The Molecular Basis for Nestmate Recognition in the Eusocial Ant *Camponotus floridanus*

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Abstract: In eusocial ants, aggressive behaviors require a sophisticated ability to detect, discriminate, and display robust responses to pheromonal and other chemical signatures that distinguish nestmate friends from non-nestmate foes. While the chemosensory requirements of maintaining a colonial lifestyle are likely to have, at least in part, driven the expansion of odorant receptor genes across hymenopteran genomes, the contextual nature of these pheromonal signals as well as the chemosensory receptors that detect them to regulate nestmate recognition and other behaviors remains largely unknown. To address this, we have developed an aggression-based bioassay incorporating a suite of highly selective odorant receptor modulators to characterize the role of olfaction in nestmate recognition in the formicine ant *Camponotus floridanus*. Our studies provide direct evidence that the recognition of non-nestmates and the subsequent triggering of aggressive responses towards them is an active process dependent on odorant-receptor based detection of precise and unambiguous chemical signatures.

Significance Statement: Despite a longstanding interest in the chemical ecology, evolution, and molecular neuroethology of nestmate recognition in eusocial ants, the mechanistic basis for this process and the specific role of chemosensory receptors in mediating these responses remains largely unknown. To address these questions, we now report studies using an ant nestmate/non-nestmate recognition bioassay incorporating a highly selective suite of odorant receptor (OR) modulators. Our data indicates that acute ablation of olfactory appendages along with specific inhibition or activation of OR signaling significantly decreases aggression between non-nestmate ants. Our results are consistent with a model of nestmate recognition in which triggering of aggression towards foes is dependent upon the detection of precise, unambiguous non-nestmate signatures that specifically requires OR-based signaling.

Main Text:

Introduction

Aggression comprises a range of biologically salient social interactions with implications for individual behavior as well as the collective integrity of animal societies. While aggressive and/or hostile behaviors can be observed throughout the Metazoa (Ayre & Grosberg, 1995; Blanchard & Blanchard, 1977; Hölldobler & Wilson, 1990; Mitani, Watts, & Amsler, 2010; Scheel, Godfrey-Smith, & Lawrence, 2016), recently established experimentally tractable eusocial insect models present an opportunity to investigate the mechanistic basis of aggression within a social context. In this regard, ants provide a compelling model for the study of aggression and its triggering mechanisms within a social context. Ant colonial lifestyles and reproductive hierarchies are maintained by archetypal aggressive social interactions that are modulated by their ability to detect, discriminate, and respond to a large array of chemical cues often known as pheromones (Endler et al., 2004; Hölldobler & Wilson, 1990; Moore & Liebig, 2010; Morel, Vandermeer, & Lavine, 1988). Moreover, recent studies (Trible et al., 2017; Yan et al., 2017) have demonstrated the value of applying novel genetic and molecular techniques that have restricted availability/utility in the study of humans and other social primates.

The formicine ant *Camponotus floridanus* live in colonies that are founded by a single reproductive queen that produces at least two morphologically distinct sterile worker groups: smaller minor workers that comprise the majority of ants within a colony and larger major workers (Gadau, Heinze, Holldobler, & Schmid, 1996; Hölldobler & Wilson, 1990). Workers nurse the queen's offspring, forage for food, and defend nest and territory from non-nestmates (nNMs)—tasks that are necessary for colony cohesion and survival (Hölldobler & Wilson, 1990). Although individual workers contribute to broader colony-level phenotypes, the integrity

of social behaviors depends on the collective actions of the colony (Gordon, 2015). Among these social behaviors, nestmate (NM) recognition—which refers to the process whereby colonies rigorously discriminate between NMs and nNMs, the latter of which are often met with highly aggressive responses—is especially important for establishing and maintaining discrete societal boundaries for *C. floridanus* and many other species of ant (Hölldobler & Wilson, 1990).

