

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CHAPTER 1

Introduction

Social insects have flourished for the last hundred million years, colonizing nearly every habitat on every continent. The most well-known social insects have evolved from either solitary wasps, in the case of ants, bees, and social wasps, or solitary cockroaches, in the case of termites (Hölldobler and Wilson 1990; Lo et al. 2000). These two distantly related lineages of highly cooperative insects have independently evolved altruistic tendencies and certain life history traits to form colonies consisting of hundreds to millions of individuals working together. The cooperative behaviors and self-sacrifice these super-organisms exhibit contrasts with the self-serving nature of other solitary organisms, whose primary drive is to reproduce and maximize the number and survival of offspring. Rather, social insects have specialized to form castes that focus on reproduction, support, or defense. Non-reproductive individuals (workers and soldiers) forego reproduction and invest their energy in the success of egg laying castes, usually represented by only one or a few individuals (queens in hymenopterans, queens and kings in termites).

Kin selection is the predominant principle explaining how social behaviors like these evolved and are successful. This theory states that individuals will choose to help the offspring of a close relative rather than reproduce themselves if the cost of not reproducing is outweighed by the benefit of helping (Hamilton 1972; Boomsma 2007). Working under the umbrella of kin selection, social insect biologists endeavor to understand how insect societies operate and why social behaviors evolved as they did. Much of the inspiration to study these animals derives from parallels observed between insect and human societies. With a relatively simple brain, social insects form cohesive and cooperative groups with a clear division of labor that emulates human organization (Strassmann and Queller 2010). We compare human cities to the astonishing feats of architecture in social insects, find joy in

their efficiency, and empathize with the lone soldier facing certain death in defense of the colony. Researchers strive to understand how altruism and different forms of cooperation succeed and create such complex societies within the context of kin and natural selection.

Communication is a key feature of insect societies (Richard and Hunt 2013). Honeybees, arguably the most well studied social insect, use tactile stimuli, descriptive dances, and specific chemicals, or pheromones, to communicate within the colony. Pheromones are a particularly influential aspect of social insect behavior. They mediate foraging, aggregation, defense, reproduction, and other essential processes (Hölldobler and Wilson 1990; Blum 1996). By mediating reproduction, pheromones maintain the reproductive division of labor, which is one of the three biological factors defining eusociality. In practice, this means limiting egg production to one or a few individuals in the colony. While various mechanisms have evolved to prevent unwanted reproduction in other castes, including aggressive physical interactions (Monnin and Peeters 1999) and visual patterns signaling fertility (Tibbetts 2002), pheromones are often used as signals of reproductive status (Slessor, Kaminski et al. 1988; Vargo 1997; Endler, Liebig et al. 2004). Royal pheromones affect other castes by either eliciting immediate behaviors (releaser pheromones) or by inducing long-term physiological changes in sterile castes (primer pheromones). Identifying these compounds and elucidating their effects and glandular origins have received increasing attention in social insect biology.

Termites, defined as a group of about 3,000 species nested within the solitary and sub-social cockroaches, are all eusocial and form colonies that feed on decomposing plant matter, soil, or wood (Inward et al. 2007). Colonies are initially founded by a single pair of mated termites, i.e., the king and queen. As colonies mature, they diversify to include

workers, soldiers, and nymphs. Workers forage, build the nest, and care for any offspring in the colony, while soldiers defend the colony from attackers. Nymphs typically develop yearly and disperse as winged alates to mate and begin new colonies. The founding royal pair is of primary importance to the colony, and must be tended carefully by workers. However, when a queen or king is lost or dies, neotenic reproductives, defined as any reproductive individual not derived from a winged termite (Thorne 1996), act as a safety net to supplement or replace lost or dying queens and kings. Workers and brachypterous nymphs are facultatively sterile, meaning they retain the ability to develop a mature reproductive system once an established king or queen is removed. Unlike hymenopterans, where males are transient and typically die immediately after mating, royal castes of both sexes are maintained in termite colonies. This creates a unique situation in which kings and queens both need to control reproduction and solicit workers for care.

Despite their prominent status as pest species within human structures and their key functions in decomposition and nutrient cycling in forest and grassland ecosystems, the life history and nature of royal pheromones in termites is poorly understood. Termites are generally blind, living either underground or within wood. In these environments, non-visual signals such as vibration, touch, and pheromones are important for communication. Vibrations and tactile interactions between termites can signal alarm, indicate a valuable food source, or stimulate aggressive responses, but pheromones are the most prominent and diverse signals within termite colonies (Kirchner et al. 1994; Evans et al. 2005, 2009; Hager and Kirchner 2013). Foraging, colony defense, nestmate recognition, nest building, and other tasks are mediated by pheromones (Bignell et al. 2014). Royal pheromones and recognition behaviors have been well studied in ants, bees, and wasps, but have received little attention in

termites and little progress has been made identifying pheromones that are unique to queens and kings.

Insect pheromones are typically excreted from specialized glands on the cuticular surface (Vander Meer et al. 1998). Social hymenopterans have a variety of exocrine glands that generate and distribute these queen-specific signals, including the poison sac and postpharyngeal gland in ants and the mandibular glands in honeybees (Vander Meer et al. 1980; Slessor et al. 2005). Termite royal pheromones have been identified in only one species, *Reticulitermes speratus*, where a volatile blend of 2-methyl-1-butanol and n-butyl-n-butyrate is produced by queens to prevent the differentiation of female workers into neotenic queens (Matsuura et al. 2010). While this volatile pheromone significantly alters the physiology of other termites (i.e., a primer effect), it was not shown to elicit any recognition response or release any behaviors. Other studies have found royal-specific compounds in both kings and queens in a few termite species, but the functions of these compounds have not been elucidated (Liebig et al. 2009; Hanus et al. 2010; Himuro et al. 2011). Additionally, no king pheromones have been identified to date and, although a number of queen pheromones in hymenopterans are glandular, several queen recognition compounds in ants, bees and wasps have been found to be cuticular in origin (Van Oystaeyen et al. 2014).

The insect cuticle is covered in a thin layer of cuticular lipids, with cuticular hydrocarbons (CHCs) being most prominent (Blomquist and Bagneres 2010). Long-chain CHCs are highly hydrophobic compounds that serve to prevent water loss and protect against pathogens. These compounds are used by essentially all insects and some groups, including termites, have co-opted CHCs for use as pheromones. Termite CHC pheromones mediate nestmate recognition and other intra-colony interactions (Bagneres et al. 1991; Darrouzet et

al. 2014). Termite queens and kings produce a number of different compounds that differentiate them from other castes (Hanus et al. 2010; Himuro et al. 2011), including multiple examples of reproductive-specific CHCs (Weil et al. 2009; Liebig et al. 2009; Darrouzet et al. 2014). CHCs can successfully help researchers discriminate castes and species through statistical analysis, but it remains unclear whether the termites themselves use these compounds to communicate status.

This series of studies examines the behaviors associated with royal recognition in termites and the chemicals responsible for eliciting these behaviors. Recognition of queens and kings is a crucial step in the development of sociality and discovering the signals that encode royal recognition will help us understand the mysterious life history of an important pest species. Initial observations of the subterranean termite, *Reticulitermes flavipes*, revealed a very strong behavioral response to queens and kings in field-collected termite colonies, in which workers shake intensely when near these individuals. To further understand the nature of this behavior, we describe and quantify the behavior and establish it as an authentic metric of queen and king recognition. We also analyzed the cuticular chemical profiles of royal and non-royal termites to look for caste-specific candidate pheromones that could elicit the behavior. Finally, we identify a queen- and king-specific compound, heneicosane, as a royal recognition pheromone.

It is our hope that these studies advance the field of termite royal recognition and build a context for future work to fully understand how queens and kings function in termite colonies. Further, this work joins a growing body of literature supporting the co-option of CHCs as royal pheromones, uncovers an independent evolution of royal CHC pheromones outside of the social Hymenoptera, and suggests that CHCs may have been used to signal

royal status since the origin of termites, at least 150 million years ago. Although the overall function of the shaking behavior in various social contexts is not fully understood, the development of an effective metric for royal recognition should prove to be a valuable tool in evaluating termite worker response to royal castes in other species and to other stimuli. This research will contribute to the field of basic termite biology and inspire others to further unravel how complex termite societies evolved into a successful and globally distributed taxon.

REFERENCES

Bagneres A-G, Killian A, Clement J-L, Lange C (1991) Interspecific recognition among termites of the genus *Reticulitermes*: Evidence for a role for the cuticular hydrocarbons. J Chem Ecol 17:2397–2420.

Bignell DE, Roisin Y, Lo N (2014) Biology of Termites: a Modern Synthesis, 2nd edn. Springer, Netherlands (doi: 10.1007/978-90-481-3977-4)

Blomquist GJ, Bagneres AG (2010) Insect Hydrocarbons: Biology, Biochemistry, and Chemical Ecology. Cambridge Univ Press, Cambridge

Blum MS (1996) Semiochemical parsimony in the arthropoda. Annu Rev Entomol 41:353–374. doi: 10.1146/annurev.en.41.010196.002033

Boomsma JJ (2007) Kin selection versus sexual selection: why the ends do not meet. Curr Biol 17:R673–R683. doi: 10.1016/j.cub.2007.06.033

Darrouzet E, Labédan M, Landré X, et al (2014) Endocrine control of cuticular hydrocarbon profiles during worker-to-soldier differentiation in the termite *Reticulitermes flavipes*. J Insect Physiol 61:25–33. doi: 10.1016/j.jinsphys.2013.12.006

Evans TA, Inta R, Lai JCS, et al (2009) Termites eavesdrop to avoid competitors. Proc R Soc B Biol Sci 276:4035–4041. doi: 10.1098/rspb.2009.1147

Evans TA, Lai JC, Toledano E, et al (2005) Termites assess wood size by using vibration signals. Proc Natl Acad Sci U S A 102:3732–3737.

Hager FA, Kirchner WH (2013) Vibrational long-distance communication in the termites *Macrotermes natalensis* and *Odontotermes sp*. J Exp Biol 216:3249–3256. doi: 10.1242/jeb.086991

Hamilton WD (1972) Altruism and related phenomena, mainly in social Insects. Annu Rev Ecol Syst 3:193–232. doi: 10.1146/annurev.es.03.110172.001205

Hanus R, Vrkoslav V, Hrdy I, et al (2010) Beyond cuticular hydrocarbons: evidence of proteinaceous secretion specific to termite kings and queens. Proc R Soc B Biol Sci 277:995–1002. doi: 10.1098/rspb.2009.1857

Himuro C, Yokoi T, Matsuura K (2011) Queen-specific volatile in a higher termite *Nasutitermes takasagoensis* (Isoptera: Termitidae). J Insect Physiol 57:962–965. doi: 10.1016/j.jinsphys.2011.04.012

Hölldobler B, Wilson EO (1990) The Ants. Belknap Press of Harvard Univ. Press, Cambridge, MA

Inward DJG, Vogler AP, Eggleton P (2007) A comprehensive phylogenetic analysis of termites (Isoptera) illuminates key aspects of their evolutionary biology. Mol Phylogenet Evol 44:953–967. doi: 10.1016/j.ympev.2007.05.014

Kirchner WH, Broecker I, Tautz J (1994) Vibrational alarm communication in the damp-wood termite *Zootermopsis nevadensis*. Physiol Entomol 19:187–190. doi: 10.1111/j.1365-3032.1994.tb01041.x

Liebig J, Eliyahu D, Brent CS (2009) Cuticular hydrocarbon profiles indicate reproductive status in the termite *Zootermopsis nevadensis*. Behav Ecol Sociobiol 63:1799–1807. doi: 10.1007/s00265-009-0807-5

Lo N, Tokuda G, Watanabe H, et al (2000) Evidence from multiple gene sequences indicates that termites evolved from wood-feeding cockroaches. Curr Biol 10:801–804.

Matsuura K, Himuro C, Yokoi T, et al (2010) Identification of a pheromone regulating caste differentiation in termites. Proc Natl Acad Sci 107:12963–12968. doi: 10.1073/pnas.1004675107

Richard F-J, Hunt JH (2013) Intracolony chemical communication in social insects. Insectes Sociaux 60:275–291. doi: 10.1007/s00040-013-0306-6

Slessor KN, Winston ML, Conte YL (2005) Pheromone Communication in the Honeybee (*Apis mellifera* L.). J Chem Ecol 31:2731–2745. doi: 10.1007/s10886-005-7623-9

Strassmann JE, Queller DC (2010) The social organism: congresses, parties, and committees. Evolution 64:605–616. doi: 10.1111/j.1558-5646.2009.00929.x

Thorne BL (1996) Termite terminology. Sociobiology 28:253–263.

Van Oystaeyen A, Oliveira RC, Holman L, et al (2014) Conserved class of queen pheromones stops social insect workers from reproducing. Science 343:287–290. doi: 10.1126/science.1244899

Vander Meer RK, Breed MD, Espelie KE, Winston ML (1998) Pheromone communication in social insects: Ants, Wasps, Bees, and Termites. Westview Press, Boulder, Colorado

Vander Meer RK, Glancey BM, Lofgren CS, et al (1980) The poison sac of red imported fire ant queens: source of a pheromone attractant. Ann Entomol Soc Am 73:609–612.

Weil T, Hoffmann K, Kroiss J, et al (2009) Scent of a queen—cuticular hydrocarbons specific for female reproductives in lower termites. Naturwissenschaften 96:315–319. doi: 10.1007/s00114-008-0475-8

CHAPTER 2

Cuticular hydrocarbons of the termite *Reticulitermes flavipes*: Reproductive-specific compounds and inter-caste variation

ABSTRACT

Cuticular hydrocarbons (CHCs) are an invaluable tool for understanding social insect communication and behavioral ecology. Species, colony, and caste specific patterns in CHCs have been well documented as pheromones mediating important behavioral and physiological aspects of social insects, including social hymenopterans and termites. Termite hydrocarbon profiles play a significant role in nestmate recognition, but have more recently been reported as potential royal pheromones. In the current study, cuticular extracts from *Reticulitermes flavipes* collected in North Carolina were analyzed across caste and colony. Our analysis focused on qualitative and quantitative differences between queens, kings, male workers, and female workers from three colonies. Principal component analysis of the 21 most prominent compounds successfully distinguished both caste and colony in our samples and revealed suites of compounds associated with royal or worker status. Closer inspection of the suite of royal compounds revealed several previously un-reported and highly enriched long chain alkanes and a royal specific compound, heneicosane, which may function as a queen pheromone.

INTRODUCTION

Colonies of social insects generally consist of at least two castes with individuals who reproduce and those who do not. But colonies of some species, especially termites, can also comprise more diverse castes such as soldiers, pseudergates, major and minor workers, and replacement reproductives (Grueter and Keller, 2016; Hölldobler and Wilson, 1990; Le Conte and Hefetz, 2008; Thorne and Traniello, 2003; Vander Meer et al., 1998; Zucchi et al., 2004). Chemical communication, mediated by volatile or non-volatile compounds, is the foundation of cooperative behavior in social insects. Caste recognition via pheromones has three primary functions: (1) maintaining the reproductive division of labor, (2) maintaining optimal proportions of non-reproductive castes for proper colony function, and (3) mediating courtship between females and males (Grueter and Keller, 2016). Of these, pheromonal regulation of the division of labor has received less attention, despite strong empirical evidence suggesting that pheromones inhibit reproduction in sterile castes.

Major sources of these pheromonal compounds are specialized glands and lipids on the cuticular surface (Vander Meer et al., 1998). Social hymenopterans have a variety of exocrine glands that generate and distribute these queen-specific signals, including the poison sac and postpharyngeal gland in ants and the mandibular glands in honeybees (Slessor et al., 2005; Vander Meer et al., 1980). Honeybee queen mandibular pheromone (QMP) and fire ant queen pheromone consist of multiple compounds that induce several behavioral and physiological effects on workers in the colony (Rocca et al., 1983; Slessor et al., 2005, 1988; Vander Meer et al., 1980; Vargo and Hulsey, 2000). Although these hallmarks of royal pheromones are glandular, several queen recognition compounds have been found to be cuticular in origin.

Cuticular hydrocarbons (CHCs) are the main class of insect cuticular compounds and play an important role in protection from desiccation and pathogens (Blomquist and Bagneres, 2010). As certain insect lineages developed sociality across evolutionary time, distinctive variations in their CHC composition were co-opted to play a role in communication with a cascading effect on recognition, behavior, and reproductive regulation. CHCs are increasingly reported as pheromones in termites, hymenopterans, and other social insects, and they have been identified as intra- and inter-specific recognition cues, including as royal caste recognition pheromones (Dietemann et al., 2003; Holman et al., 2016; Liebig et al., 2009; Van Oystaeyen et al., 2014).

In social Hymenoptera, the role of CHCs in recognition has been documented many times. CHCs acting as pheromones have been shown to both release behaviors and prime physiological changes important to maintaining reproductive division of labor. In *Myrmecia gulosa* ants, long-chain hydrocarbons have been identified as fertility signals eliciting tending behavior in queens and strong policing responses in workers (Dietemann et al., 2003). Similar signals have been found in *Harpegnathos* and *Lasius* ants and *Polistes* wasps (Espelie et al., 1994; Holman et al., 2016; Liebig et al., 2000). CHCs acting as primers for physiological changes in social insect colonies most commonly inhibit maturation of the ovaries to prevent unwanted reproduction. Recently, straight-chain alkanes of varying length and methylation were proposed to be a conserved class of royal primer pheromones inhibiting ovarian development in ants, bees, and wasps (Van Oystaeyen et al., 2014). The consistent use of similar compounds across a diverse phylogeny of insects suggests either an ancient origin of this pheromonal mechanism or convergence on CHCs as efficient

recognition signals. In either case, these patterns might suggest even broader use of CHCs as fertility signals in unrelated social insects.

CHCs have been used by chemotaxonomists to successfully help discriminate castes and species in subterranean termites (Blattodea: Isoptera: Rhinotermitidae), and unique CHC blends are perceived by termites and used to recognize nestmates and defend against foreign intruders (Bagneres et al., 1991; Bagnères et al., 1990). Early studies examining within-species differences in *Reticulitermes* sp. showed strong qualitative similarities across worker, soldier, and alate castes and distinct quantitative blend ratios of those shared CHCs, but no evidence supporting active recognition of these differences by the termites (Howard et al., 1978). Other studies have found similar qualitative similarities in *Reticulitermes virginicus*, *Coptotermes formosanus*, and *Zootermopsis angusticollis* and described identifiable differences in various CHC proportions in workers versus soldiers (Blomquist et al., 1979; Haverty et al., 1996; Howard et al., 1982). More recently, Darrouzet et al. (2014) analyzed CHC profiles from workers, soldiers, and neotenic queens, which are replacement reproductives deriving from worker or nymphal lineages, and found them to be statistically distinct. The main distinguishing feature across three castes was the proportion of 23-carbon and 24-carbon *n*-alkanes. In addition, they found that cuticular profiles shifted when termites were exposed to juvenile hormone, showing a gradual transition from worker-like to soldier-like CHCs.

Royal recognition pheromones are pivotal to monopolizing reproduction and maintaining a proper royal-to-worker ratio. The presence and activity of these chemicals in termites has been proposed since the coining of the term “pheromone”, but successfully identifying compounds and showing their effects has eluded all but a few termite researchers.

Matsuura et al. (2010) were able to identify a volatile queen pheromone that inhibited reproductive differentiation in *Reticulitermes speratus*. This pheromone consists of a blend of 2-methyl-1-butanol and n-butyl-n-butyrate, both highly volatile compounds. Queen-specific volatile compounds and CHCs have also been identified in *Nasutitermes takasagoensis* and *Zootermopsis nevadensis*, respectively, but their function has not been evaluated (Himuro et al., 2011; Liebig et al., 2009). The CHCs unique to *Z. nevadensis* reproductives appear to be identical in queens and kings, which could imply a mode of royal recognition shared across the sexes in a basal termite. Thus far no CHC royal pheromones have been identified in termites.

The dearth of termite exocrine glands (compared to hymenopterans) suggests that CHCs might have filled an important role in royal recognition in termites. In this study, we analyzed the CHC profiles across two castes and both sexes in the subterranean termite, *Reticulitermes flavipes*. We identified a reproductive-specific *n*-alkane and several other compounds that are significantly enriched in reproductives, exhibiting quantitative differences across castes. These hydrocarbons could serve as an important signal to recognize and distinguish royal castes and contribute significantly to the maintenance of reproductive activity in these termites.

MATERIALS AND METHODS

Termite Collection. Colonies of *Reticulitermes flavipes* were collected in Raleigh, NC from three wooded locations (Schenk Forest, Yates Mill Pond, or Lake Johnson) between 2010 and 2015. Whole tree limbs or logs were split into smaller pieces and set out in shallow pans to dry. Using either plastic container lids with moist paper towels underneath or ~10 cm PVC piping containing coils of moistened corrugated cardboard, the termites passively moved out of the drying wood and into the moist substrate. Fully extracted colonies were kept either in clear plastic boxes lined with moist sand and strips of pine wood (shims) for food or in 9 cm Petri dishes with an autoclaved lab substrate consisting of 70% sawdust and 30% α-cellulose.