NM recognition is a dynamic behavior that has been postulated to occur when an individual ant compares chemically encoded "labels" that it encounters with potentially multiple neural-encoded "templates" that represent its own particular global colony chemosensory signature (Neupert, Hornung, Grenwille Millar, & Kleineidam, 2018; Obin & Vandermeer, 1989; R. Vander Meer & Morel, 1998). Subtle variations in the profile of cuticular hydrocarbons (CHCs) distinguish nNMs from NMs (Guerrieri et al., 2009; Morel et al., 1988; Neupert et al., 2018). Early genetic models provided a framework for understanding the criteria required to assess colony membership status when comparing the recognition template to a respective label (Crozier & Dix, 1979). These have been broadly organized into two categories: the gestalt model, in which label sharing between individuals yields a distinct template based on a blend; and individualistic models, which include requiring the exact matching of the label to the template ("genotype matching"), rejection of any labels containing cues not found in the template ("foreign-label rejection"), and the acceptance of labels that overlap with the template ("habituated-label acceptance"). Similarly, there have been efforts to elucidate the rules governing label-template matching within a phenotypic context (Guerrieri et al., 2009; Neupert et al., 2018; Sherman et al., 1997). These models suggest that ants discriminate between friends and foes based on the presence and/or absence of NM ("desirable") cues or nNM ("undesirable") cues. While it was initially proposed that ants accept individuals if they possess desirable cues

(D-present) or if they lack undesirable cues (U-absent) to the exclusion of all others (Sherman et al., 1997), more recent evidence suggests that ants actively detect foes but not friends through the detection of nNM odor cues (simple U-present model) (Guerrieri et al., 2009). Importantly however, discrimination may also occur when critical components of the CHC profile are missing (Neupert et al., 2018). These studies suggest that there are multiple templates being used to assess different labels, and that there is variability in the importance of a given component of the label, whether in absence or in abundance, when determining nNM or NM status.

While the importance of olfactory responses to CHCs in mediating NM recognition among ants is well established, several alternative hypotheses have been proposed for the neuronal and molecular mechanisms required for ants to distinguish friends (NMs) from foes (nNMs) (A. Brandstaetter, Rössler, & Kleineidam, 2011; A. S. Brandstaetter & Kleineidam, 2011; Crozier & Dix, 1979; Guerrieri et al., 2009; Neupert et al., 2018; Ozaki et al., 2005; Sherman, Reeve, & Pfennig, 1997). In all of these models, CHCs and other semiochemicals are detected initially by the peripheral olfactory sensory system which, in *C. floridanus* and indeed other insects, relies on three major classes of peripheral chemosensory receptors—odorant receptors (ORs), gustatory receptors (GRs) and ionotropic receptors (IRs). In previous studies, we have revealed a large expansion of the *OR* gene family in ants as well as other eusocial insects (Zhou et al., 2015; Zhou et al., 2012), leading to the suggestion that this class of chemoreceptors is largely responsible for the detection of many socially relevant chemical cues, including CHCs and general odorants (Pask et al., 2017; Slone et al., 2017). Insect ORs are expressed in olfactory receptor neurons (ORNs) housed within sensilla on the antennae (reviewed in (Suh, Bohbot, & Zwiebel, 2014)), where they function as heteromeric complexes consisting of an obligate and conserved OR co-receptor (Orco) and at least one “tuning” OR that

100 determines odorant (ligand) specificity (Benton, Sachse, Michnick, & Vosshall, 2006; P. L.
101 Jones, Pask, Rinker, & Zwiebel, 2011; Larsson et al., 2004; Pask, Jones, Rutzler, Rinker, &
102 Zwiebel, 2011; Sato, Pellegrino, Nakagawa, Vosshall, & Touhara, 2008; Wicher et al., 2008;
103 Zhou et al., 2012).

104 Despite the long-held appreciation for the role of CHCs and other chemical cues in
105 mediating NM recognition and social behaviors in ants, little is known about the specific
106 molecular components of olfactory signal transduction that are active in regulating NM
107 recognition and the triggering of aggression toward nNMs as well as other social behaviors.
108 Electrophysiological studies of *Camponotus japonicus* first suggested that a dedicated
109 multiporous NM recognition sensilla exhibited an all-or-none response to nNM CHC blends but,
110 importantly, did not respond to NM CHC blends—thus leading to a model in which ants are
111 desensitized and ultimately anosmic to their own odor cues (Ozaki et al., 2005). In contrast,
112 recent studies using both antennal electrophysiology and antennal lobe calcium imaging in the
113 related ant species *C. floridanus* demonstrate these ants are capable of detecting both nNM and
114 NM odors (A. Brandstaetter et al., 2011; A. S. Brandstaetter & Kleineidam, 2011; Sharma et al.,
115 2015). It has been proposed these seemingly contradictory findings support a model in which
116 two sensilla subtypes—one broadly tuned to hydrocarbons and the other tuned to specific
117 hydrocarbons—facilitate coarse habituation to different labels (Bos & d'Ettorre, 2012).

118 The paucity of data in this regard may be attributed, at least in part, to the challenges of
119 molecular targeting approaches currently available in the study of Hymenopteran insects. The
120 development of these techniques represents an important step towards understanding the function
121 and evolution of the molecular mechanisms involved in complex social behaviors such as
122 NM/nNM recognition with the potential to shed light on longstanding questions within the field

of social insect biology. To begin to address this, we recently carried out a series of behavioral, physiological, and gene knockout studies to characterize the relationship between ant ORs and CHCs as well as other biologically salient chemical cues. These studies demonstrated that CHCs and other general odorants were broadly detected across the various OR subclades while CRISPR-mediated gene knockout of *orco* resulted in alterations in both solitary and social behaviors as well as profound neuroanatomical disruptions in the antennal lobe (Pask et al., 2017; Slone et al., 2017; Yan et al., 2017). Taken together, these studies suggest that ORs play a critical role not only in a diversity of behaviors but also importantly in ant neural development.

Here, we report studies that specifically address the mechanistic basis for NM recognition by utilizing a suite of highly selective Orco agonists and antagonists to acutely and globally impact OR-based pathways in the context of a novel NM/nNM aggression bioassay. In this manner, we are able to directly examine NM recognition to test the hypotheses that aggression is triggered by the active detection and decoding of discrete chemosensory stimuli that are dependent upon olfaction and more specifically the functionality of the OR-Orco ion channel complex is necessary for nNM recognition.

Results

Nestmate Recognition Requires Antennal-based Signaling

Initially, we took a broad approach to assess the requirement for *C. floridanus* antennae (as the principal location of olfactory signaling) to modulate NM/nNM aggression in trials conducted using adult minor worker ants with either unilateral or bilateral antennal ablations. To this end, we have developed an aggression-based NM recognition bioassay in which two ants—NMs from the same home colony or nNMs from two different field collected colonies—were able to interact with one another after an acclimation period (Fig. 1A). In these studies, both

control *C. floridanus* workers as well as those having undergone unilateral ablations were able to routinely discriminate nNMs from NMs and display only nNM aggression. In contrast, ants with bilateral antennal ablations displayed a significant and indeed near-complete reduction in aggression against nNMs (Fig. 1B). These data are consistent with the widely reported ability of *C. floridanus* workers to robustly discriminate between nNMs and NMs and supports the hypothesis that their chemosensory apparatus is required to recognize and trigger aggression against nNMs (A. Brandstaetter et al., 2011; Guerrieri et al., 2009; Hölldobler & Wilson, 1990; Leonhardt, Brandstaetter, & Kleineidam, 2007; Morel et al., 1988; Neupert et al., 2018; Ozaki et al., 2005; Pask et al., 2017; Slone et al., 2017).