Secondary Reproductive Production. To produce neotenic queens and kings, colonies were subdivided into 5 cm Petri dishes with ~500 workers and ~10 soldiers but no reproductives. Newly emerged neotenics typically appeared within 2-3 weeks and were removed to prevent inhibition of queen and king differentiation in the neotenic-generating dishes. Newly-emerged neotenics were then held in 9 cm dishes containing ~500 termite workers until used in experiments.

Cuticular Extracts and GC-MS. Individual termites from every caste were sexed and freeze-killed for 15 min at -20°C, followed by extraction in 200 µL of hexane containing 100 ng *n*-C28 as an internal standard. Extraction lasted for 2 min with intermittent gentle mixing. Extracts were removed to a new vial, evaporated under a gentle stream of nitrogen, re-dissolved in 50 µL of hexane and transferred to a 100 µL glass insert in a 1.5 mL GC autoinjection vial. Two of 50 µL were injected in splitless mode using a 7683B Agilent autosampler into a DB-5 column (20 m x 0.18 mm internal diameter x 0.18 µm film thickness, J&W Scientific, Folsom, CA, USA) in an Agilent 7890 series gas chromatograph

(Agilent Technologies, Santa Clara, CA, USA) connected to a flame ionization detector (FID) with ultra-high purity hydrogen as carrier gas (0.75 mL/min constant flow rate). The column was held at 50°C for 1 min, ramped to 320°C at 10°C/min, and held at 320°C for 10 min.

Peak Identification. A subset of termite CHC samples were run on an Agilent 5975 mass selective detector coupled to an Agilent 6890 GC for GC-MS analyses. The GC was operated in splitless injection mode and fitted with a DB-5MS column (30 m × 0.25 mm × 0.25 μm; Agilent). The oven was programmed from 50–310°C at 15°C/min after an initial delay of 2 min and held at 310°C for 10 min. Injector temperature was 280°C; MS quadrupole temperature was 150°C; MS source temperature was 230°C; and transfer line temperature was 300°C. CHCs were identified primarily on their EI mass spectra and their Kovats indices on the DB-5 column. Methyl branch positions of mono-, and dimethylalkanes were determined from characteristic even- and odd-mass fragments of their respective mass spectra(Nelson, 2001) as well as by calculated retention indices(Carlson et al., 1998). We did not determine the positions of double bonds.

Principal Component Analysis (PCA). Chromatograms for queens, kings, male and female workers and male and female soldiers were exported from Agilent ChemStation (OpenLab CDS C.01.06). Using queen chromatograms, 21 relevant peaks were selected to discriminate among castes. Excluded peaks were typically < 1% of total chromatogram area for all samples. To include samples from multiple GC runs, retention times were converted to Kovats retention indices using the formula for temperature programmed chromatography. All peaks were normalized to the *n*-C28 internal standard and area percentages were input into

the PCA matrix. PCA was conducted in JMP (JMP®, Version 12. SAS Institute Inc., Cary, NC, 1989-2007).

RESULTS AND DISCUSSION

Comparison of Cuticular Extracts. Hexane extracts of neotenic queens, kings, and female and male workers were run on a GC-MS to identify common hydrocarbons and find reproductive-specific compounds that might be involved in a recognition response. Averaging the total mass of CHCs from individual termites, we found that termite workers generally had one third the total mass of CHCs compared to neotenic queens and kings (Table 1). The most prominent chemicals in the termite extracts were normal and monomethyl alkanes ranging from 23 to 25 carbons; they made up > 40% of the total mass of CHCs for all termite castes. Most of the shorter-chain hydrocarbons (< 26 carbons), except heneicosane, have been found in *R. flavipes*, but several 31+ carbon compounds that appeared in our extracts have not been identified previously (Bagnères et al., 1990; Clément et al., 2001; Dronnet et al., 2006). Several CHCs that have been identified in *R. flavipes* were present in our samples in only trace quantities: 9-tricosene, 2-methyltetracosane, 9-tetracosene, 9-pentacosene, 7, 9-pentacosadiene, and 2-methylpentacosane; these compounds were not included in further analysis.

Our GC-MS identification revealed 20 compounds shared across all castes and one reproductive-specific compound (Fig. 1). Eleven of the shared compounds were enriched in kings and queens and 9 were enriched in workers (Fig. 2). Differences in total quantities of worker-enriched compounds ranged from just 1.65% to > 2-fold increase, in the case of pentacosane and x,y,z-pentacosatriene. Queens and kings had increases ranging from ~10% to > 3-fold in hentriacontane and 13- and 11-methylheptatriacontane (Table 1, Fig. 2). Previous studies describing *R. flavipes* cuticular profiles found similar results regarding tricosane (more in queens and kings) and tetracosane (more in workers) quantities across

castes (Bagnères et al., 1990; Clément et al., 2001; Darrouzet et al., 2014; Howard et al., 1978).

Heneicosane (*n*-C21) appeared to be a royal-specific compound and the long-chain mono- and dimethyl alkanes, although present in workers in trace amounts, are much more represented in royals (Table 1, Fig. 2). These compounds could be caste-specific metabolites, vital ingredients for the maintenance of these castes, or possible reproductive pheromones.

Principal Component Analysis. The percentages of identified compounds from GC-MS were used in principal component analysis to statistically discriminate castes and colonies. PCA of 21 peaks from termites revealed a strong separation across reproductive and non-reproductive castes (Fig. 3A). Quantitative variation in the CHC dataset showed that the first two principal components accounted for 68.3% of total chemical variation. The eigenvectors that contributed most to separation between workers and reproductives suggested that heneicosane, tricosane, and the suite of compounds with chain length of > 35 carbons were primary indicators of reproductive status, while tetracosane, 2-methyltetracosane, pentacosane, 2-methylpentacosane, and 3 methylpentacosane were the primary indicators of a worker-type cuticular profile (Fig. 3B). One queen sample was situated within the worker group, but given overall similarity of other queens across colonies and the sometimes difficult process of identifying newly emerged neotenic queens, it is highly likely that this was a misidentified worker labeled as a reproductive.

Queens and kings were not distinguished from each other in the PCA, but they do possess several compounds with differential proportions (Fig. 2). Compounds with ≤ 25 carbons were a higher percentage of the total profile in kings except for heneicosane (*n*-C21),

while queens had higher proportions of compounds > 31 carbons. Finally, there was a clear separation of colonies in the PCA. Tricosane, tetracosane, 2-methyltetracosane, pentacosane, 2-methylpentacosane, and (13) 11-methylpentatriacontane appeared to be compounds linking colonies 2 and 3, while separating them from colony 1.

The royal-specific and reproductive-enriched compounds we found have rarely been reported in *R. flavipes*. Heneicosane was found in trace unquantifiable amounts in every caste of *R. flavipes*, and also in workers of *R. lucifugus* and *R. banyulensis*, but this CHC has not been shown to have caste-related patterns (Bagneres et al., 1991; Howard et al., 1978). Darrouzet et al. (2014) showed similar chemical profile discrimination across castes and an intriguing age-based pattern of separation. They identified several of the same *n*-alkanes as distinguishing worker from neotenic profiles, which aligns with our findings, but heneicosane and the long-chain CHCs that we found were not reported in their analysis. Interestingly, in the drywood termite *Cryptotermes secundus*, heneicosane appears to be unique to workers, while the long-chain alkanes remain unique to queens (Weil et al., 2009).

The differences between royal and worker castes in *R. flavipes* may relate to (a) functional differences (e.g., desiccation resistance, pathogen mitigation) associated with division of labor, (b) functional differences necessary to successfully reproduce (queens), or (c) caste recognition. In our view, *n*-C21 should be pursued as a possible queen recognition pheromone component in *R. flavipes* due to recent work in social hymenopterans showcasing a conserved class of queen pheromones consisting largely of straight chain alkanes (Van Oystaeyen et al., 2014),. A critical step toward assessing whether *n*-C21 is a pheromone is the development of discriminating bioassays of releaser pheromone activity. In many other social insects, including fire ants and honeybees, queen retinue behavior is elicited by queen-

specific compounds, representing a clear behavioral assay (Slessor et al., 1988; Vander Meer et al., 1980). In contrast, termites do not form retinues around the queen and they largely do not show any obvious queen tending behavior. Instead, head-butting and other aggressive-like behaviors have been correlated with reproductive output (Korb et al., 2009; Penick et al., 2013), and these behaviors could be the basis for developing a new royal-recognition behavioral assay.

Conclusions. Distinguishing reproductives from other castes is of pivotal importance to the success of social insect colonies, and delineating which chemicals mediate these interactions will help to understand how these complex societies evolved. This study reveals differences in CHCs between royal (queens and kings) and worker castes in *Reticulitermes flavipes*. We demonstrated the presence of heneicosane (*n*-C21) as a reproductive caste-specific CHC. Although regional variation of cuticular lipid profiles may be responsible, it is relatively rare that a new compound would appear as part of geographic polymorphism of CHCs. The possibility that this compound serves as a queen pheromone is a compelling argument for future investigations. Investigating the conservation of CHCs that distinguish royal and colony recognition could reveal distinct mechanisms mediating communication at the caste and colony levels. Teasing these two levels apart could lead to a deeper understanding of how and why termites communicate using CHCs.

REFERENCES

Bagnères AG, Clément JL, Blum MS, et al (1990) Cuticular hydrocarbons and defensive compounds of *Reticulitermes flavipes* (Kollar) and *R. santonensis* (Feytaud): Polymorphism and chemotaxonomy. J Chem Ecol 16:3213–3244.

Bagneres A-G, Killian A, Clement J-L, Lange C (1991) Interspecific recognition among termites of the genus *Reticulitermes*: Evidence for a role for the cuticular hydrocarbons. J Chem Ecol 17:2397–2420.

Blomquist GJ, Bagneres AG (2010) Insect Hydrocarbons: Biology, Biochemistry, and Chemical Ecology. Cambridge Univ Press, Cambridge

Blomquist GJ, Howard RW, McDaniel CA (1979) Structures of the cuticular hydrocarbons of the termite *Zootermopsis angusticollis* (Hagen). Insect Biochem 9:365–370. doi: 10.1016/0020-1790(79)90083-0

Carlson DA, Bernier UR, Sutton BD (1998) Elution Patterns from Capillary GC for Methyl-Branched Alkanes. J Chem Ecol 24:1845–1865. doi: 10.1023/A:1022311701355

Clément J-L, Bagnères A-G, Uva P, et al (2001) Biosystematics of *Reticulitermes* termites in Europe: morphological, chemical and molecular data. Insectes Sociaux 48:202–215. doi: 10.1007/PL00001768

Darrouzet E, Labédan M, Landré X, et al (2014) Endocrine control of cuticular hydrocarbon profiles during worker-to-soldier differentiation in the termite *Reticulitermes flavipes*. J Insect Physiol 61:25–33. doi: 10.1016/j.jinsphys.2013.12.006

Dietemann V, Peeters C, Liebig J, et al (2003) Cuticular hydrocarbons mediate discrimination of reproductives and nonreproductives in the ant *Myrmecia gulosa*. Proc Natl Acad Sci U S A 100:10341–10346.

Dronnet S, Lohou C, Christides J-P, Bagnères A-G (2006) Cuticular Hydrocarbon Composition Reflects Genetic Relationship Among Colonies of the Introduced Termite *Reticulitermes santonensis* Feytaud. J Chem Ecol 32:1027. doi: 10.1007/s10886-006-9043-x

Espelie KE, Gamboa GJ, Grudzien TA, Bura EA (1994) Cuticular hydrocarbons of the paper wasp, *Polistes fuscatus*: A search for recognition pheromones. J Chem Ecol 20:1677–1687. doi: 10.1007/BF02059889

Grueter C, Keller L (2016) Inter-caste communication in social insects. Curr Opin Neurobiol 38:6–11. doi: 10.1016/j.conb.2016.01.002

Haverty MI, Grace JK, Nelson LJ, Yamamoto RT (1996) Intercaste, intercolony, and temporal variation in cuticular hydrocarbons of *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae). J Chem Ecol 22:1813–1834. doi: 10.1007/BF02028506

Himuro C, Yokoi T, Matsuura K (2011) Queen-specific volatile in a higher termite *Nasutitermes takasagoensis* (Isoptera: Termitidae). J Insect Physiol 57:962–965. doi: 10.1016/j.jinsphys.2011.04.012

Hölldobler B, Wilson EO (1990) The Ants. Belknap Press of Harvard Univ. Press, Cambridge, MA

Holman L, Hanley B, Millar JG (2016) Highly specific responses to queen pheromone in three *Lasius* ant species. Behav Ecol Sociobiol 70:387–392. doi: 10.1007/s00265-016-2058-6

Howard RW, McDaniel CA, Blomquist GJ (1978) Cuticular hydrocarbons of the eastern subterranean termite, *Reticulitermes flavipes* (Kollar)(Isoptera: Rhinotermitidae). J Chem Ecol 4:233–245.

Howard RW, McDaniel CA, Nelson DR, et al (1982) Cuticular hydrocarbons of *Reticulitermes virginicus* (Banks) and their role as potential species- and caste-recognition cues. J Chem Ecol 8:1227–1239. doi: 10.1007/BF00990755

Korb J, Weil T, Hoffmann K, et al (2009) A Gene Necessary for Reproductive Suppression in Termites. Science 324:758–758. doi: 10.1126/science.1170660

Le Conte Y, Hefetz A (2008) Primer pheromones in social hymenoptera. In: Annual Review of Entomology. Annual Reviews, Palo Alto, pp 523–542

Liebig J, Eliyahu D, Brent CS (2009) Cuticular hydrocarbon profiles indicate reproductive status in the termite *Zootermopsis nevadensis*. Behav Ecol Sociobiol 63:1799–1807. doi: 10.1007/s00265-009-0807-5

Liebig J, Peeters C, Oldham NJ, et al (2000) Are variations in cuticular hydrocarbons of queens and workers a reliable signal of fertility in the ant *Harpegnathos saltator*? Proc Natl Acad Sci U S A 97:4124–4131. doi: 10.1073/pnas.97.8.4124

Nelson DR (2001) Discovery of novel trimethylalkanes in the internal hydrocarbons of developing pupae of *Heliothis virescens* and *Helicoverpa zea*. Comp Biochem Physiol B Biochem Mol Biol 128:647–659. doi: 10.1016/S1096-4959(00)00336-5

Penick CA, Trobaugh B, Brent CS, Liebig J (2013) Head-butting as an early indicator of reproductive disinhibition in the termite *Zootermopsis nevadensis*. J Insect Behav 26:23–34. doi: 10.1007/s10905-012-9332-x

Rocca JR, Tumlinson JH, Glancey BM, Lofgren CS (1983) The queen recognition pheromone of *Solenopsis invicta*, preparation of (E-6-(1-pentenyl)-2H-pyran-2-one. Tetrahedron Lett 24:1889–1892. doi: 10.1016/S0040-4039(00)81798-0

Slessor KN, Kaminski L-A, King GGS, et al (1988) Semiochemical basis of the retinue response to queen honey bees. Nature 332:354–356. doi: 10.1038/332354a0

Slessor KN, Winston ML, Conte YL (2005) Pheromone Communication in the Honeybee (*Apis mellifera* L.). J Chem Ecol 31:2731–2745. doi: 10.1007/s10886-005-7623-9

Thorne BL, Traniello JFA (2003) Comparative Social Biology of Basal Taxa of Ants and Termites. Annu Rev Entomol 48:283–306. doi: 10.1146/annurev.ento.48.091801.112611

Van Oystaeyen A, Oliveira RC, Holman L, et al (2014) Conserved class of queen pheromones stops social insect workers from reproducing. Science 343:287–290. doi: 10.1126/science.1244899

Vander Meer RK, Breed MD, Espelie KE, Winston ML (1998) Pheromone communication in social insects: Ants, Wasps, Bees, and Termites. Westview Press, Boulder, Colorado

Vander Meer RK, Glancey BM, Lofgren CS, et al (1980) The poison sac of red imported fire ant queens: source of a pheromone attractant. Ann Entomol Soc Am 73:609–612.

Vargo EL, Hulsey CD (2000) Multiple glandular origins of queen pheromones in the fire ant Solenopsis invicta. J Insect Physiol 46:1151–1159. doi: 10.1016/S0022-1910(99)00226-7

Weil T, Hoffmann K, Kroiss J, et al (2009) Scent of a queen—cuticular hydrocarbons specific for female reproductives in lower termites. Naturwissenschaften 96:315–319. doi: 10.1007/s00114-008-0475-8

Zucchi R, Wenzel JW, Noll FB (2004) Evolution of caste in neotropical swarm-founding wasps (Hymenoptera, Vespidae, Epiponini).

Table 1. Cuticular hydrocarbons of *Reticulitermes flavipes* workers, queens and kings.

Peak #	Hydrocarbon name	Kovats index	Workers (n = 57)		Neotenic Queens (n = 7)		Neotenic Kings (n = 7)	
			% Total profile	± SEM	% Total profile	± SEM	% Total profile	± SEM
1	heneicosane	2100	0.00	0.00	2.62	0.66	1.20	0.00
2	tricosane	2301	7.96	0.25	9.22	0.70	12.15	7.96
3	11-methyltricosane	2336	0.77	0.04	1.51	0.24	1.78	0.77
4	tetracosane	2400	5.35	0.14	3.40	0.49	3.05	5.35
5	12- and 11-methyltetracosane	2435	1.02	0.05	1.30	0.16	1.52	1.02
6	2-methyltetracosane	2464	9.41	0.23	7.02	0.79	7.71	9.41
7	3-methyltetracosane and x-pentacosene	2476	3.14	0.19	1.69	0.36	1.73	3.14
8	pentacosane	2501	28.17	0.99	14.02	2.54	14.00	28.17
9	13- and 11-methylpentacosane	2535	13.71	0.31	15.33	1.24	18.88	13.71
10	x,y-pentacosadiene	2549	3.85	0.19	4.83	0.98	4.81	3.85
11	2-methylpentacosane (and x,y-pentacosadiene)	2563	5.75	0.14	2.88	0.39	3.58	5.75
12	3-methylpentacosane	2574	5.30	0.19	3.12	0.51	3.45	5.30
13	x,y,z-pentacosatriene	2610	4.48	0.24	2.09	0.40	2.38	4.48
14	heptacosane	2700	0.36	0.02	0.19	0.02	0.18	0.36
15	octacosane *	2801	NA	NA	NA	NA	NA	NA
16	hentriacontane	3100	0.72	0.18	3.46	1.79	2.86	0.72
17	(13-) 11-methylpentatriacontane	3525	2.23	0.12	5.74	2.15	4.47	2.23
18	13-, 12-, and 11-methylhexatriacontane	3622	1.49	0.34	1.47	0.19	1.26	1.49
19	13- and 11-methylheptatriacontane	3723	2.35	0.09	9.93	1.41	7.28	2.35
20	11,15-dimethylheptatriacontane	3745	1.42	0.10	3.12	0.61	2.44	1.42
21	15-, 13-, and 11-methylnonatriacontane	3922	1.19	0.07	4.16	0.53	3.39	1.19
22	11,15-dimethylnonatriacontane	3944	0.74	0.06	1.99	0.27	1.55	0.74
Average mass of integrated CHCs (ng)**			301.88	10.90	1201.84	362.46	1182.25	110.09

% Total profile represents the average percent of the total chromatogram area for the 21 compounds from the cuticular profiles of *R. flavipes* workers, queens, and kings. The 21 compounds were selected by setting a 1% representation in the total area integrated in the chromatogram.

Peak # corresponds to peak labeled in Fig. 1.

*Octacosane (*n*-C28) was used as internal standard and does not naturally occur in any sample.

** Average mass calculated from the integrated area of 21 extracted peaks in relation to the internal standard using 48 workers, 4 queens, and 4 kings. Some samples were omitted because they were not spiked with the internal standard.

Figure 1. Gas chromatograms of reproductive and worker castes in *R. flavipes*. Females are marked in red and males are marked in blue. The reproductive-specific compound heneicosane (*n*-C21) and the internal standard octacosane (*n*-C28) are marked. Numbered peaks correspond with peaks listed in Table 1. The inset shows ~100X of the *n*-C21 region in the chromatogram of a female worker, showing no *n*-C21.

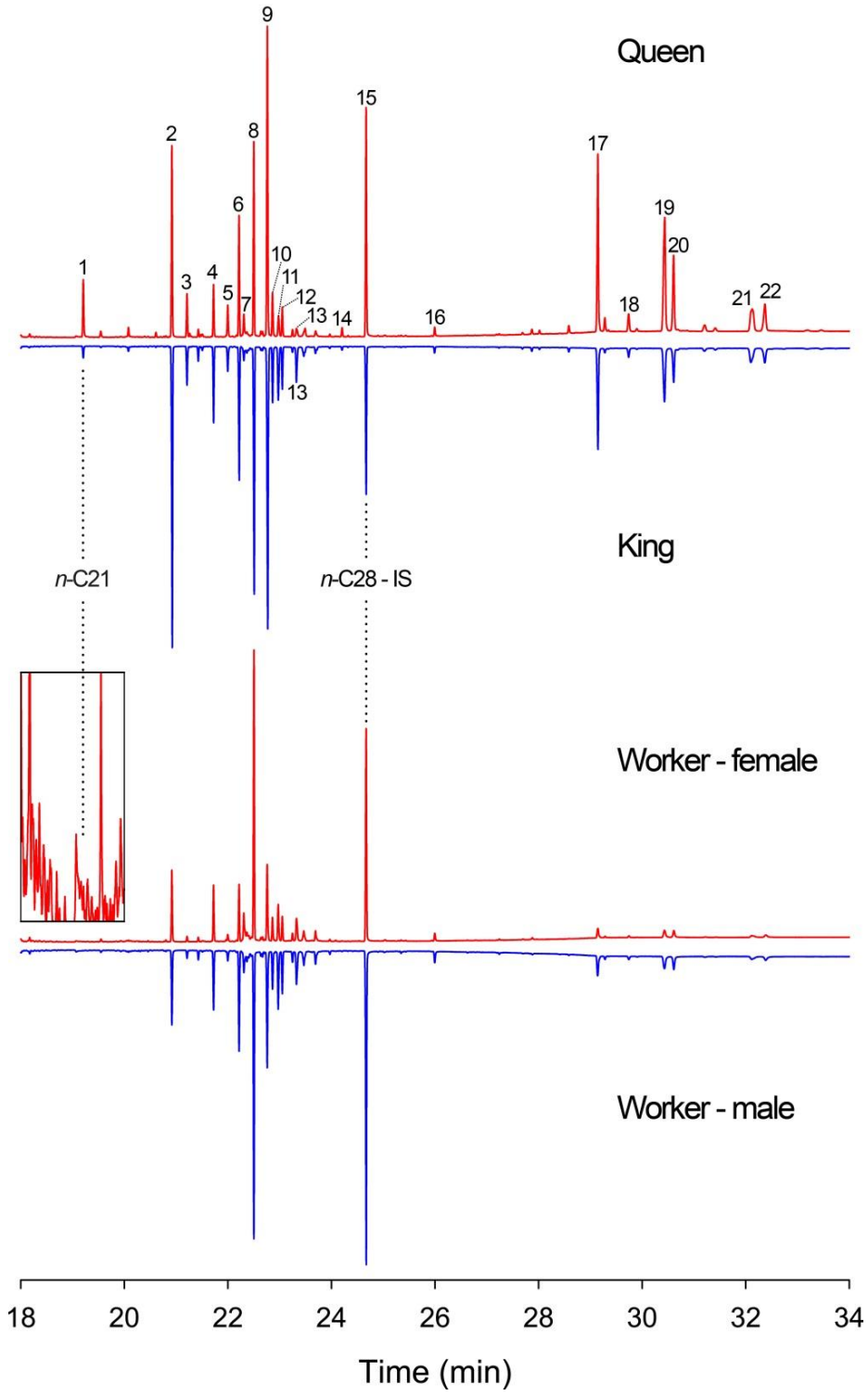

Queen
King
Worker - female
Worker - male
n-C21
n-C28 - IS
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
18
20
22
24
26
28
30
32
34
Time (min)

Figure 2. Average percent (± SEM) of total cuticular profile for 21 analyzed hydrocarbons across caste. Workers of both sexes are averaged together in this figure. Samples sizes are 57 workers, 7 queens, and 7 kings.

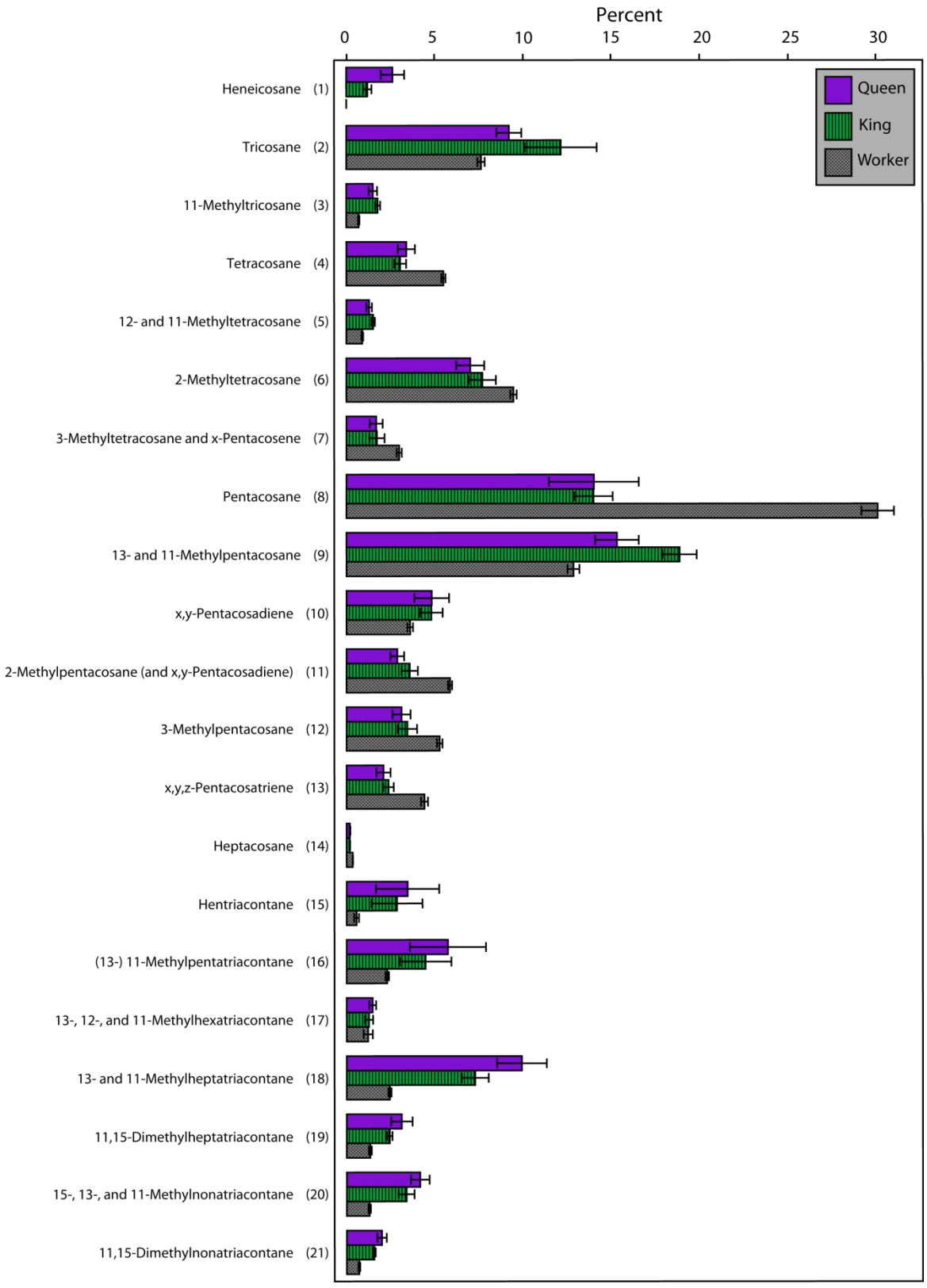
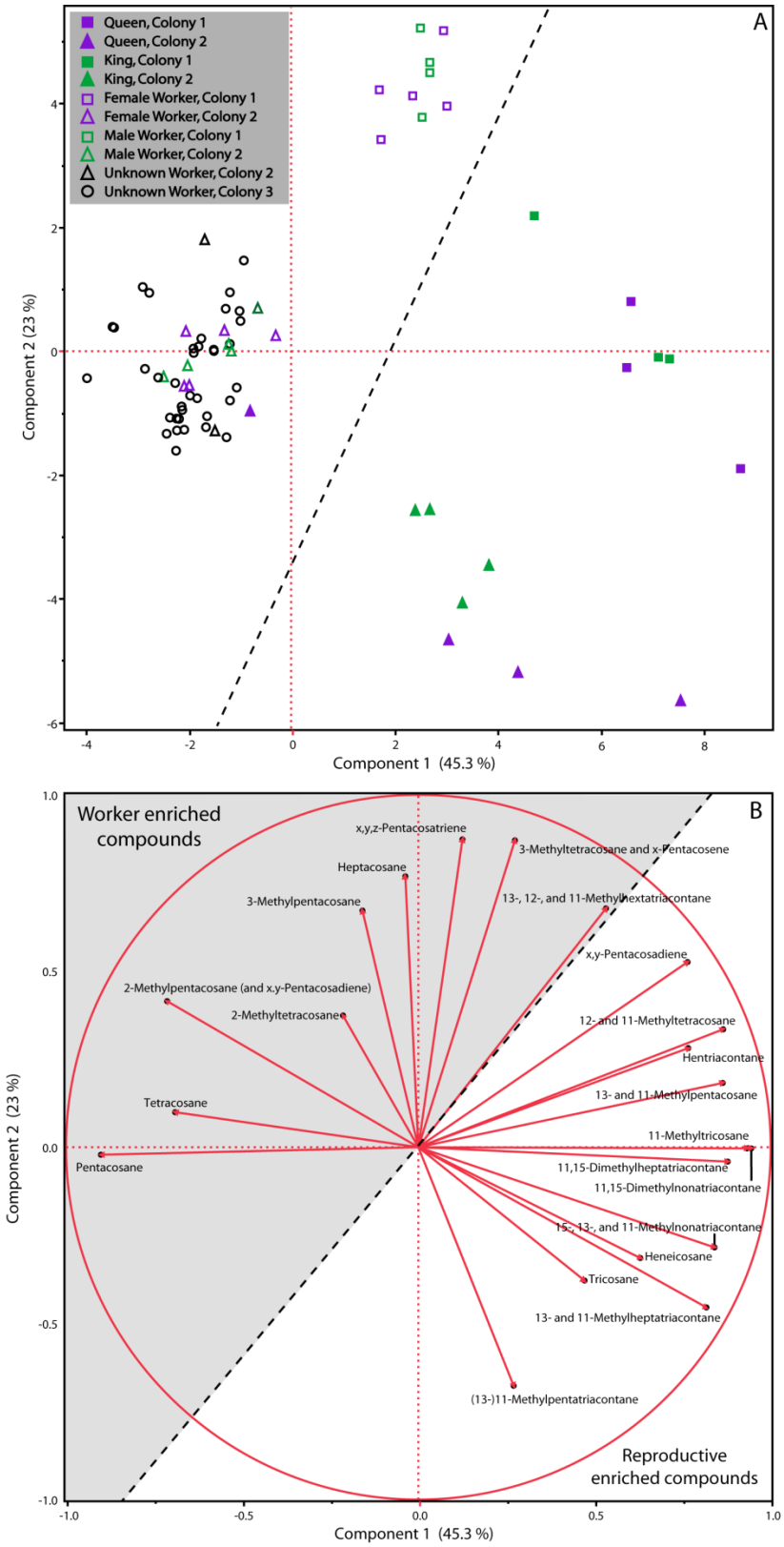

Percent
0
5
10
15
20
25
30
Queen
King
Worker
Heneicosane (1)
Tricosane (2)
11-Methyltricosane (3)
Tetracosane (4)
12- and 11-Methyltetracosane (5)
2-Methyltetracosane (6)
3-Methyltetracosane and x-Pentacosene (7)
Pentacosane (8)
13- and 11-Methylpentacosane (9)
x,y-Pentacosadiene (10)
2-Methylpentacosane (and x,y-Pentacosadiene) (11)
3-Methylpentacosane (12)
x,y,z-Pentacosatriene (13)
Heptacosane (14)
Hentriacontane (15)
(13-) 11-Methylpentatriacontane (16)
13-, 12-, and 11-Methylhexatriacontane (17)
13- and 11-Methylheptatriacontane (18)
11,15-Dimethylheptatriacontane (19)
15-, 13-, and 11-Methylnonatriacontane (20)
11,15-Dimethylnonatriacontane (21)

Figure 3. **A.** Principal component analysis of gas chromatograms from worker, queen, and king termites of *R. flavipes*. Red markers represent females, blue markers represent males, and black markers are workers of unknown sex. Shapes correspond to colony #1 (■), colony #2 (▲), and colony #3 (●). Filled shapes are active reproductives (i.e., kings or queens) while empty shapes represent termite workers. The dotted diagonal line separates all royal individuals from workers in the score plot. The percent of variance explained by each PC is indicated on the *x*- and *y*-axes. 21 compounds were used in the PCA analysis. **B.** PC1 and PC2 eigenvectors, showing in the un-shaded section that compounds enriched in reproductives, such as *n*-C21, *n*-C23, and ≥ 35 carbon alkanes, weigh toward the reproductive phenotype. Worker-enriched compounds present in the shaded portion of the figure weigh toward the worker phenotype.


A
Queen, Colony 1
Queen, Colony 2
King, Colony 1
King, Colony 2
Female Worker, Colony 1
Female Worker, Colony 2
Male Worker, Colony 1
Male Worker, Colony 2
Unknown Worker, Colony 2
Unknown Worker, Colony 3
Component 2 (23 %)
Component 1 (45.3 %)
B
Worker enriched compounds
x,y,z-Pentacosatriene
3-Methyltetracosane and x-Pentacosene
Heptacosane
3-Methylpentacosane
13-, 12-, and 11-Methylhextatriacontane
x,y-Pentacosadiene
2-Methylpentacosane (and x,y-Pentacosadiene)
2-Methyltetracosane
12- and 11-Methyltetracosane
Hentriacontane
13- and 11-Methylpentacosane
Tetracosane
11-Methyltricosane
Pentacosane
11,15-Dimethylheptatriacontane
11,15-Dimethylnonatriacontane
13-, 12-, and 11-Methylnonatriacontane
Heneicosane
Tricosane
13- and 11-Methylheptatriacontane
(13-)11-Methylpentatriacontane
Reproductive enriched compounds
Component 2 (23 %)
Component 1 (45.3 %)

CHAPTER 3

Queen and king recognition in the Eastern Subterranean termite, *Reticulitermes flavipes*: Evidence for royal recognition pheromones

ABSTRACT

Royal recognition is a central feature of insect societies, allowing them to maintain reproductive division of labor and regulate colony demography. Queen recognition has been broadly demonstrated and queen pheromones have been identified in social hymenopterans, but not in termites. Here we describe behaviors that are elicited in workers and soldiers by queens and kings of the subterranean termite, *Reticulitermes flavipes*, and investigate the chemical basis for the behavior. Workers and soldiers readily perform a lateral or longitudinal shaking behavior upon antennal contact with queens and kings. When royal cuticular chemicals are transferred to live workers or inert glass dummies, they elicit antennation and shaking in a dose-response manner. The striking response to reproductives and their cuticular extracts suggests that royal-specific cuticular compounds act as recognition pheromones and that shaking behavior is a clear and measurable queen recognition response in this termite species.

INTRODUCTION

Social insects rely on chemical communication to function effectively; within the colony, pheromones mediate foraging, aggregation, defense, reproduction, and other essential processes (Blum, 1996; Hölldobler and Wilson, 1990). Recognizing reproductive castes (queens in social hymenopterans, queens and kings in termites) is especially important to preserve the royal-worker division of labor and to ensure proper care for these high-value individuals. Royal pheromones and recognition behaviors have been well studied in ants, bees, and wasps, but have received little attention in termites. Pheromones largely mediate and guide the behavior and physiology of sterile castes in social hymenopteran colonies, with some notable exceptions that include visual signals and tactile/physical interactions (Liebig et al., 1999; Tibbetts and Dale, 2004; West, 1967). Queen pheromones are generally classified to either elicit immediate behaviors (releaser pheromones) or induce long-term physiological changes in sterile worker castes (primer pheromones). Identifying these compounds and elucidating their effects and glandular origins have received increasing attention in social insect biology.

Distantly related to the hymenopteran social insects, termites share life history traits and ecologically important roles with ants, bees, and wasps. Termites exhibit a more flexible developmental pathway than their hymenopteran counterparts, as most individuals within a termite colony retain the ability to develop functional gonads and molt into worker- or nymph-derived reproductives, often called neotenics (Bignell et al., 2014; Yamamoto and Matsuura, 2012). Active queens and kings inhibit reproductive development in other colony members and most likely use chemical signals to do so. Additionally, because reproductively

active males (kings) stay within the nest, termites most likely employ both queen- and king-specific pheromones to preserve the reproductive division of labor in each sex and elicit care from workers (Light and Weesner, 1951; Lüscher, 1961; Matsuura et al., 2010).

The first termite queen primer pheromone was identified in the Japanese subterranean termite (*Reticulitermes speratus*) as a blend of two highly volatile compounds—2-methyl-1-butanol and n-butyl-n-butyrate—which inhibit the reproductive differentiation of female workers and nymphs into supplementary reproductives (Matsuura et al., 2010). Although reproductive-specific volatile compounds and long-chain hydrocarbons have been found in *Nasutitermes takasagoensis* and *Zootermopsis nevadensis*, respectively, their functions have not been evaluated (Himuro et al., 2011; Liebig et al., 2009). Thus, no releaser pheromones involved in royal recognition or other behaviors have been described in termites. It is possible that the search for these compounds has been impeded by the rarity and fragility of reproductives, the paucity of termite researchers, or the lack of robust bioassays to measure the physiological or behavioral effects of presumptive queen and king pheromones.

Foundational work on queen-recognition in bees and fire ants involves ketones, esters, alcohols, and fatty acids (Rocca et al., 1983; Slessor et al., 1988). However, queens from a number of other social hymenopteran species possess unique cuticular hydrocarbons (CHCs) that correlate with ovary activation and often elicit queen recognition (Espelie et al., 1994; Holman et al., 2016; Van Oystaeyen et al., 2014). CHCs are the dominant class of most insects' cuticular lipid layer and help to prevent desiccation and act as a barrier against pathogens. Also known to be important cues in nestmate and interspecific recognition in both solitary and social species, CHCs are highly variable and responsive to different physiological and environmental inputs (Blomquist and Bagneres, 2010). CHCs are thus

hypothesized to have been co-opted over the course of evolution as reliable signals for fertility because of the relationship between their composition and the insect's physiology and metabolism.

Cuticular hydrocarbons also show promise as termite royal pheromones. They have been found to be involved in caste- and species-recognition in termites, especially within the subterranean termites (Blattodea: Isoptera: Rhinotermitidae) (Bagnères et al., 1998; Batista-Pereira et al., 2004; Darrouzet et al., 2014; Haverty et al., 1996; Weil et al., 2009). The literature remains divided on the role of CHCs in recognition and aggression, but it is generally held that CHC blends are important in mediating behavior and agonistic interactions in certain termite species (Bagneres et al., 1991; Bagnères et al., 1990). Additionally, in Chapter 2 of this dissertation we report clear caste differences in the CHC profiles of *R. flavipes*, where a queen- and king-specific straight-chain C21 alkane (*n*-heneicosane) could mediate royal recognition.

Pivotal to the identification of queen-recognition pheromones are behavioral assays that can be used to test worker responses to queens. Yet, queen recognition behaviors have not been well described in termites, and there is no clear retinue response around reproductive individuals, as commonly observed in hymenopterans (Dietemann et al., 2003; Nunes et al., 2014; Slessor et al., 1988; Vander Meer et al., 1980). Aggressive intracolony interactions establishing reproductive dominance have been described and are loosely related to queen recognition, but it is unclear whether a chemical signal is involved (Korb, 2005; Penick et al., 2013). Behaviors such as head-butting, tremulations, jerking, and oscillatory behavior have been described in several termite species, but never in relation to reproductive

individuals (Howse, 1965, 1964; Kirchner et al., 1994; Ohmura et al., 2009; Whitman and Forschler, 2007).

Upon observing a distinct oscillatory/shaking behavior in workers of the Eastern subterranean termite *R. flavipes* in close proximity to queens, we hypothesized that this might represent a royal recognition response. To complement a growing literature describing queen fertility and recognition signals in social Hymenoptera, and to understand the mechanisms dictating royal recognition in termites, we investigated behavioral responses of workers in response to queens and kings of *R. flavipes* and the chemical signals that elicit them.

MATERIALS AND METHODS

Termite collection. Colonies of *Reticulitermes flavipes* were collected in Raleigh, North Carolina, from three wooded locations (Schenck Forest, Yates Mill Pond, Lake Johnson) between 2010 and 2015. Whole tree limbs or logs were split into smaller pieces and set out in shallow pans to dry. Using either plastic container lids with moist paper towels underneath or ~10 cm PVC pipes containing coils of moistened corrugated cardboard, the termites passively moved out of the drying wood and into the moist substrate. Fully extracted colonies were kept either in clear plastic boxes lined with moist sand and pine shims for food or in 9-cm petri dishes with an autoclaved lab substrate consisting of 70% sawdust and 30% α-cellulose. Colonies were maintained in opaque plastic containers in a 24^{o}C incubator under 14:10 L:D cycle with lights-on at 0600.