To further control for potentially confounding variables—including the outright death or incapacitation of the ants due to the damage sustained from the ablations—we measured a number of other behavioral indicators including total distance traveled, percentage of time spent moving/not moving, and the frequency of rotations using an automated tracking program (see Materials and Methods). Here, the activity of a single ant was recorded for three minutes immediately following the 10-minute acclimation period and preceding the ablation aggression bioassays. These assays revealed no significant difference between the sham control and either of the ablation treatments (Fig. 1C-E). That treated ants were able to recover from the injury and retain fundamental aspects of mobility coupled with the observation that unilaterally ablated workers maintained the ability to discriminate between NMs and nNMs suggests that the decrease in aggression was likely due to the absence of antennae-mediated signaling as opposed to confounding variables introduced by the ablation treatment. However, as the removal of the antennae disrupts a broad range of both mechanoreceptors as well as chemoreceptors (Nakanishi,

Nishino, Watanabe, Yokohari, & Nishikawa, 2009), a more targeted approach is required to assess the specific function of OR-dependent chemoreceptor signaling in this context.

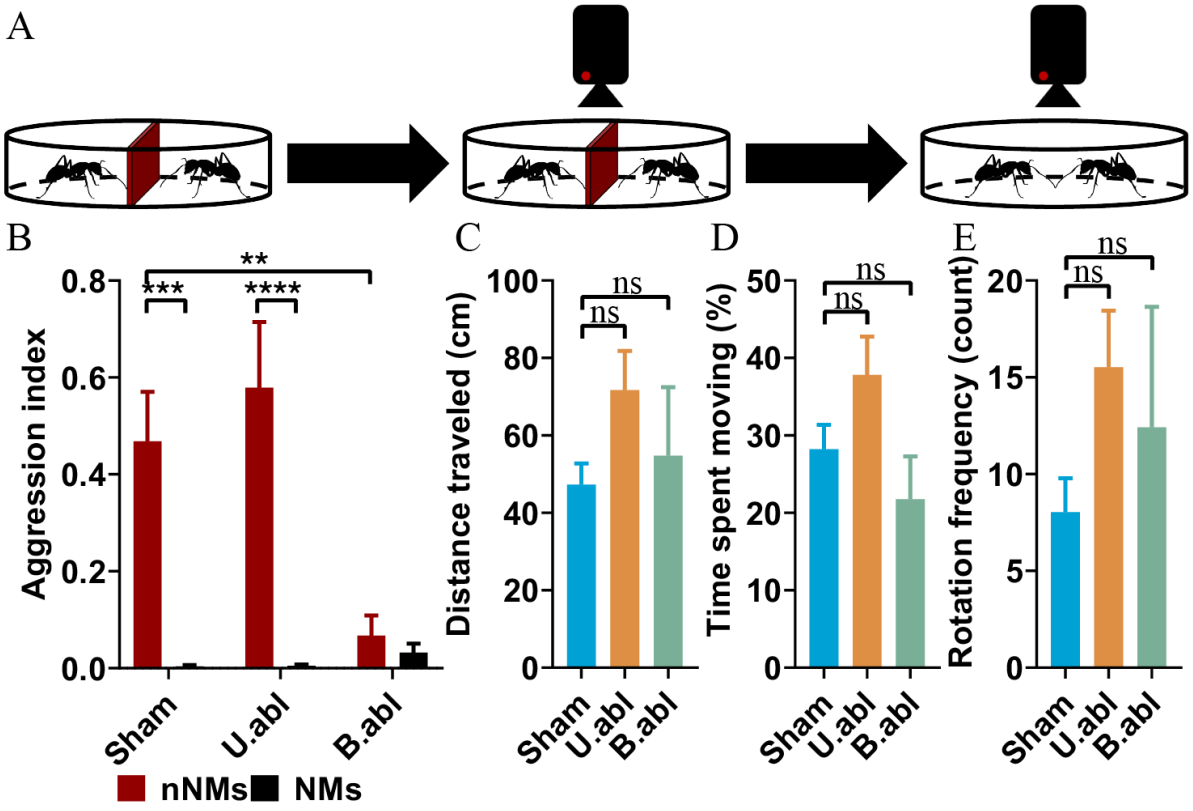

Fig. 1. Aggression and mobility responses of adult minor workers following antennal ablation (Sham = control; U.abl = unilateral ablation; B.abl = bilateral ablation). (**A**) Schematic of the ablation bioassay depicting the acclimation period (left), mobility controls (center), and aggression bioassay (right). (**B**) Bilateral antennal ablations significantly reduce nNM aggression compared to the sham control (Two-Way ANOVA, N=6-10). (**C-E**) There is no significant difference across the mobility parameters tested between the sham control and either of the ablation treatments (Kruskal-Wallis Test, N=24-29). Error bars display S.E.M. Asterisks indicate P-value: **<0.01, ***<0.001, ****<0.0001.

Nestmate Recognition is an Active, OR-dependent Process

In order to further examine this process within the narrow context of assessing the role of ORs in nNM recognition and aggression, we adapted our bioassay to incorporate the acute volatile administration of a suite of highly specific Orco allosteric modulators (Fig. 2A). The first member of this unique class of pharmacological agents (VUAA1) was initially identified through high-throughput screening for small molecule activators of Orco/OR complexes expressed in