Production of secondary reproductives. To produce neotenic queens and kings, colonies of 2,000 to 5,000 individuals were subdivided into 5 cm petri dishes without reproductives. Newly emerged neotenics typically appeared within 2–3 weeks and were removed to prevent inhibition of queen and king differentiation in the neotenic-generating dishes. Newly-emerged neotenics were then held in 9-cm dishes containing ~500 workers and 20–50 soldiers until used in experiments.

General bioassay procedure. Unless noted otherwise, termites were divided into dishes of 30 workers and two soldiers, allowed to acclimate for at least 7 days and observed once. In order to lessen the effects of repeated measures and to more efficiently use the available

termites, we assayed 10 replicate dishes per treatment and then returned the termites into a larger colony. Then we re-distributed the termites from the larger colony back into new replicate dishes. This allowed us to use the termites several times by randomizing the termites across replicates and treatments.

Royal recognition bioassay. All queens and kings used in recognition assays were neotenics. Primary reproductives were too rare to provide effective replication. One hundred workers were placed into a 5-cm petri dish with moist unwoven paper towels or filter paper. Two soldiers were added to each dish to discourage soldier differentiation during the experiment. To differentiate the introduced (focal) worker from resident workers, the introduced worker was dyed blue by feeding on substrate impregnated with 0.1% Nile Blue. Workers turned blue in one to two weeks of consuming dyed diet and appeared to show no decrease in activity or survivorship, as previously described (Su et al., 1991). A single dyed worker was introduced to each assay dish at least 24 h before the assay. Assays consisted of removing the lid of the petri dish, wiping off any condensation to improve visibility, waiting at least 2 min to rest the termites, and observing the dyed focal worker for 10 min. Measured parameters included total time spent moving by the focal worker and the total number of distinct allogrooming sessions, shaking responses, and antennations by the focal workers and resident workers that interacted with it. All observed behaviors were divided into either active (actions performed by the focal termite) or reactive (reactions elicited by the focal termite from others) categories.

Foreign queen recognition bioassay. We performed assays similar to our royal recognition assays to test the responses of nestmate termites to unrelated intruders in dishes with 30 nestmate workers and two nestmate soldiers. These assays were designed to assess the queen recognition activity of foreign queens versus native queens and to support observations that *R. flavipes* colonies show little aggression toward foreign queens. Petri dish lids were removed and condensation was wiped from the lid before one of four treatments was added: nestmate neotenic queen, nestmate worker, foreign neotenic queen, or foreign worker. Observations began immediately after a focal termite was introduced. Replicates were observed for 7 min and cumulative antennation and shaking elicited in resident termites were recorded each min. Introduced workers were dyed blue for tracking purposes. All treatments had 10 replicate petri dishes. Dishes were assayed once and then re-distributed into a larger colony. Because only five queens were available at the time of this assay, each queen was observed in two replicate assays 1 wk apart.

Transfer of cuticular compounds to live termites. To test whether the recognition behaviors elicited by queens were mediated by cuticular compounds and whether these compounds could be transferred to non-reproductive termites, we "perfumed" workers by tumbling queens with workers in various ratios. We included queen:worker ratios of 7:15, 1:1, 5:1, and 10:1 with a 40:15 worker:dyed worker negative control and a live tumbled queen as positive control. Each treatment (queen:worker ratio) and control was replicated 10 times. For the 7:15 queen:worker and 40:15 worker:worker experiments, 15 blue-dyed workers were tumbled in glass vials with either 7 queens or 40 workers, respectively. After perfuming, five blue-dyed workers were frozen for extraction and cuticular analysis and ten

were removed to a clean petri dish for use in the bioassay. For all other ratios (1:1, 5:1, and 10:1), a blue-dyed worker was tumbled alone with 1, 5, or 10 queens and then observed in the bioassay. Queens were derived from the native colony in all treatments except for the 10:1 queen:worker treatment, where three of the 10 queens were from a foreign colony. Cuticular compounds were transferred by placing live termites into 4 mL glass vials and gently rotating the vials to tumble them for 3 min. Efforts were made to maximize contact among termites. Queens were rested in the dark while running the assay and 5 tumbling sessions were performed each day to minimize stress in the queens. After tumbling, a single queen-"perfumed" dyed worker was added to each petri dish with 30 workers and two soldiers and immediately assayed for 7 min, recording cumulative antennation and shaking responses elicited in resident termites. As above, termites were assayed once and then re-distributed into a larger colony.

Transfer of cuticular compounds to glass dummies. We designed an experiment similar to the previous queen compound transfer bioassay to test whether queen cuticular compounds could be extracted in hexane and effectively transferred to glass dummies. We melted Pasteur pipette tips into roughly the length and diameter of a termite queen. Neotenic queens, neotenic kings, and workers were extracted in hexane (200 µL/individual) for 2 min with gentle mixing. Hexane was transferred to new vials and evaporated under a gentle stream of high purity nitrogen. Final concentrations of 0.1, 0.3, 1, and 3 queen- or king-equivalents (QEs or KEs) per 20 µL were created from the initial extract for a dose-response study of royal compounds. Worker controls were tested using 6 worker equivalents (WE) because worker body mass and CHC mass (Chapter 2) were approximately half those of queens and

this would be equivalent to our highest concentration in royals. The bioassays tested one dummy per petri dish with two colonies (n=10 dishes) for each queen treatment and controls and n=5 dishes per colony for all king treatments due to limited extract availability. First, glass dummies were rinsed in hexane and allowed to dry before applying 20 µL of extract onto each in a glass petri dish. Hexane was allowed to evaporate from treated dummies for 5 min before introduction into assay dishes. Observations began 2 min after introducing the dummy to allow the termites to settle. We measured antennation, shaking responses, and presence/absence of aggression towards the dummies for 5 min. Aggression was defined as a repetitive lunging motion toward the dummy. In these assays, each group of termites in a dish was observed once per treatment, but then observed again in five other treatments, with a rest period of at least 24 h between assays.

Behavioral assays in light and dark conditions. An experiment was conducted to examine the behavioral responses of workers to queens in the photophase and scotophase, and under light and dark conditions. Termites (30 workers and 2 soldiers) were placed in a 5-cm petri dish, observed only once and returned to the colony for re-use. A queen was introduced into each petri dish and observed either in a fully lit laboratory (~450 lux at the assay dishes) or within a dark box with only a red headlamp. Antennation and shaking elicited in resident termites were measured for 7 min.

RESULTS

Behaviors elicited by different castes. We assessed the differential responses of worker and soldier termites to royals (queens and kings), workers, and soldiers. Behaviors that were readily observed and quantified included total time spent moving by the focal termite and the total number of allogrooming sessions, shaking responses, and antennations by both the focal termite and by resident workers and soldiers that interacted with it. Of these, shaking by resident termites in response to the introduced focal termite emerged as the most discriminating and showed a clear and significant difference between reproductive and non-reproductive individuals (below). Shaking behavior was defined as repetitive lateral oscillatory movements. All shaking behaviors performed by the focal termite were recorded, while shaking behaviors in resident termites were only recorded when they were within approximately 1 mm of the focal individual. Whereas shaking responses by the resident termites significantly discriminated royal and non-royal castes (Figs. 1, 2), shaking responses by the focal termite varied across castes, but failed to discriminate workers and soldiers from queens (Table S1).Therefore, only shaking responses by resident termites in response to the introduced focal termite were used in subsequent assays. Resident termite antennation responses were also informative, though to a lesser extent than shaking responses. Nevertheless, in all subsequent results we report on both shaking and antennation responses. Antennation was defined as placing both antennae on another individual and any continuous contact was counted as one session. Termites had to move more than ~3 mm away from the focal termite before a second antennation session was recorded. Allogrooming behavior and time spent moving also varied with caste, but neither resolved royals from workers and

soldiers, and allogrooming sessions occurred rarely and proved difficult to discretely quantify (Table S1).

Effects of time of day and light vs. dark conditions. There were no significant differences in the shaking (Fig. 1A) or antennation responses (Fig. 1B) between the photophase and scotophase. Observations under dark conditions in both photophase and scotophase, however, yielded significantly lower rates of shaking and antennation toward live neotenic queens than in a lit room. Dummies coated with worker extracts elicited much less total shaking and antennation than live queens, but demonstrated similar differences in rates of shaking and antennation between lit and dark conditions. Therefore, all subsequent assays were conducted under ambient light conditions during the photophase.

Workers recognize native and foreign royals. Shaking behavior occurred ~5–8-fold more in response to a neotenic queen or king than to a worker or soldier (Fig. 2A). Differences in antennation were less pronounced, showing a ~2–3-fold increase in response to reproductives (Fig. 2B). Though not pursued in other assays, allogrooming and movement rates both showed patterns across caste. Grooming by the focal termite (active grooming) was almost exclusively performed by workers and the introduced focal workers groomed resident termites significantly more than other castes (Table S1). Queens elicited significantly more allogrooming (reactive allogrooming) than workers, but not more than soldiers or kings (Table S1). Workers moved around the assay dish significantly more than soldiers and kings, and both workers and queens spent ~2X more time moving in the assay dish than other castes. These results are summarized in the supplementary table (Table S1).

In assays comparing responses to native and foreign workers and neotenic queens, workers and soldiers showed no overt aggression toward queens or workers introduced to dishes during the assay (not shown). However, significantly more shaking responses were evident toward nestmate and foreign neotenic queens than toward nestmate and foreign workers, respectively (Fig. 3A). Likewise, more antennation responses were elicited by nestmate neotenic queens than by nestmate workers, and foreign queens elicited more antennation responses than foreign workers (Fig. 3B). Therefore, in some subsequent assays, foreign neotenic queens were used as noted when nestmate queens were not available.

Queen and king cuticular extracts elicit royal recognition. To test whether queen recognition compounds could be transferred from the queen to other workers, we tumbled workers with neotenic queens in glass vials to "perfume" workers with royal scent. As negative controls, we tumbled 40:15 undyed workers with blue-dyed "focal" workers (i.e., 2.7X) to account for the greater body mass of queens. Dose-response assays included 0.5X to 10X queen : worker ratios, and a tumbled live queen represented the positive control. Queen-coated workers elicited significantly more shaking responses (Fig. 4A) and antennation (Fig. 4B) than worker-perfumed control workers. Notably, there was a clear dose-response relationship between the queen : worker ratio per tumbled worker and shaking responses (Fig. 4A). However, despite the effective transfer of queen compounds to workers, none of the queen-perfumed workers elicited as much shaking responses as live neotenic queens. Kings were not tested in this assay.

Finally, to control for the presence of non-chemical cues on workers that might facilitate the queen recognition responses, we transferred hexane extracts of workers and

neotenic queens and kings to glass dummies, which were introduced into assay dishes. We used the extract of 6 workers (6WE) as negative control and 0.1 to 3 neotenic queen- or king-equivalents in a dose-response study. Shaking responses increased significantly with the dose of either queen- (Fig. 5A) or king extracts (Fig. 6A), with both 1 and 3 QE treatments and the 1 KE treatment being significantly higher than the respective worker extract controls. Antennation responses to introduced glass dummies were uninformative in these assays. Although termites responded to queen-extracts in a dose-dependent manner (Fig. 5B), their antennation responses to 3 QE and worker extracts were not significantly different. Antennation responses to king extracts on glass dummies were not significantly different across all treatments (Fig. 6B).

The presence or absence of aggression was also recorded in all assays. More aggression (65%) was directed at the control dummies coated with hexane than at workers (20%), kings (0–30% across concentrations), and queens (5–25% across concentrations). All extracts elicited significantly less worker aggression than the control dummies (Chi-square test, workers: df = 1 $p < 0.017$, kings: df = 5, $p < 0.0001$, queens: df = 5, $p < 0.0001$) (Fig. 7).

DISCUSSION

Lateral shaking as a royal recognition behavior. Our aim was to develop a royal recognition bioassay in *Reticulitermes flavipes* to facilitate future isolation and identification of queen and king recognition pheromones. We evaluated four response parameters that could be readily quantified in behavioral assays: time spent moving by the focal termite, number of allogrooming events, shaking responses, and reciprocal antennations by the focal termite and resident workers and soldiers that interacted with it. Of these, only antennation and shaking responses were significantly elevated when neotenic queens or kings were present or in response to their cuticular extracts. Our bioassay results strongly support the conclusion that lateral or longitudinal shaking behavior is a strong indicator of neotenic queen and king recognition. Lateral shaking is different from head-drumming, which is used primarily by soldiers either to recruit termites or to send alarm signals through the nest substrate (Evans et al., 2005; Hager and Kirchner, 2013; Kirchner et al., 1994; Whitman and Forschler, 2007).

This is the first empirical evidence of behavioral royal recognition in termites. The lateral shaking behavior was differentially elicited by queens and kings more than by workers in all of our assays. Still, the functions of the lateral shaking behaviors remain unclear because it occurs in several contexts. First, this behavior is expressed away from reproductives and it is performed by all castes, including queens and kings. Secondly, the prevalence of lateral shaking behavior is highly correlated with alarm or disturbance in the colony(Reinhard and Clément, 2002). Our dark/light assays suggest that this response is intensified under lit conditions and all recognition assays were performed in a lit lab and

involved some disturbance as the focal termite or glass dummy was added to the assay dish. Shaking behavior might communicate a rapid local mechanical signal in disturbed or excited conditions to ensure the safety of high-value reproductives or begin repair of damaged areas of the nest. In the drywood termite *Cryptotermes secundus*, workers and nymphs exhibit increased aggression among nestmates after disturbance and an increase in shaking behavior in food-limited situations (Korb, 2005; Korb and Schmidinger, 2004). In both of these cases, the shaking behavior is interpreted as aggressive and it signals a transition from a cooperative to a more self-serving disposition in the study termites.

Shaking most likely does not elicit aggressive behavior in *R. flavipes* in the context of our bioassay. Indeed, aggression in this termite species is less frequent in general than in drywood termites as contests for replacement reproductives are rarer, the colony is much larger than in drywood termites, and the nest habitat is larger and more prone to disturbance in colonies with satellite nests and vulnerable areas outside a single piece of wood. Overall, higher rates of shaking directed toward royals in all our assays, and also toward queens in undisturbed dishes (CF, personal observation), strongly support the notion that while shaking may serve multiple functions in *R. flavipes*, it is a major and predictable queen and king recognition response. Most significant was the observation that shaking responses increased with the dose of royal extract, whereas aggression responses declined. These results suggest that shaking in the context of this bioassay is a response to royal semiochemicals and not an aggressive response. In other contexts (e.g., foreign workers or soldiers, interspecific interactions) shaking behavior might elicit aggression, but in these situations the shaking response should increase with the dose of the intruder semiochemicals. Because shaking behavior may convey different information in different contexts, it is also plausible that it

was co-opted from ancestral alarm or agitation responses that elicited aggressive behaviors to be a royal-recognition response that modulates colony-wide behavior.

Although shaking behavior likely conveys information over relatively short range, as it is typically elicited from physical contact with a reproductive, workers are often observed shaking repeatedly after they move away from the queen or king. Therefore, this behavior could be amplified and dispersed over a longer distance through a chain of workers.

It is also possible that shaking responses in *R. flavipes* vary in response to different stimuli. Our real-time visual observations could not resolve nuances in this behavior, but it is possible that the frequency, amplitude and other features of the behavior may be context-specific. Physical measurements of termite jerking or drumming behavior have been recorded before with few conclusive statements about their purpose (Howse, 1965; Kirchner et al., 1994; Ohmura et al., 2009). Forschler et al. (2007) described four general types of shaking behavior distinguished by speed and frequency in *R. flavipes*. Our assays did not differentiate between these movements but included three of the four described.

Honey bees exhibit a behavior similar to termite shaking, called the vibration response, where individuals shake rapidly, leading their nestmates to change tasks within the colony (Hyland et al., 2007; Schneider and Lewis, 2004). Bees which receive these vibration stimuli are typically less active and show increased task performance after receiving the signal. Other royal recognition responses in social insects are typically chemically mediated and include retinue responses or other aggregations around royal castes (Dietemann et al., 2003; Nunes et al., 2014; Slessor et al., 1988; Vander Meer et al., 1980), queen tending behaviors such as grooming or feeding, and strong aggressive responses to establish

reproductive dominance or prevent unwanted reproduction in the colony (Liebig et al., 2000; Smith et al., 2012).

Royal recognition is chemically mediated in *R. flavipes.* Lateral shaking is readily elicited in *R. flavipes* by cuticular chemicals of neotenic queens and kings (Figs. 1, 4–6). We transferred cuticular compounds from queens to worker termites by tumbling them in various queen : worker ratios. We also transferred hexane extracts of queen and king cuticular lipids to glass dummies. In both experiments the royal-perfumed workers and glass dummies elicited significantly more shaking responses than the respective controls, indicating that royal-recognition pheromones were contained in the transferred chemicals. CHCs are most likely responsible, as they are the dominant feature of insect cuticular lipids, but fatty acids, esters, waxes, or other lipids may be involved. In previous research (Chapter 2 of this dissertation) we identified a suite of CHCs that are highly enriched in *R. flavipes* queens and kings as well as a royal-specific saturated hydrocarbon, heneicosane. In addition, aggressive behaviors were significantly associated with hexane controls and low concentrations of termite extracts, but not with higher concentrations of queen, king or worker extracts (Fig. 7), suggesting that these extracts likely contain colony recognition cues and can mitigate aggressive behaviors toward foreign objects. The behavioral assays we developed and validated in this Chapter should facilitate experiments to test whether any of these candidate royal compounds serve as a recognition pheromone in this species.

Other species of termites possess CHCs that researchers have linked to reproductive status, but their functions in royal recognition have not been evaluated (Liebig et al., 2009; Weil et al., 2009). In contrast, CHC recognition pheromones have been demonstrated in

many social hymenopterans, including various ant species and *Polistes* wasps (Dietemann et al., 2003; Espelie et al., 1994; Holman et al., 2016, 2010; Liebig et al., 2000; Smith et al., 2015, 2013). Van Oystaeyen et al. (2014) found that species from across the hymenopteran phylogeny (ant, bee, and wasp) used similar CHCs as queen pheromones, which acted to reduce or suppress ovary development. They also compared fertility signals across 64 species of social Hymenoptera to conclude that saturated CHCs are a conserved class of pheromones that function similarly across a diverse assemblage of species (but see Amsalem et al. (2015) countered by Holman et al. (2017). The wide phylogenetic distance between the eusocial hymenopterans and termites, and their shared use of CHCs as fertility signals, could indicate an intriguing case of convergent evolution that would push the use of CHCs as queen pheromones from ~100 million years ago (evolution of bees, ants and wasps) to ~150 million years ago, when eusocial termites evolved from within the cockroaches.

In conclusion, this is the first demonstration of queen and king recognition in termites. We report a highly discriminating bioassay that quantitatively related shaking behavior in workers and soldiers to presence of a neotenic queen or king. We further showed that queen and king cuticular compounds elicited this behavior. Our bioassay should prove to be useful for future research to identify specific royal pheromones, the social status of newly emerging reproductives, and the activity of candidate volatile and non-volatile royal pheromones. Queens and kings possess similar cuticular profiles in *R. flavipes* and both sexes elicit increased lateral shaking and antennation. By examining caste-specific differences in cuticular profiles, future work may be able to identify the chemical basis for this behavior and describe the first queen recognition pheromones and perhaps the first ever king pheromone in termites. Finally, the function of this behavior should also be the target of

future research to understand how this behavior changes in different contexts within the colony and whether the shaking behavior consists of different elements that require closer scrutiny with high speed photography and laser Doppler vibrometry.

REFERENCES

Amsalem E, Orlova M, Grozinger CM (2015) A conserved class of queen pheromones? Re-evaluating the evidence in bumblebees (*Bombus impatiens*). Proc R Soc B 282:20151800. doi: 10.1098/rspb.2015.1800

Bagnères AG, Clément JL, Blum MS, et al (1990) Cuticular hydrocarbons and defensive compounds of *Reticulitermes flavipes* (Kollar) and *R. santonensis* (Feytaud): Polymorphism and chemotaxonomy. J Chem Ecol 16:3213–3244.

Bagneres A-G, Killian A, Clement J-L, Lange C (1991) Interspecific recognition among termites of the genus *Reticulitermes*: Evidence for a role for the cuticular hydrocarbons. J Chem Ecol 17:2397–2420.

Bagnères A-G, Rivière G, Clément J-L (1998) Artificial neural network modeling of caste odor discrimination based on cuticular hydrocarbons in termites. Chemoecology 8:201–209. doi: 10.1007/s000490050026

Batista-Pereira LG, Dos Santos MG, Corrêa AG, et al (2004) Cuticular hydrocarbons of *Heterotermes tenuis* (Isoptera: Rhinotermitidae): analyses and electrophysiological studies. Z Für Naturforschung C 59:135–139.

Bignell DE, Roisin Y, Lo N (2014) Biology of Termites: a Modern Synthesis, 2nd edn. Springer, Netherlands (doi: 10.1007/978-90-481-3977-4)

Blomquist GJ, Bagneres AG (2010) Insect Hydrocarbons: Biology, Biochemistry, and Chemical Ecology. Cambridge Univ Press, Cambridge

Blum MS (1996) Semiochemical parsimony in the arthropoda. Annu Rev Entomol 41:353–374. doi: 10.1146/annurev.en.41.010196.002033

Darrouzet E, Labédan M, Landré X, et al (2014) Endocrine control of cuticular hydrocarbon profiles during worker-to-soldier differentiation in the termite *Reticulitermes flavipes*. J Insect Physiol 61:25–33. doi: 10.1016/j.jinsphys.2013.12.006

Dietemann V, Peeters C, Liebig J, et al (2003) Cuticular hydrocarbons mediate discrimination of reproductives and nonreproductives in the ant *Myrmecia gulosa*. Proc Natl Acad Sci U S A 100:10341–10346.

Espelie KE, Gamboa GJ, Grudzien TA, Bura EA (1994) Cuticular hydrocarbons of the paper wasp, *Polistes fuscatus*: A search for recognition pheromones. J Chem Ecol 20:1677–1687. doi: 10.1007/BF02059889

Evans TA, Lai JC, Toledano E, et al (2005) Termites assess wood size by using vibration signals. Proc Natl Acad Sci U S A 102:3732–3737.

Hager FA, Kirchner WH (2013) Vibrational long-distance communication in the termites *Macrotermes natalensis* and *Odontotermes sp*. J Exp Biol 216:3249–3256. doi: 10.1242/jeb.086991

Haverty MI, Grace JK, Nelson LJ, Yamamoto RT (1996) Intercaste, intercolony, and temporal variation in cuticular hydrocarbons of *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae). J Chem Ecol 22:1813–1834. doi: 10.1007/BF02028506

Himuro C, Yokoi T, Matsuura K (2011) Queen-specific volatile in a higher termite *Nasutitermes takasagoensis* (Isoptera: Termitidae). J Insect Physiol 57:962–965. doi: 10.1016/j.jinsphys.2011.04.012

Hölldobler B, Wilson EO (1990) The Ants. Belknap Press of Harvard Univ. Press, Cambridge, MA

Holman L, Hanley B, Millar JG (2016) Highly specific responses to queen pheromone in three *Lasius* ant species. Behav Ecol Sociobiol 70:387–392. doi: 10.1007/s00265-016-2058-6

Holman L, Jorgensen CG, Nielsen J, d'Ettorre P (2010) Identification of an ant queen pheromone regulating worker sterility. Proc R Soc B Biol Sci 277:3793–3800. doi: 10.1098/rspb.2010.0984

Holman L, van Zweden JS, Oliveira RC, et al (2017) Conserved queen pheromones in bumblebees: a reply to Amsalem et al. PeerJ. doi: 10.7717/peerj.3332

Howse PE (1964) The significance of the sound produced by the termite *Zootermopsis angusticollis* (Hagen). Anim Behav 12:284-IN8. doi: 10.1016/0003-3472(64)90015-6

Howse PE (1965) On the significance of certain oscillatory movements of termites. Insectes Sociaux 12:335–345. doi: 10.1007/BF02222723

Hyland KM, Cao TT, Malechuk AM, et al (2007) Vibration signal behaviour and the use of modulatory communication in established and newly founded honeybee colonies. Anim Behav 73:541–551. doi: 10.1016/j.anbehav.2006.10.006

Kirchner WH, Broecker I, Tautz J (1994) Vibrational alarm communication in the damp-wood termite *Zootermopsis nevadensis*. Physiol Entomol 19:187–190. doi: 10.1111/j.1365-3032.1994.tb01041.x

Korb J (2005) Regulation of sexual development in the basal termite *Cryptotermes secundus*: mutilation, pheromonal manipulation or honest signal? Naturwissenschaften 92:45–49. doi: 10.1007/s00114-004-0589-6

Korb J, Schmidinger S (2004) Help or disperse? Cooperation in termites influenced by food conditions. Behav Ecol Sociobiol 56:89–95. doi: 10.1007/s00265-004-0757-x

Liebig J, Eliyahu D, Brent CS (2009) Cuticular hydrocarbon profiles indicate reproductive status in the termite *Zootermopsis nevadensis*. Behav Ecol Sociobiol 63:1799–1807. doi: 10.1007/s00265-009-0807-5

Liebig J, Peeters C, Hölldobler B (1999) Worker policing limits the number of reproductives in a ponerine ant. Proc R Soc Lond B Biol Sci 266:1865–1870. doi: 10.1098/rspb.1999.0858

Liebig J, Peeters C, Oldham NJ, et al (2000) Are variations in cuticular hydrocarbons of queens and workers a reliable signal of fertility in the ant *Harpegnathos saltator*? Proc Natl Acad Sci U S A 97:4124–4131. doi: 10.1073/pnas.97.8.4124

Light SF, Weesner FM (1951) Further studies on the production of supplementary reproductives in *Zootermopsis* (Isoptera). J Exp Zool 117:397–414. doi: 10.1002/jez.1401170302

Lüscher M (1961) Social control of polymorphism in termites. In: Insect Polymorphism. Royal Entomological Society of London, London, UK, pp 57–67

Matsuura K, Himuro C, Yokoi T, et al (2010) Identification of a pheromone regulating caste differentiation in termites. Proc Natl Acad Sci 107:12963–12968. doi: 10.1073/pnas.1004675107

Nunes TM, Mateus S, Favaris AP, et al (2014) Queen signals in a stingless bee: suppression of worker ovary activation and spatial distribution of active compounds. Sci Rep 4:srep07449. doi: 10.1038/srep07449

Ohmura W, Takanashi T, Suzuki Y (2009) Behavioral analysis of tremulation and tapping of termites (Isoptera). Sociobiology 54:269–274.

Penick CA, Trobaugh B, Brent CS, Liebig J (2013) Head-butting as an early indicator of reproductive disinhibition in the termite *Zootermopsis nevadensis*. J Insect Behav 26:23–34. doi: 10.1007/s10905-012-9332-x

Reinhard J, Clément J-L (2002) Alarm reaction of European *Reticulitermes* termites to soldier head capsule volatiles (Isoptera, Rhinotermitidae). J Insect Behav 15:95–107. doi: 10.1023/A:1014436313710

Rocca JR, Tumlinson JH, Glancey BM, Lofgren CS (1983) The queen recognition pheromone of *Solenopsis invicta*, preparation of (E-6-(1-pentenyl)-2H-pyran-2-one. Tetrahedron Lett 24:1889–1892. doi: 10.1016/S0040-4039(00)81798-0

Schneider SS, Lewis LA (2004) The vibration signal, modulatory communication and the organization of labor in honey bees, *Apis mellifera*. Apidologie 35:117–131. doi: 10.1051/apido:2004006

Slessor KN, Kaminski L-A, King GGS, et al (1988) Semiochemical basis of the retinue response to queen honey bees. Nature 332:354–356. doi: 10.1038/332354a0

Smith AA, Millar JG, Hanks LM, Suarez AV (2013) A conserved fertility signal despite population variation in the cuticular chemical profile of the trap-jaw ant *Odontomachus brunneus*. J Exp Biol 216:3917–3924. doi: 10.1242/jeb.089482

Smith AA, Millar JG, Hanks LM, Suarez AV (2012) Experimental evidence that workers recognize reproductives through cuticular hydrocarbons in the ant *Odontomachus brunneus*. Behav Ecol Sociobiol 66:1267–1276. doi: 10.1007/s00265-012-1380-x

Smith AA, Millar JG, Suarez AV (2015) A social insect fertility signal is dependent on chemical context. Biol Lett 11:20140947. doi: 10.1098/rsbl.2014.0947

Su N, Ban P, Scheffrahn R (1991) Evaluation of 12 dye markers for population studies of the eastern and formosan subterranean termite (Isoptera, Rhinotermitidae). Sociobiology 19:349–362.

Tibbetts EA, Dale J (2004) A socially enforced signal of quality in a paper wasp. Nature 432:218–222. doi: 10.1038/nature02949

Van Oystaeyen A, Oliveira RC, Holman L, et al (2014) Conserved class of queen pheromones stops social insect workers from reproducing. Science 343:287–290. doi: 10.1126/science.1244899

Vander Meer RK, Glancey BM, Lofgren CS, et al (1980) The poison sac of red imported fire ant queens: source of a pheromone attractant. Ann Entomol Soc Am 73:609–612.

Weil T, Hoffmann K, Kroiss J, et al (2009) Scent of a queen—cuticular hydrocarbons specific for female reproductives in lower termites. Naturwissenschaften 96:315–319. doi: 10.1007/s00114-008-0475-8

West MJ (1967) Foundress associations in polistine wasps: dominance hierarchies and the evolution of social behavior. Science 157:1584–1585.

Whitman JG, Forschler BT (2007) Observational notes on short-lived and infrequent behaviors displayed by *Reticulitermes flavipes* (Isoptera: Rhinotermitidae). Ann Entomol Soc Am 100:763–771. doi: 10.1603/0013-8746(2007)100[763:ONOSAI]2.0.CO;2

Yamamoto Y, Matsuura K (2012) Genetic influence on caste determination underlying the asexual queen succession system in a termite. Behav Ecol Sociobiol 66:39–46. doi: 10.1007/s00265-011-1249-4

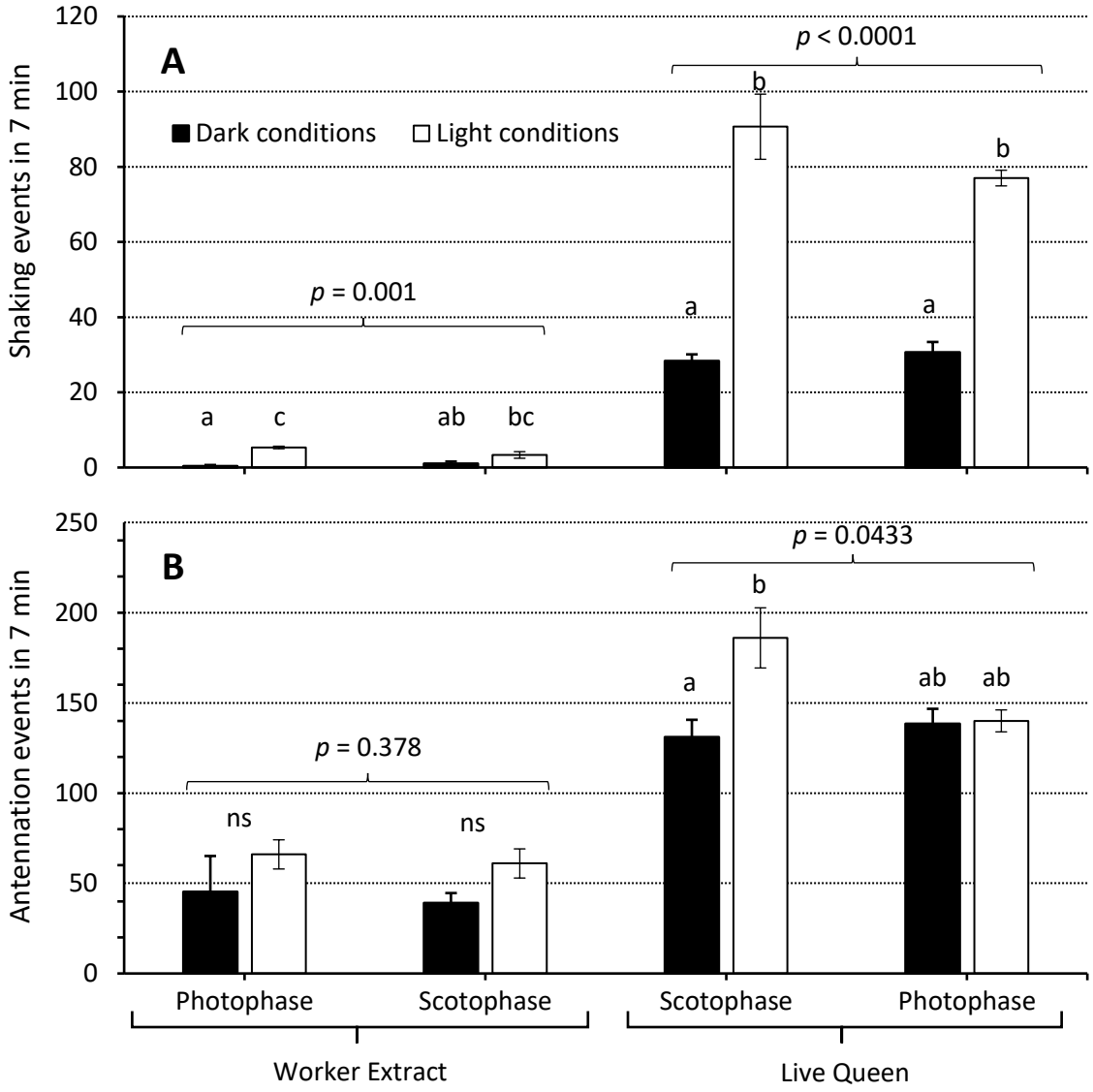


Figure 1. Shaking (**A**) and antennation (**B**) responses of termites to a live neotenic queen or a glass dummy coated with hexane extract of 2 worker-equivalents. Each assay dish consisted of 30 workers, 2 soldiers, and either an introduced glass dummy or live queen. Termites were assayed in their photophase and scotophase and under light and dark conditions. Number of replicate assays was 3. Letters indicate significantly different values using one way ANOVA ($p < 0.05$) and Tukey's HSD within live queen or worker extract, respectively. Error bars represent standard error of the mean.

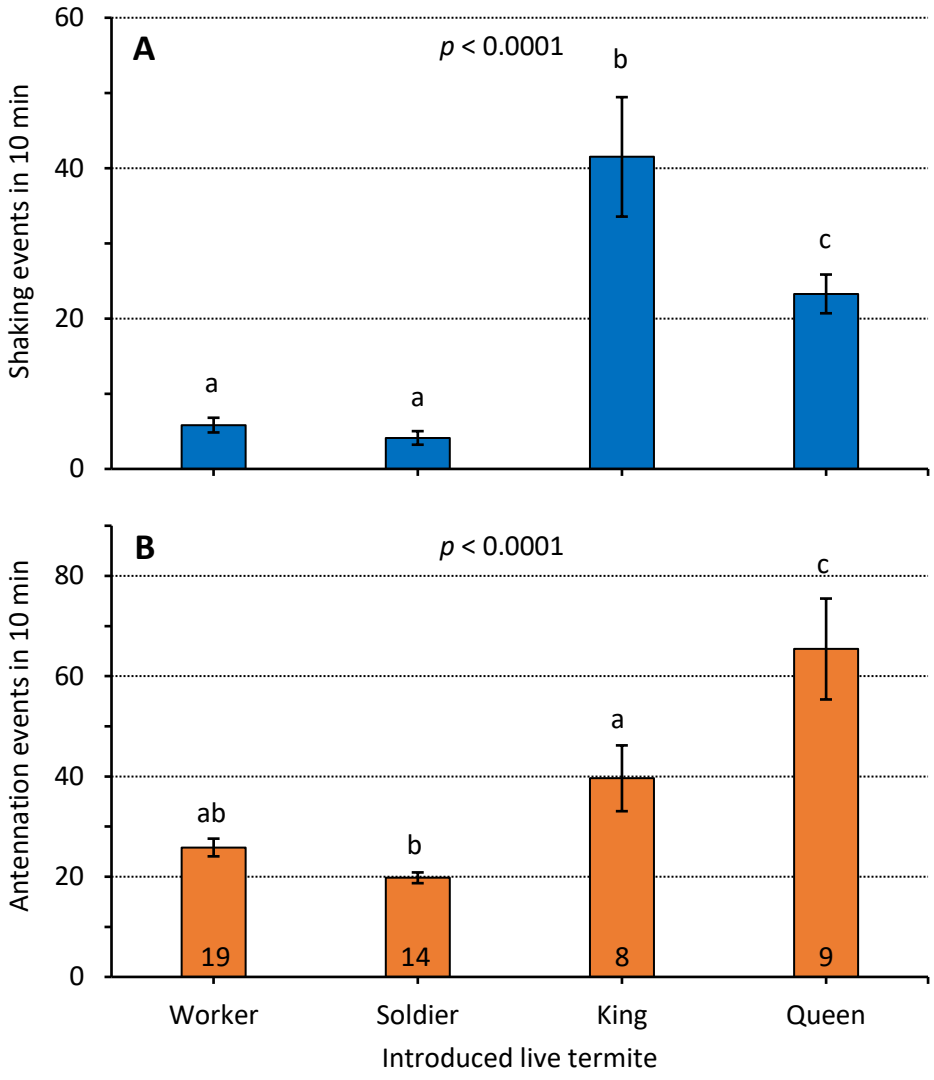


Figure 2. Behaviors exhibited by groups of 100 termites toward a worker, soldier, neotenic king, and neotenic queen, measured by lateral shaking (**A**) and antennation (**B**) during a 10 min observation period. Queens and kings in all assays were neotenic (secondary) reproductives generated within the lab. Each assay dish consisted of 30 workers, 2 soldiers, and an introduced live focal termite. Number of replicate assays is indicated within each bar for each caste. Letters indicate significantly different values using one way ANOVA ($p < 0.05$) and Tukey's HSD. Error bars represent standard error of the mean.

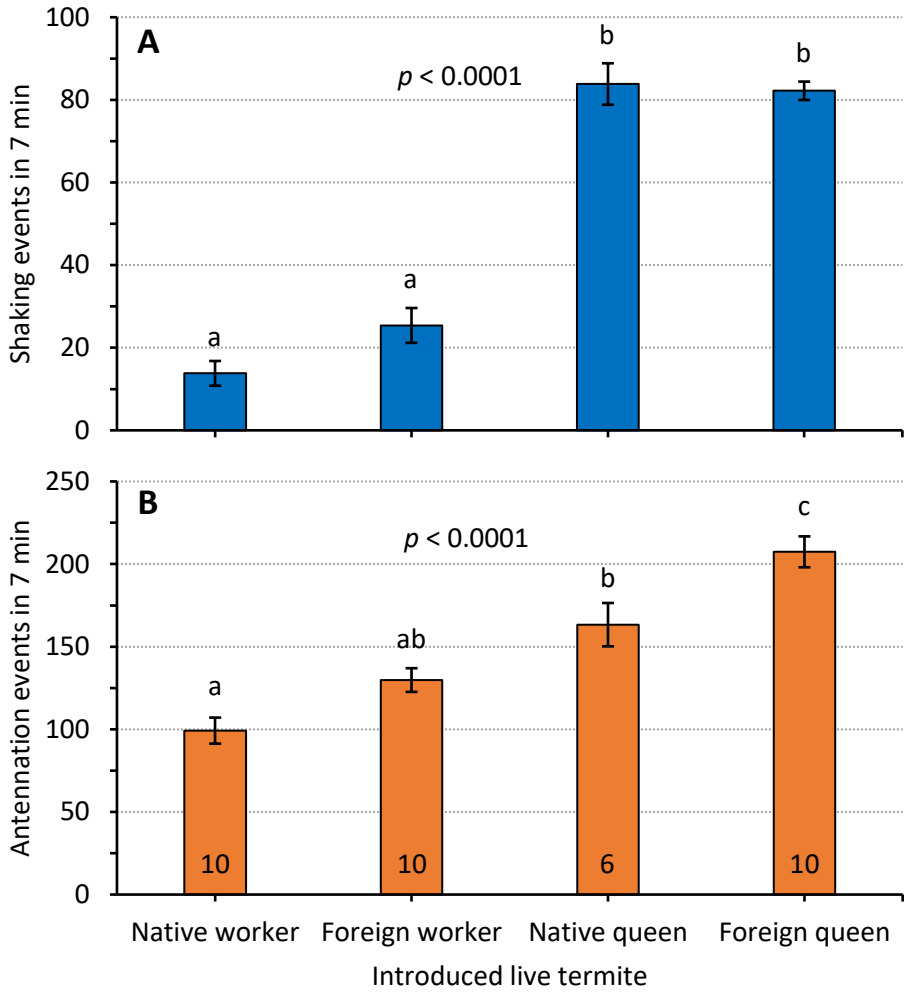


Figure 3. Assays measuring behavior elicited by a native or foreign neotenic queen with foreign and native worker controls. Shaking (**A**) and antennation (**B**) were measured in 7 min assays. Each assay dish consisted of 30 workers, 2 soldiers, and an introduced live focal termite. Number of replicate assays is indicated within each bar for each caste. Letters indicate significantly different values using one way ANOVA ($p < 0.05$) and Tukey's HSD. Error bars are standard error of the mean.

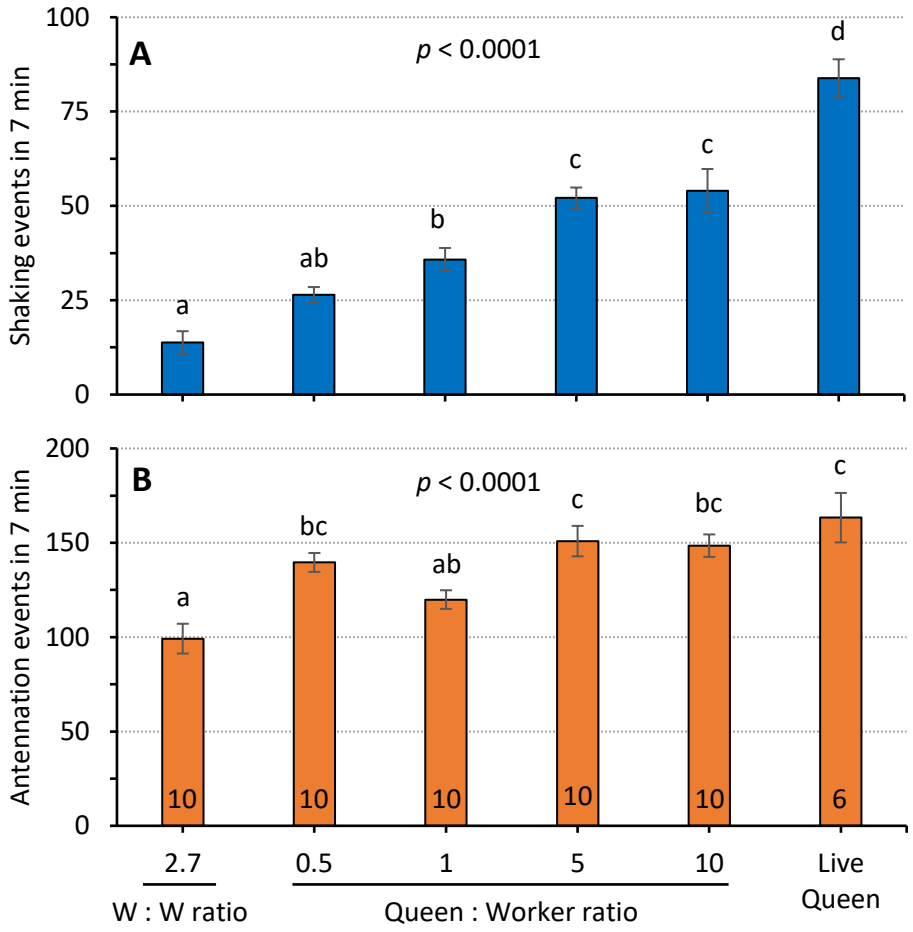


Figure 4. Live workers coated with neotenic queen cuticular compounds elicit queen recognition behavior as measured by shaking (**A**) and antennation (**B**) responses. Workers were tumbled in clean glass vials in the following proportions: With other workers: 40:15 (2.7X) dyed workers : undyed workers. With queens: 7:15 (0.5X), 1:1 (1X), 5:1 (5X), and 10:1 (10X) Queen : Worker treatments. A live neotenic queen was tumbled in a vial as a positive control. Each assay dish consisted of 30 workers, 2 soldiers, and a tumbled test individual. For all treatments number of replicate assays is indicated within each bar. Letters indicate significantly different values using one way ANOVA ($p < 0.05$) and Tukey's HSD. Error bars represent standard error of the mean.

Figure 5. Termite responses to glass dummies treated with hexane extracts of neotenic queens. Lateral shaking (**A.**) and antennation (**B.**) were measured during 5 min assays for each treatment. Glass dummies were coated with hexane only (0), 0.1, 0.3, 1, and 3 queen equivalents along with worker extracts dissolved in hexane. Worker extracts were created by pooling 6 workers with mass approximately equal to 3 neotenic queens to approximate the highest queen concentration. Each assay dish consisted of 30 workers, 2 soldiers, and an introduced glass dummy. Letters indicate significantly different values using one way ANOVA ($p < 0.05$) and Tukey's HSD. Error bars are standard error of the mean. For all treatments number of replicate assays is indicated within each bar.

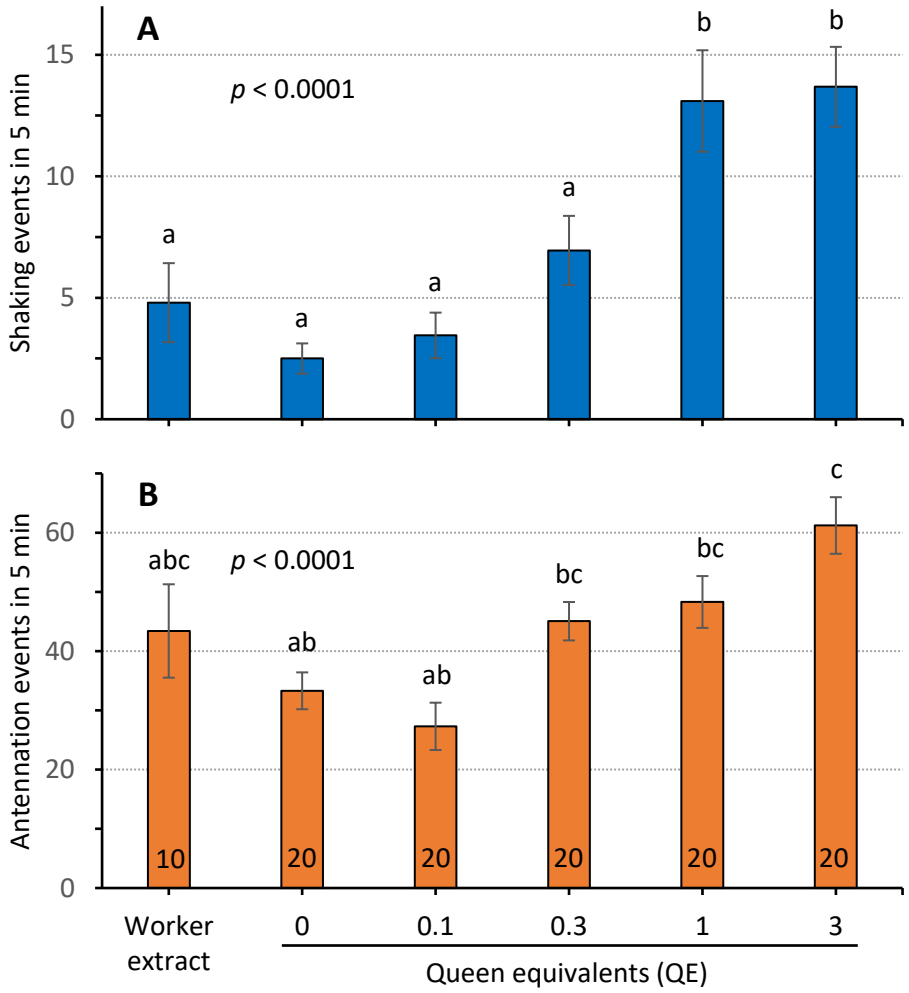
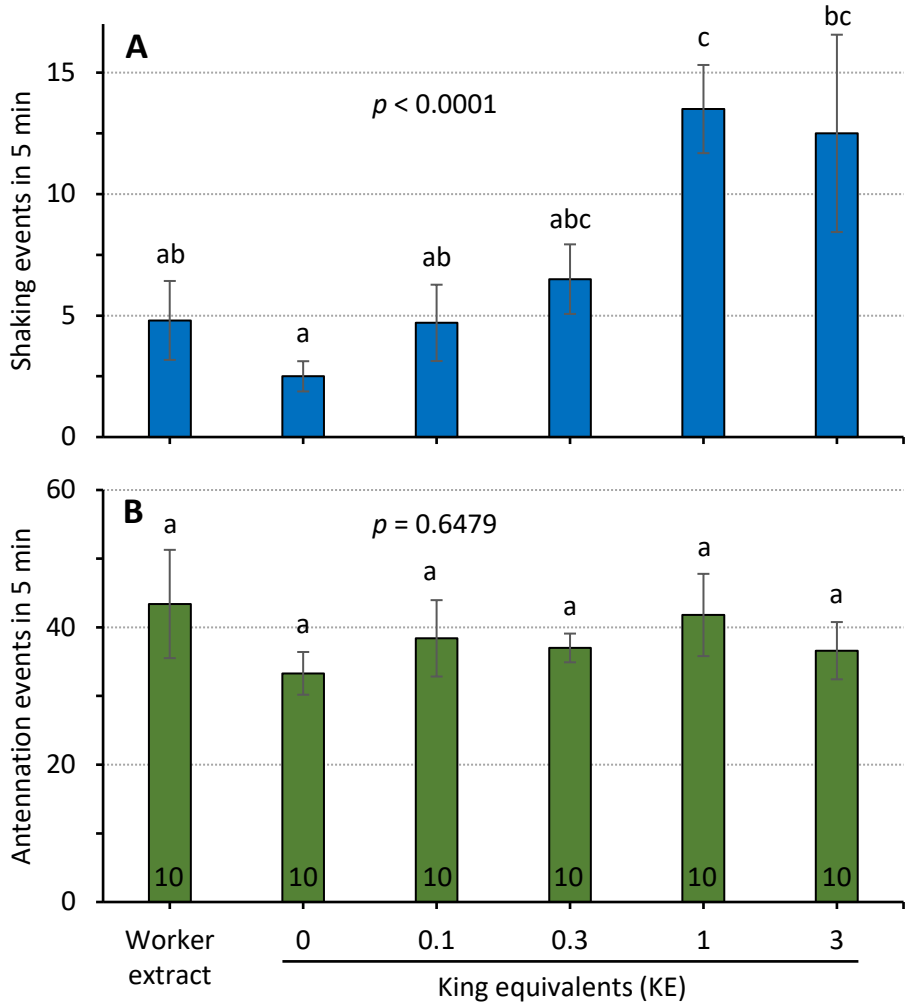

A
p < 0.0001
Shaking events in 5 min
0
5
10
15
a
a
a
a
b
b
B
p < 0.0001
Antennation events in 5 min
0
20
40
60
abc
ab
ab
bc
bc
c
10
20
20
20
20
20
Worker extract
0
0.1
0.3
1
3
Queen equivalents (QE)

Figure 6. Termite responses to glass dummies treated with hexane extracts of neotenic kings. Lateral shaking (**A**) and antennation (**B**) were measured during 5 min assays for each treatment. Glass dummies were coated with hexane only (0), 0.1, 0.3, 1, and 3 king equivalents along with worker extracts dissolved in hexane. Worker extracts were created by pooling 6 workers with mass approximately equal to 3 neotenic kings to approximate the highest king concentration. Each assay dish consisted of 30 workers, 2 soldiers, and an introduced glass dummy. Letters indicate significantly different values using one way ANOVA ($p < 0.05$) and Tukey's HSD. Error bars are standard error of the mean. For all treatments number of replicate assays is indicated within each bar.


A
p < 0.0001
Shaking events in 5 min
0
5
10
15
ab
a
ab
abc
c
bc
B
p = 0.6479
Antennation events in 5 min
0
20
40
60
a
a
a
a
a
a
10
10
10
10
10
10
Worker extract
0
0.1
0.3
1
3
King equivalents (KE)

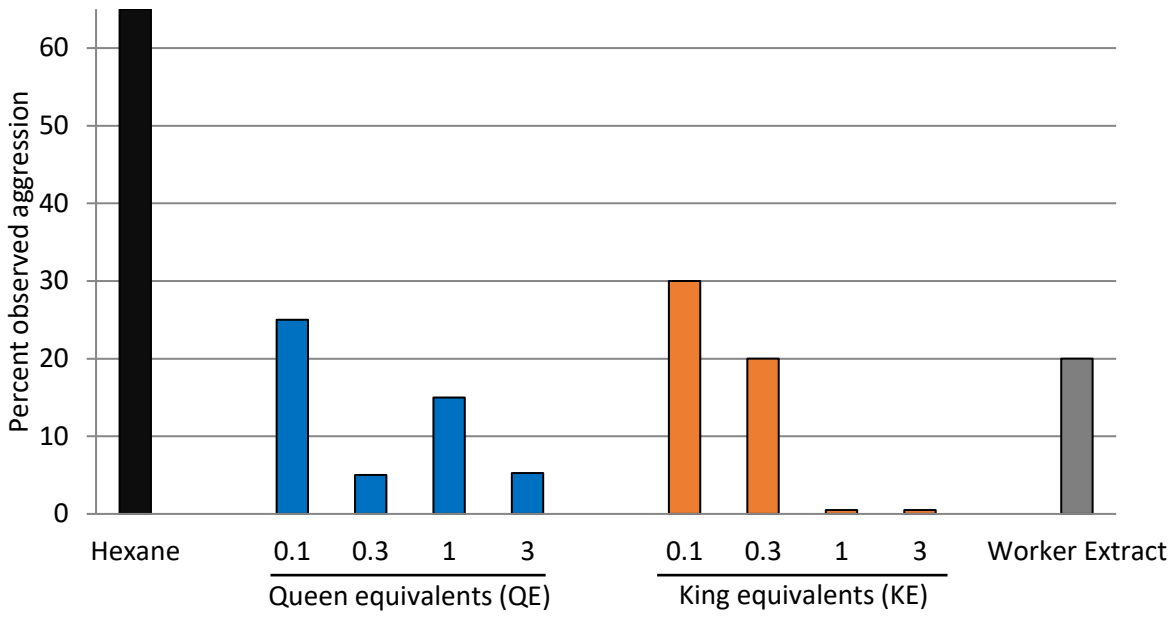


Figure 7. Relationship between cuticular extract concentrations of queens (**A**), kings (**B**), and workers (**C)** and aggressive behavior. Concentrations are denoted in queen- and king-equivalents applied to glass dummies. Worker extracts were created by pooling 6 workers with mass approximately equal to 3 queens or kings. Glass dummies (n = 20 for all queen concentrations and hexane controls, n = 10 for all king concentrations and the worker extract) were observed over a 5 min period. Chi square tests for each caste show a significant effect on worker aggression for queens (df = 5, $p < 0.0001$), kings (df = 5, $p < 0.0001$), and workers (df = 1 $p < 0.017$).

APPENDIX

Appendix A

Table S1. Behaviors exhibited by termites during a 10 min observation period. Queens and kings in all assays were neotenic (secondary) reproductives generated within the lab. Each assay dish consisted of 100 workers, 2 soldiers, and an introduced live focal termite. Number of replicate assays is indicated for each caste. Letters indicate significantly different values using one way ANOVA ($p < 0.05$) and Tukey's HSD within rows (specific behavior). Error bars represent standard error of the mean.

Metric	Worker ± SEM *n* = 19		Soldier ± SEM *n* = 14		King ± SEM *n* = 8		Queen ± SEM *n* = 9	
% Time moving	39.6 a	4.1	21.1 b	2.2	17.4 b	9.7	36.0 ab	3.9
Active antennation	27.6 a	2.0	17.2 bc	1.9	9.1 c	2.4	21.1 ab	1.9
Reactive antennation	25.8 ab	1.8	19.8 b	1.1	39.6 a	6.6	65.4 c	10.1
Active shaking	6.5 a	1.1	5.3 a	1.1	0.4 b	0.3	4.1 ab	0.9
Reactive shaking	5.8 a	1.0	4.1 a	0.9	41.5 b	7.9	23.3 c	2.6
Active allogrooming	1.1 a	0.3	0.03 b	0.04	0 b	0	0 b	0
Reactive allogrooming	1.1 a	0.2	1.3 ab	0.3	1.7 ab	1.0	2.8 b	0.2

Active refers to actions performed by the focal termite.

Reactive refers to reactions elicited by the focal termite from others.

CHAPTER 4

Identification of a queen- and king-recognition pheromone in the subterranean termite, *Reticulitermes flavipes*

ABSTRACT

Chemical communication is fundamental to success in social insect colonies. More specifically, royal pheromones used by queens (and kings in termites) maintain the reproductive division of labor and help workers recognize and care for these vital individuals. Termites are highly dependent on pheromones to communicate and effectively recognize different castes within the colony, but to date queen and king recognition pheromones have not been identified. We use a recently developed queen recognition behavioral assay to investigate the pheromonal role of a queen- and king-specific *n*-alkane, heneicosane. We found that when applied to glass dummies, heneicosane elicited a behavioral response in workers identical to that elicited by live termite queens, including increased antennation and vibratory shaking. Further, the effects of heneicosane were amplified when presented with termite cuticular extracts, underscoring the importance of chemical context in termite royal recognition. Heneicosane is the first identified royal recognition pheromone in termites and the first king pheromone discovered in social insects.

INTRODUCTION

Social insect societies depend on nestmate communication to thrive as cohesive groups in challenging environments. Recognition of nestmates helps defend the colony, while recognition of different castes enables proper regulation of colony demography through caste differentiation, and maintains the social and reproductive division of labor.

Chemical communication, mediated by volatile or non-volatile pheromones, is the foundation of nestmate and caste recognition, though other sensory modalities can also be involved, including visual and tactile signals. The chemical basis of nestmate recognition has been described in many species of ants, bees, wasps, and termites (Richard and Hunt, 2013), but caste recognition signals, and specifically queen recognition signals, are not as well studied. Some hymenopterans (ants, bees, wasps) use cuticular hydrocarbons (CHCs) as contact-based signals that mediate queen recognition and colony organization. Complex mixtures of colony- or species-specific blends of CHCs can identify a variety of status conditions in hymenopterans, including nestmate status, mating status, age, caste, and fertility (Penick and Liebig, 2017; Polidori et al., 2017; Richard and Hunt, 2013; Smith et al., 2013; Van Oystaeyen et al., 2014). In addition, compounds other than CHCs can code this information, for example the queen mandibular pheromone of honey bees, which consists of a blend of carboxylic acids, carboxylic esters, phenols, and alcohols (Keeling et al., 2003; Slessor et al., 1988).

Queen recognition pheromones regulate colony behavior and organization and consolidate reproduction by royal castes, whose pheromones elicit two major types of responses: Immediate behavioral responses (releaser effects) that consist of aggregation (i.e., retinue response), queen tending, and policing behaviors toward rival reproductives, and

physiological responses of worker castes (primer responses) to prevent reproduction and maintain harmony within the colony. Queen pheromones have been characterized in a handful of hymenopterans (Dietemann et al., 2003; Holman et al., 2016, 2010; Kocher and Grozinger, 2011; Slessor et al., 1988; Smith et al., 2013; Van Oystaeyen et al., 2014), but have received scant attention in termites.

Termites, nested within the order Blattodea, developed sociality independently from Hymenoptera, but share many of the biological features that maintain cooperative and altruistic interactions within the colony. Thus royal recognition is equally vital to social cohesion and maintaining the division of labor in termites. In social hymenopterans, males are present in the colony only transiently and queens are the only permanent reproductive caste. In subterranean termites, however, colonies are founded by a monogamous queen and king pair (primary reproductives), but secondary or replacement reproductives (neotenics) can differentiate within the colony. Thus both the king and queen and neotenics are permanent members of the colony, so royal recognition must extend to both sexes.

Living either underground or within wood, termites are generally blind and must rely on olfactory, vibratory, and tactile signals to communicate. Vibrations carried through the air or substrate can raise an alarm, indicate the quality of a food source, or identify the presence of a predator or competitor (Evans et al., 2009, 2005; Hager and Kirchner, 2013; Kirchner et al., 1994; Oberst et al., 2017). Termite-termite tactile interactions in *Cryptotermes secundus* also convey information, and these behaviors intensify when queens are removed from the colony or genes linked to reproductive suppression are silenced (Korb, 2005; Korb et al., 2009; Weil et al., 2009). Chemical communication, however, is the primary mediator of recognition in termites. In addition to their functions in preventing water loss and protecting

the insects from pathogens, termite CHCs have been associated with nestmate recognition and other intra-colony interactions (Bagneres et al., 1991; Darrouzet et al., 2014). Termite queens and kings produce a number of different compounds that differentiate them from other castes (Hanus et al., 2010; Himuro et al., 2011), including multiple examples of reproductive-specific CHCs (Darrouzet et al., 2014; Liebig et al., 2009; Weil et al., 2009). Compounds that mediate reproductive inhibition of other castes have been reported in only one termite species, *Reticulitermes speratus*, in which queens release volatile compounds to inhibit the reproductive differentiation of female workers into supplementary reproductives (Matsuura et al., 2010).

Royal recognition pheromones have yet to be reported in any termite species, and few royal recognition behaviors have been described. In the dampwood termite, *Zootermopsis nevadensis*, the removal of reproductives induces increased head-butting, but the behaviors have not been linked to the unique CHC shared by kings and queens (Penick et al., 2013). Others have reported similar aggressive interactions upon suppression of a gene highly expressed in queens (Hoffmann et al., 2014; Korb et al., 2009), but few behaviors have been empirically tested to be queen or king specific. In Chapter 2 we developed an effective termite queen recognition bioassay. The bioassay measures the rapid lateral (or longitudinal) shaking behavior and increased antennation from workers and soldiers elicited by queens and kings. These two behaviors are common throughout the colony, but are especially common in close proximity to reproductives. In addition, the shaking behavior, sometimes referred to as oscillatory movements (Howse, 1965; Whitman and Forschler, 2007), and categorized separately from head drumming or vertical oscillatory movement (Howse, 1964), has been documented in many termite species, but has never been evaluated in the context of royal

caste recognition (Ohmura et al., 2009). Experiments by us demonstrated that queen cuticular compounds could be transferred to non-reproductive workers and inert dummies and they elicited queen-recognition behavior. These experiments supported the notion that there is a chemically mediated queen recognition system in *R. flavipes* and that it can be reliably observed. In Chapter 1 of this dissertation we identified a saturated hydrocarbon, heneicosane, which is specific to queens and kings, and several other high molecular weight hydrocarbons that are highly enriched in royal castes. Here we present evidence that heneicosane is a queen and king pheromone that releases a royal-specific recognition behavior. Heneicosane represents the first identified royal recognition pheromone in termites, offering a unique opportunity to further understand termite social systems and their chemical mediation.

MATERIALS AND METHODS

Termite Collection. Colonies of *Reticulitermes flavipes* were collected in Raleigh, North Carolina, from two wooded locations (Yates Mill Pond and Lake Johnson) in 2015. Whole tree limbs or logs on the ground were split into smaller pieces and dried in shallow plastic pans. We placed ~10 cm PVC pipes containing coils of moistened corrugated cardboard in the pans and the termites passively moved out of the drying wood and into the moist substrate. Fully extracted colonies were kept either in clear plastic boxes lined with moist sand and pine shims for food or in 9-cm petri dishes with an autoclaved lab substrate consisting of 70% sawdust and 30% α-cellulose. Boxes and dishes were kept in opaque plastic containers inside a 24ºC incubator under 14:10 L:D cycle with lights-on at 0600.

Cuticular Extracts and GC-MS. Individual termites from every caste were sexed and freeze-killed for 15 min at -20°C, followed by extraction in 200 µL of hexane containing 100 ng octacosane (*n*-C28) as an internal standard. Extraction lasted for 2 min with intermittent gentle mixing. Extracts were removed to a new vial, evaporated under a gentle stream of high-purity nitrogen, re-dissolved in 50 µL of hexane and transferred to a 100 µL glass insert in a 1.5 mL GC autoinjection vial. Two of 50 µL were injected in splitless mode using a 7683B Agilent autosampler into a DB-5 column (20 m x 0.18 mm internal diameter x 0.18 µm film thickness, J&W Scientific, Folsom, CA, USA) in an Agilent 7890 series gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) connected to a flame ionization detector (FID) with ultra-high purity hydrogen as carrier gas (0.75 mL/min

constant flow rate). The column was held at 50°C for 1 min, ramped to 320°C at 10°C/min, and held at 320°C for 10 min.

A subset of termite CHC samples was run on an Agilent 5975 mass selective detector coupled to an Agilent 6890 GC for GC-MS analyses, as reported in Chapter 1. The GC was operated in splitless injection mode and fitted with a DB-5MS column (30 m × 0.25 mm × 0.25 μm; Agilent). The oven was programmed from 50–310°C at 15°C/min after an initial delay of 2 min and held at 310°C for 10 min. Injector temperature was 280°C; MS quadrupole temperature was 150°C; MS source temperature was 230°C; and transfer line temperature was 300°C. CHCs were identified as described in Chapter 1. Heneicosane was identified by its EI mass spectrum, Kovats index on a DB-5 column, and co-injection with an authentic standard.

Royal Recognition Bioassay. Thirty workers were placed into a 5-cm petri dish with moist unwoven paper towels or filter paper. Two soldiers were added to each dish to discourage soldier differentiation during the experiment. To test the response of the workers and soldiers in the petri dish to different castes, we introduced a queen, king, soldier, or marked worker. This individual was the "focal termite" in the assay. All queens and kings used in recognition assays were neotenics. Queens, kings, and soldiers were easily tracked because of their distinct morphology, while introduced workers were dyed blue to facilitate tracking by feeding on substrate impregnated with 0.1% Nile Blue. Workers turned blue in one to two weeks of consuming dyed diet and appeared to show no decrease in activity or survivorship, as previously described (Su et al., 1991). The focal termite (dyed worker, soldier, queen or king) was introduced to each assay dish at least 24 h before the assay. Assays consisted of

removing the lid of the petri dish, wiping off any condensation to improve visibility, waiting at least 2 min to rest the termites, and observing the focal termite for 10 min. Measured parameters included shaking responses and antennations by resident workers that interacted with the focal termite. Shaking responses were defined as any oscillatory movements within ~3 mm of the focal termite; antennation was defined as placing both antennae on the focal termite. For shaking behaviors, all repetitive behaviors by an individual within range were recorded. For antennation, any continuous contact was counted as one session and termites had to move more than ~3 mm away from the focal termite before a second antennation session would be recorded.

Transfer of Cuticular Compounds to Glass Dummies. We designed an experiment to demonstrate the behavioral response of workers toward queen extracts applied to glass dummies. Following methods from Chapter 3, workers and neotenic queens were extracted in hexane (200 µL/individual) and extracts were applied to glass dummies. Final concentrations were 3 queen equivalents (QEs) per 20 µL and 6 worker equivalents (WEs) for worker controls, with pure hexane as a negative control. We used 6 WEs because this represents an equivalent total mass of hydrocarbons, as worker body mass and cuticular hydrocarbon mass (Chapter 2) are approximately half that of queens. The bioassays tested one dummy per petri dish from one colony (n = 5 dishes for queens, 10 dishes for controls). We melted Pasteur pipette tips into roughly the length and diameter of a termite queen to form the dummies. Then they were rinsed in hexane and dried before applying 20 µL of extract onto each in a glass petri dish (Chapter 3). Hexane was allowed to evaporate from treated dummies for 5 min before introduction into assay dishes. To allow the termites to settle after adding the

dummy, our observations began 2 min after its introduction. We measured antennation and shaking responses towards the dummies for 5 min. In these assays, dishes were observed once per treatment, but then observed again in worker and hexane controls, with a rest period of at least 24 h between assays. King extracts were not tested in these assays because we did not have sufficient numbers of kings.

Application of Heneicosane and Cuticular Compounds to Glass Dummies. We tested whether heneicosane applied to glass dummies could elicit shaking behavior and antennation. We included a tetracosane (*n*-C24) treatment as a control because it is present at similar relative amounts in the cuticular profiles of all castes (Chapter 2). Each of the two hydrocarbons (heneicosane and tetracosane) was tested alone and in combination with termite worker extracts. We hypothesized that worker extracts might enhance any behavioral response by providing a familiar termite or nestmate chemical context to termites in the assay. We tested worker extract alone and pure hexane as negative controls. Extracts were created as previously described and applied to dummies for the assay. For heneicosane and tetracosane, we used final concentrations of 0.1, 1, and 10 μg per dummy, and in treatments with worker extract we used a concentration of 2 WEs per dummy to emulate the mass of hydrocarbons typically found on a queen (Chapter 2).

The bioassays tested one dummy per petri dish with two colonies (n = 10 dishes) for each treatment. Dummies were coated with extracts as described above and observed for 5 min after a 2 min resting period. All dishes were observed once per treatment, but then observed again in all other treatments and controls, with a rest period of at least 24 h between assays.

RESULTS AND DISCUSSION

Comparison of Cuticular Extracts. The most prominent hydrocarbons across all termite extracts were normal and monomethyl alkanes ranging from 23 to 25 carbons; they made up > 40% of the total mass of CHCs (Chapter 2). Heneicosane (*n*-C21) appears to be a royal-specific compound and several long-chain mono- and dimethyl alkanes, although present in workers in small amounts, are much more represented in royals (Fig. 1). These compounds could be caste-specific metabolites, vital ingredients for the maintenance of royal castes, or possible reproductive pheromones. Because of its royal-specific status and its ease of use in the laboratory, we targeted heneicosane as a possible queen and king recognition compound.

Behaviors Elicited by Cuticular Extracts of Different Castes. We first performed bioassays on live workers, soldiers, and neotenic queens and kings to observe typical recognition behaviors. The most relevant observed behaviors indicating royal recognition were shaking, or lateral oscillatory movements, and antennation. Both behaviors were elevated in the presence of royal castes and effectively discriminated royal and non-royal termites when observing live individuals (Fig. 2). These results support a strong behavioral royal recognition response in *R. flavipes*.

Our further bioassays using hexane extracts from queens and workers applied to glass dummies also showed a significant difference between reproductive and non-reproductive extracts (Fig. 3), suggesting that cuticular compounds mediate the royal recognition response. CHCs are most likely responsible for at least some of the recognition response and,

combined with analytical chemistry identifying reproductive-specific CHCs, we hypothesized that heneicosane is a termite royal pheromone.

Heneicosane as a Royal-Recognition Pheromone. We tested the efficacy of heneicosane to elicit royal-recognition behavior. Tetracosane treatments controlled for a generalized response to high doses of straight chain alkanes. We also included glass dummies coated with nestmate worker extracts as well as each of the two hydrocarbons to control for any general nestmate recognition cues whose absence might induce aggression, policing or other alarm behaviors. Our results demonstrated consistent behaviors and significant elevations of both shaking and antennation in the presence of *n*-C21 (Fig. 4).

Shaking responses displayed a clear pattern of royal recognition. To begin, *n*-C21 applied alone to dummies stimulated a clear positive dose-response, with shaking events doubling between 0.1 μg and 10 μg (Fig. 4A). The 1 μg and 10 μg treatments of *n*-C21 alone were also significantly different from the control hexane extract (Dunnett's test, $p = 0.0187$ for 1 μg, $p = 0.0008$ for 10 μg). Tetracosane alone also elicited shaking responses, but there was no evidence of a dose-dependent response.

Stimulation with *n*-C21 in the presence of termite cuticular extracts significantly elevated the shaking and antennation responses compared to *n*-C21 alone ($p < 0.05$, Tukey's HSD). However, both shaking and antennation responses appeared to be at their maximum levels even at 0.1 μg, as evidenced by the lack of dose-response patterns. Nevertheless, the combination of worker extract with any of the three doses of *n*-C21 stimulated significantly greater shaking responses than the worker extract control (Dunnett's Test, $p = 0.0063$ for 0.1 μg, $p = 0.0351$ for 1 μg, and $p < 0.0001$ for 10 μg). Stimulation with *n*-C24 alone or in

combination with worker extract failed to stimulate greater shaking or antennation, except at a high dose, where we observed increased antennation responses. Our behavioral studies (Chapter 3) indicate that shaking responses are much more predictive than antennation responses of the presence of queens, kings or their cuticular extracts. Across all treatments, *n*-C21 within a termite chemical background elicited a significantly stronger shaking response than *n*-C24 or controls and closely approached the frequency of shaking responses seen in response to actual termite queens (Fig. 2) and queen extracts (Fig. 3).

Our findings strongly support the conclusion that heneicosane is a royal recognition pheromone in *R. flavipes*. Comparisons of the CHC profiles of four termite castes show clear differences between reproductive and non-reproductive castes, and heneicosane emerged as a reproductive-specific hydrocarbon. Pairing this finding with a discriminating bioassay that quantitatively related shaking behavior to the presence of a neotenic queen or king, we were able to directly test the activity of this CHC as a candidate recognition pheromone. We showed that heneicosane stimulates termites to antennate inert glass dummies and perform a shaking response as they normally do toward queens and kings. Further, termites responded to heneicosane in a dose-dependent manner and largely ignored a similar hydrocarbon that is not specific to royal cuticular profiles. Interestingly, the royal-recognition response to heneicosane was significantly elevated within the background of a termite cuticular extract, indicating that chemical context is important for effective communication of the recognition signal. It remains unknown however, whether the chemical context requires a colony-specific odor, or if more general species-level profiles might be as effective. Nevertheless, our demonstration that workers respond equally to native and foreign queens (Chapter 3) would

suggest that, in *R. flavipes*, *n*-C21 and related royal-specific pheromones might suppress the aggressive responses that are normally elicited by foreign colony CHCs.

Chemical context was previously reported as a major factor in queen recognition for the trap-jaw ant, *Odontomachus brunneus*, in which queen-specific compounds elicit submissive postures in workers, but only when a blend of familiar ant cuticular compounds is included (Smith et al., 2015). As in *R. flavipes*, the ant queen recognition signal is conserved across populations, but otherwise the ant and termite systems appear quite divergent. In *R. flavipes* heneicosane is unique to queens and kings and by itself can elicit royal recognition responses, whereas in the ant (*Z*)-9-nonacosene occurs in both workers and queens, is relatively more abundant in queens, and by itself does not stimulate any behaviors. Ants also appear to readily distinguish between this queen-enriched alkene in a native worker background vs. in a foreign cuticular chemistry background. Further exploration of the ability of unrelated termite extracts to elicit recognition behaviors could help understand how inter-colony or population level variation in cuticular profiles may affect royal recognition, colony fusion, and other competitive interactions in termites.

Our chemical analysis of *R. flavipes* demonstrated that heneicosane is conserved across multiple colonies and in both kings and queens (Chapter 2). It clearly encodes "royal-status", making it also the first king pheromone identified in any social insect. Although we have not thoroughly explored all differences in king and queen chemical profiles, the presence of a shared pheromone is nonetheless striking. In social insects in general, queen pheromones serve two related functions: as releasers of attendant and other behaviors by workers, and as primers that suppress reproductive activation in workers. In termites, it is conceivable that queens and kings would share releaser pheromones because they elicit

similar behavioral responses from workers and soldiers. Heneicosane appears to encode this shared royal-recognition function, and at least in theory, no other components are necessary to affect its releaser function. The primer function however, requires the differential suppression of developmental pathways for neotenic males and females, which would presumably require unique queen and king primer pheromones. Indeed, the only primer pheromone that has been reported in termites is a volatile blend of 2-methyl-1-butanol and n-butyl-n-butyrate in *Reticulitermes speratus* queens (Matsuura et al., 2010); the respective king pheromone has not been elucidated. Although the primer effects of heneicosane have not been evaluated, we suspect that it might function exclusively as a releaser because it is shared by both queens and kings. This speculation is also informed by one of the few other studies exploring termite king- and queen-specific CHCs, in which the compounds indicating reproductive status in *Z. nevadensis* were identical for males and females (Liebig et al., 2009). Although the pheromonal activity of these compounds was not tested, it suggests that queens and kings may share some compounds indicating royal status generally, while requiring other signals to differentiate the sexes and prevent unwanted reproduction. In eusocial hymenopterans, on the other hand, the presence of only a queen and not a king would enable the same signal to function as both releaser and primer pheromone, as evident across a range of bees, ants and wasps. Interestingly however, in the trap-jaw ant, (*Z*)-9-nonacosene elicits queen recognition but fails to suppress worker reproduction (Smith et al., 2015).

Heneicosane is the first royal-recognition (releaser) pheromone identified in termites. Certain hydrocarbons have been shown to be specific to or enriched in functional reproductives in other termites (Darrouzet et al., 2014; Liebig et al., 2009; Weil et al., 2009),

but their link to behavior or primer effects remain elusive. Cuticular extracts of the termite *C. secundus* include three worker-specific CHC peaks and 21 queen-specific peaks (Weil et al., 2009). Down-regulation of *Neofem4*, a cytochrome P450 gene involved in hydrocarbon biosynthesis, not only resulted in quantitative changes in queen CHCs, but also interfered with queen recognition (Hoffmann et al., 2014). However, the specific CHCs responsible for queen-recognition have not been identified in this species.

The use of cuticular hydrocarbons as a conserved class of pheromones inhibiting ovary development is widespread in social hymenopterans (Amsalem et al., 2015; Holman et al., 2017; Van Oystaeyen et al., 2014). Yet, in recent years this has become a rather contentious focus of debate, leading some to doubt the status of CHCs as queen pheromones and optimize methods to accurately test whether queen-related compounds truly inhibit reproduction (see Amsalem et al. (2015), countered by Holman et al. (2017). Sociality in insects has evolved independently multiple times. The eusocial hymenopterans (bees, ants and wasps) evolved ~100 million years ago, and the conserved use of CHCs in nestmate recognition and as queen pheromones presumably evolved around this time as well. Our finding of a CHC as a royal recognition pheromone in termites not only contributes evidence to the discussion that CHCs are a conserved class of social recognition pheromones, but also pushes their emergence in eusocial communication to ~150 million years ago, when eusocial termites evolved from within the cockroaches. CHCs have evolved multiple functions, primary among them is to prevent water loss and pathogen attack (Blomquist and Bagneres, 2010). The shared use of CHCs as recognition and fertility signals in eusocial insects appears to represent a striking example of the convergent co-option of specific CHCs to encode communication signals. Solitary insects co-opted CHCs well before social insects, by using

CHCs and their derivatives as sex pheromones and in mimicry systems. Eusocial insects presumably re-directed these signals for royal recognition and to suppress reproduction in workers.

Future work may be able to differentiate king and queen recognition, perhaps by discovering sex-specific cuticular pheromones or volatile components. Whether heneicosane has a primer effect on reproductive differentiation is an important area of investigation. The olfactory and gustatory receptors that detect the heneicosane signal, the transduction mechanisms, and central nervous integration with other CHCs requires further study. The function of the shaking response also merits further exploration. It is widely seen throughout the colony and across most termite lineages (Ohmura et al., 2009; Whitman and Forschler, 2007). It is plausible that the shaking response is nuanced and different shaking responses are elicited by different stimuli. Doppler vibrometry and related technologies may help distinguish subtle differences in the function of the shaking response.

REFERENCES

Amsalem E, Orlova M, Grozinger CM (2015) A conserved class of queen pheromones? Re-evaluating the evidence in bumblebees (*Bombus impatiens*). Proc R Soc B 282:20151800. doi: 10.1098/rspb.2015.1800

Bagneres A-G, Killian A, Clement J-L, Lange C (1991) Interspecific recognition among termites of the genus *Reticulitermes*: Evidence for a role for the cuticular hydrocarbons. J Chem Ecol 17:2397–2420.

Blomquist GJ, Bagneres AG (2010) Insect Hydrocarbons: Biology, Biochemistry, and Chemical Ecology. Cambridge Univ Press, Cambridge

Darrouzet E, Labédan M, Landré X, et al (2014) Endocrine control of cuticular hydrocarbon profiles during worker-to-soldier differentiation in the termite *Reticulitermes flavipes*. J Insect Physiol 61:25–33. doi: 10.1016/j.jinsphys.2013.12.006

Dietemann V, Peeters C, Liebig J, et al (2003) Cuticular hydrocarbons mediate discrimination of reproductives and nonreproductives in the ant *Myrmecia gulosa*. Proc Natl Acad Sci U S A 100:10341–10346.

Evans TA, Inta R, Lai JCS, et al (2009) Termites eavesdrop to avoid competitors. Proc R Soc B Biol Sci 276:4035–4041. doi: 10.1098/rspb.2009.1147

Evans TA, Lai JC, Toledano E, et al (2005) Termites assess wood size by using vibration signals. Proc Natl Acad Sci U S A 102:3732–3737.

Hager FA, Kirchner WH (2013) Vibrational long-distance communication in the termites *Macrotermes natalensis* and *Odontotermes sp*. J Exp Biol 216:3249–3256. doi: 10.1242/jeb.086991

Hanus R, Vrkoslav V, Hrdy I, et al (2010) Beyond cuticular hydrocarbons: evidence of proteinaceous secretion specific to termite kings and queens. Proc R Soc B Biol Sci 277:995–1002. doi: 10.1098/rspb.2009.1857

Himuro C, Yokoi T, Matsuura K (2011) Queen-specific volatile in a higher termite *Nasutitermes takasagoensis* (Isoptera: Termitidae). J Insect Physiol 57:962–965. doi: 10.1016/j.jinsphys.2011.04.012

Hoffmann K, Gowin J, Hartfelder K, Korb J (2014) The scent of royalty: a P450 gene signals reproductive status in a social insect. Mol Biol Evol 31:2689–2696. doi: 10.1093/molbev/msu214

Holman L, Hanley B, Millar JG (2016) Highly specific responses to queen pheromone in three *Lasius* ant species. Behav Ecol Sociobiol 70:387–392. doi: 10.1007/s00265-016-2058-6

Holman L, Jorgensen CG, Nielsen J, d'Ettorre P (2010) Identification of an ant queen pheromone regulating worker sterility. Proc R Soc B Biol Sci 277:3793–3800. doi: 10.1098/rspb.2010.0984

Holman L, van Zweden JS, Oliveira RC, et al (2017) Conserved queen pheromones in bumblebees: a reply to Amsalem et al. PeerJ. doi: 10.7717/peerj.3332

Howse PE (1965) On the significance of certain oscillatory movements of termites. Insectes Sociaux 12:335–345. doi: 10.1007/BF02222723

Howse PE (1964) The significance of the sound produced by the termite *Zootermopsis angusticollis* (Hagen). Anim Behav 12:284-IN8. doi: 10.1016/0003-3472(64)90015-6

Keeling CI, Slessor KN, Higo HA, Winston ML (2003) New components of the honey bee (Apis mellifera L.) queen retinue pheromone. Proc Natl Acad Sci 100:4486–4491. doi: 10.1073/pnas.0836984100

Kirchner WH, Broecker I, Tautz J (1994) Vibrational alarm communication in the damp-wood termite *Zootermopsis nevadensis*. Physiol Entomol 19:187–190. doi: 10.1111/j.1365-3032.1994.tb01041.x

Kocher SD, Grozinger CM (2011) Cooperation, Conflict, and the Evolution of Queen Pheromones. J Chem Ecol 37:1263–1275. doi: 10.1007/s10886-011-0036-z

Korb J (2005) Regulation of sexual development in the basal termite *Cryptotermes secundus*: mutilation, pheromonal manipulation or honest signal? Naturwissenschaften 92:45–49. doi: 10.1007/s00114-004-0589-6

Korb J, Weil T, Hoffmann K, et al (2009) A Gene Necessary for Reproductive Suppression in Termites. Science 324:758–758. doi: 10.1126/science.1170660

Liebig J, Eliyahu D, Brent CS (2009) Cuticular hydrocarbon profiles indicate reproductive status in the termite *Zootermopsis nevadensis*. Behav Ecol Sociobiol 63:1799–1807. doi: 10.1007/s00265-009-0807-5

Matsuura K, Himuro C, Yokoi T, et al (2010) Identification of a pheromone regulating caste differentiation in termites. Proc Natl Acad Sci 107:12963–12968. doi: 10.1073/pnas.1004675107

Oberst S, Bann G, Lai JCS, Evans TA (2017) Cryptic termites avoid predatory ants by eavesdropping on vibrational cues from their footsteps. Ecol Lett 20:212–221. doi: 10.1111/ele.12727

Ohmura W, Takanashi T, Suzuki Y (2009) Behavioral analysis of tremulation and tapping of termites (Isoptera). Sociobiology 54:269–274.

Penick CA, Liebig J (2017) A larval “princess pheromone” identifies future ant queens based on their juvenile hormone content. Anim Behav 128:33–40. doi: 10.1016/j.anbehav.2017.03.029

Penick CA, Trobaugh B, Brent CS, Liebig J (2013) Head-butting as an early indicator of reproductive disinhibition in the termite *Zootermopsis nevadensis*. J Insect Behav 26:23–34. doi: 10.1007/s10905-012-9332-x

Polidori C, Giordani I, Wurdack M, et al (2017) Post-mating shift towards longer-chain cuticular hydrocarbons drastically reduces female attractiveness to males in a digger wasp. J Insect Physiol 100:119–127. doi: 10.1016/j.jinsphys.2017.05.001

Richard F-J, Hunt JH (2013) Intracolony chemical communication in social insects. Insectes Sociaux 60:275–291. doi: 10.1007/s00040-013-0306-6

Slessor KN, Kaminski L-A, King GGS, et al (1988) Semiochemical basis of the retinue response to queen honey bees. Nature 332:354–356. doi: 10.1038/332354a0

Smith AA, Millar JG, Hanks LM, Suarez AV (2013) A conserved fertility signal despite population variation in the cuticular chemical profile of the trap-jaw ant *Odontomachus brunneus*. J Exp Biol 216:3917–3924. doi: 10.1242/jeb.089482

Smith AA, Millar JG, Suarez AV (2015) A social insect fertility signal is dependent on chemical context. Biol Lett 11:20140947. doi: 10.1098/rsbl.2014.0947

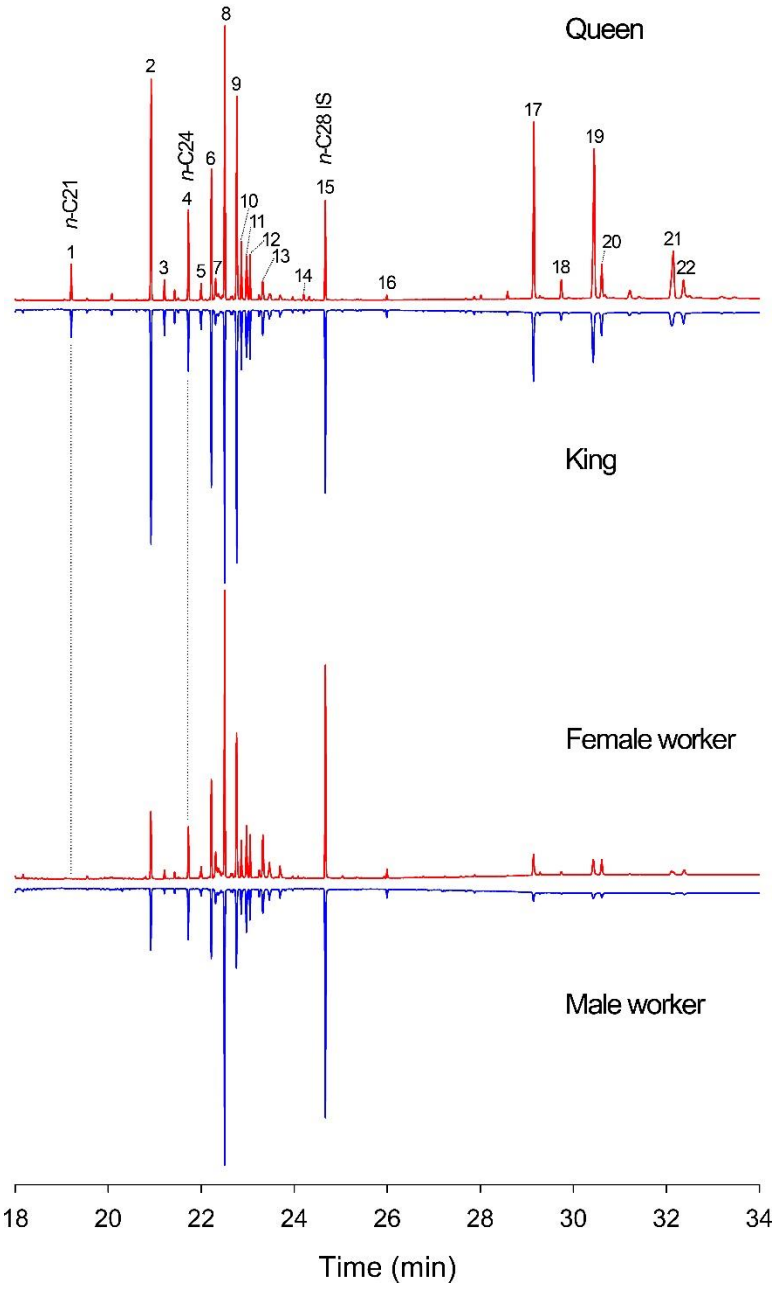
Su N, Ban P, Scheffrahn R (1991) Evaluation of 12 dye markers for population studies of the eastern and formosan subterranean termite (Isoptera, Rhinotermitidae). Sociobiology 19:349–362.

Van Oystaeyen A, Oliveira RC, Holman L, et al (2014) Conserved class of queen pheromones stops social insect workers from reproducing. Science 343:287–290. doi: 10.1126/science.1244899

Weil T, Hoffmann K, Kroiss J, et al (2009) Scent of a queen—cuticular hydrocarbons specific for female reproductives in lower termites. Naturwissenschaften 96:315–319. doi: 10.1007/s00114-008-0475-8

Whitman JG, Forschler BT (2007) Observational notes on short-lived and infrequent behaviors displayed by *Reticulitermes flavipes* (Isoptera: Rhinotermitidae). Ann Entomol Soc Am 100:763–771. doi: 10.1603/0013-8746(2007)100[763:ONOSAI]2.0.CO;2

Figure 1. Gas chromatograms of reproductive and worker castes in *R. flavipes*. Females are marked in red and males are marked in blue. The reproductive-specific compound heneicosane (*n*-C21), its experimental control (tetracosane, *n*-C24), and the internal standard octacosane (*n*-C28) are marked. Identified peaks are listed: *1* heneicosane; *2* tricosane; *3* 11-methyltricosane; *4* tetracosane; *5* 12- and 11-methyltetracosane; *6* 2-methyltetracosane; *7* 3-methyltetracosane and x-pentacosene; *8* pentacosane; *9* 13- and 11-methylpentacosane; *10* x,y-pentacosadiene; *11* 2-methylpentacosane (and x,y-pentacosadiene); *12* 3-methylpentacosane; *13* x,y,z-pentacosatriene; *14* heptacosane; *15* octacosane (added as internal standard); *16* hentriacontane; *17* 13- and 11-methylpentatriacontane; *18* 13-, 12-, and 11-methylhexatriacontane; *19* 13- and 11-methylheptatriacontane; *20* 11,15-dimethylheptatriacontane; *21* 15-, 13-, and 11-methylnonatriacontane; *22* 11,15-dimethylnonatriacontane.


Queen
King
Female worker
Male worker
n-C21
n-C24
n-C28 IS
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
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22
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32
34
Time (min)

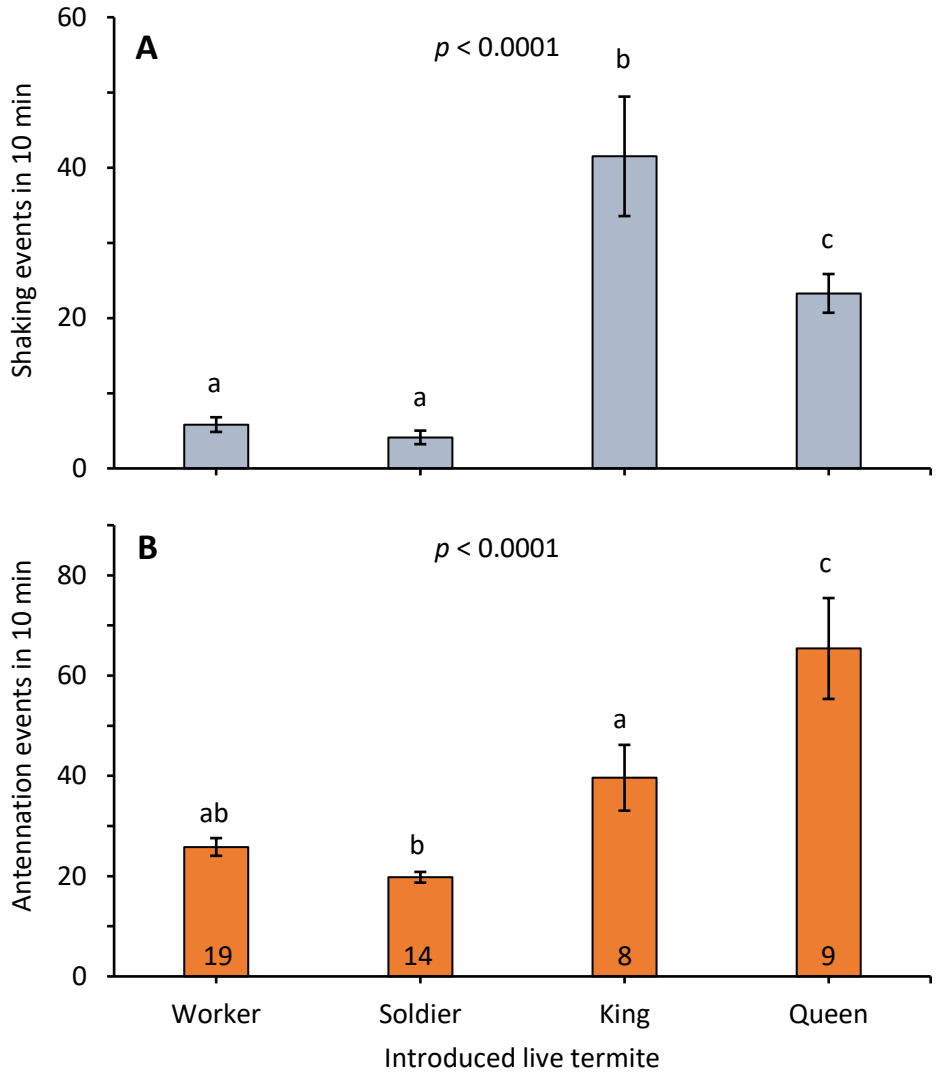


Figure 2. Behaviors exhibited by groups of 100 termites toward a worker, soldier, neotenic king, and neotenic queen, measured by lateral shaking (**A**) and antennation (**B**) during a 10 min observation period. Queens and kings in all assays were neotenic (secondary) reproductives generated within the lab. Each assay consisted of 30 workers, two soldiers, and an introduced live focal termite. Number of replicate assays is indicated within each bar for each caste. Letters indicate significantly different values using one way ANOVA ($p < 0.05$) and Tukey's HSD. Error bars represent standard error of the mean.

Figure 3. Termite responses to glass dummies treated with pure hexane and hexane extracts of workers and neotenic queens. Lateral shaking (**A**) and antennation (**B**) were measured during 5 min assays for each treatment. Glass dummies were coated with 20 μL of each solution. Worker extracts were created by pooling six workers with mass approximately equal to three neotenic queens to approximate the queen concentration. Each assay dish consisted of 30 workers, two soldiers, and an introduced glass dummy. Letters indicate significantly different values using one way ANOVA and Tukey's HSD. Error bars are standard error of the mean. For all treatments number of replicate assays is indicated within each bar.

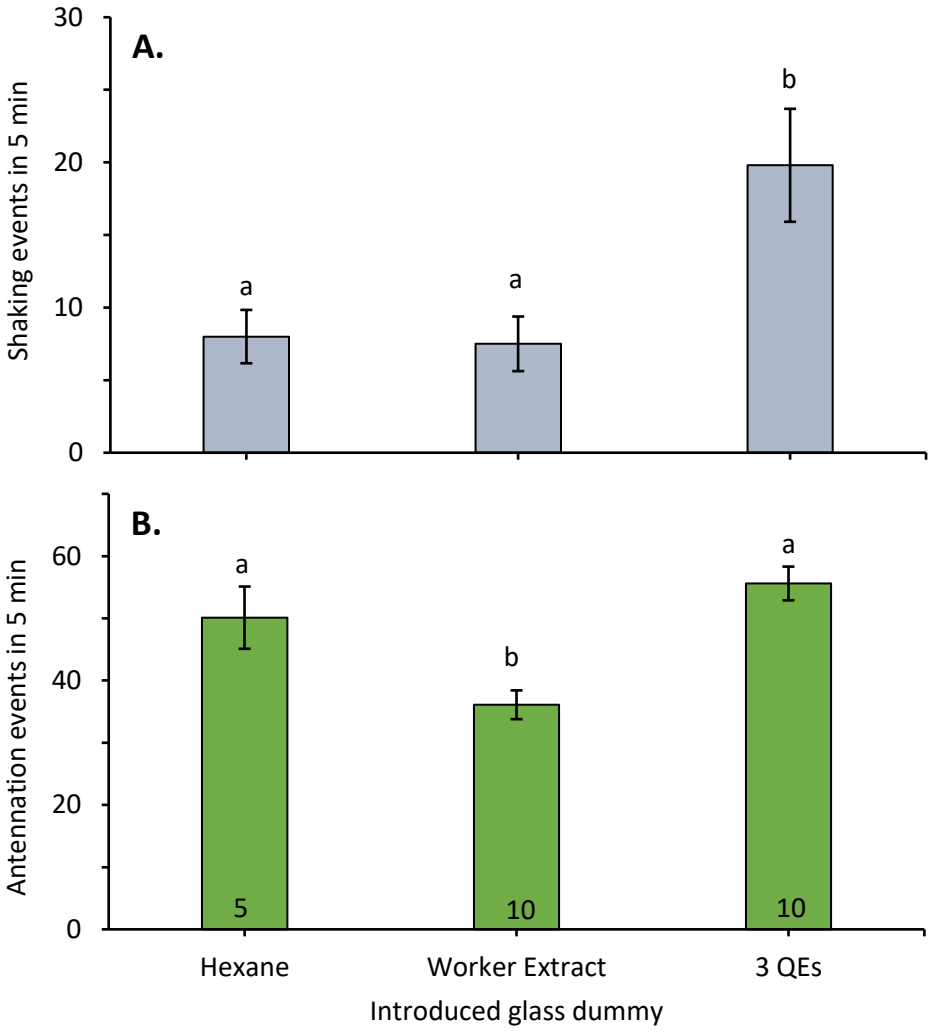
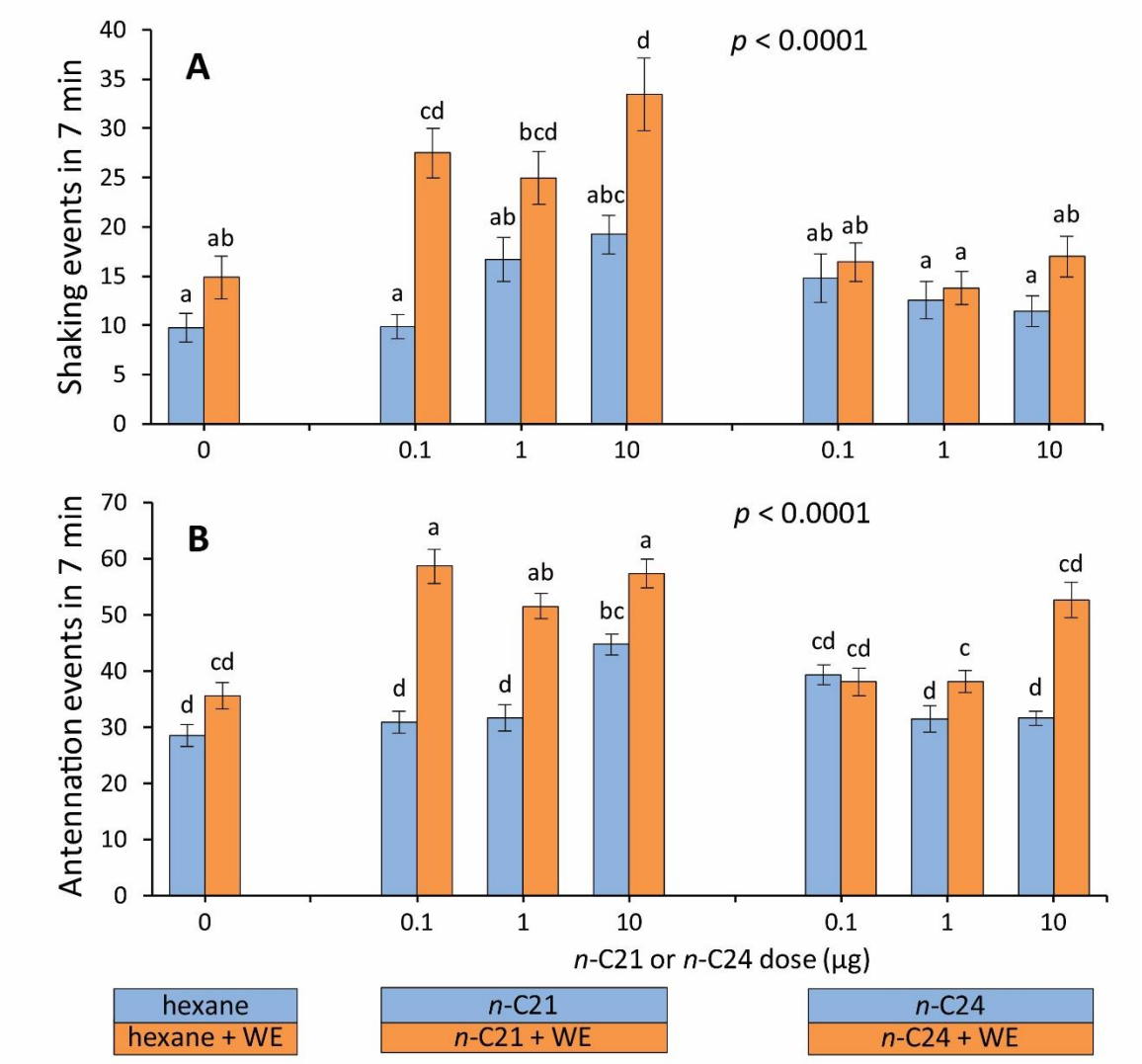

A.
Shaking events in 5 min
30
20
10
0
a
a
b
B.
Antennation events in 5 min
60
40
20
0
a
b
a
5
10
10
Hexane
Worker Extract
3 QEs
Introduced glass dummy

Figure 4. Termite responses to glass dummies treated with heneicosane (*n*-C21) or tetracosane (*n*-C24) alone (blue bars) and with workers extracts (orange bars). Lateral shaking (**A**) and antennation (**B**) were measured during 5 min assays for each treatment. Glass dummies were coated with 20 μL of each solution. Worker extracts were created by pooling six workers with mass approximately equal to three neotenic queens to approximate the queen concentration. Each assay dish consisted of 30 workers, two soldiers, and an introduced glass dummy. Letters indicate significantly different values using one way ANOVA and Tukey's HSD. Error bars are standard error of the mean. For all treatments number of replicate assays is indicated within each bar.


A
p < 0.0001
Shaking events in 7 min
40
35
30
25
20
15
10
5
0
a
ab
a
cd
ab
bcd
abc
d
ab
ab
a
a
a
ab
0
0.1
1
10
0.1
1
10
B
p < 0.0001
Antennation events in 7 min
70
60
50
40
30
20
10
0
d
cd
d
a
d
ab
bc
a
cd
cd
d
c
d
cd
0
0.1
1
10
0.1
1
10
n-C21 or n-C24 dose (µg)
hexane
hexane + WE
n-C21
n-C21 + WE
n-C24
n-C24 + WE