HEK293 cells (P. L. Jones et al., 2011; Pask et al., 2011; Rinker et al., 2012). In subsequent studies that revealed extraordinarily narrow structure-activity relationships, several additional members of this class of actives were identified and characterized that now comprise several more potent agonists (including VUAA4 used here), a non-competitive antagonist (VUANT1, used here) as well as an inactive structural analog (VUAA0, used here) (P. L. Jones et al., 2011; P. L. Jones et al., 2012; Rinker et al., 2012; Romaine et al., 2014; Taylor et al., 2012). Subsequent studies, including single-sensillum recordings of female-specific basiconic sensilla in *C. floridanus*, have demonstrated that the potency of these modulators in both volatile and non-volatile form is conserved across a wide range of insect orders (Hansen et al., 2014; P. L. Jones et al., 2012; Sharma et al., 2015; Tsitoura & Iatrou, 2016; Tsitoura, Koussis, & Iatrou, 2015). Indeed, VUAA-Orco interactions have recently been directly confirmed by cryo-electron microscopy studies characterizing the structure of an Orco tetramer from the parasitic fig wasp *Apocrypta bakeri* (Butterwick et al., 2018).

The use of these unique and highly specific chemical tools allows us to selectively pharmacologically target Orco and therefore the functionality of all OR/Orco complexes without impacting other chemosensory signaling pathways to examine NM recognition with altered OR signaling in otherwise wild-type adult *C. floridanus* workers. This is an essential aspect of our approach in light of the broad neuroanatomical alterations that have recently been observed in the development of the antennal lobes of Orco mutants in two ant species (Trible et al., 2017; Yan et al., 2017) which are reasonably likely to impact olfactory processing. Indeed, the use of volatile Orco modulators represent a novel and requisite approach for disrupting OR functionality in insects such as ants that require alternatives to CRISPR-mediated targeting of pleiotrophic genes such as *orco* (Trible et al., 2017; Yan et al., 2017). Due to the widespread and

obligate colocalization of Orco together with tuning ORs in every insect ORN (W. D. Jones, Nguyen, Kloss, Lee, & Vosshall, 2005; Larsson et al., 2004; Taylor et al., 2012) exposure to Orco modulators is expected to have profound and widespread effects. In the case of the VUAA4 Orco agonist, hyper-activation of all Orco/OR complexes is expected to generate an uninterpretable or "confused" signal while treatment with the VUANT1 antagonist is expected to silence those complexes and thereby not generate an interpretable signal (Butterwick et al., 2018; Hansen et al., 2014).

Indeed, ants taken from across nine independent colonies exposed to either Orco modulator displayed a significant reduction, and indeed a near complete elimination, of aggression towards nNMs (Fig. 2B). Importantly, in addition to the inability to aggressively respond to nNMs, ants treated with either the Orco agonist or the antagonist displayed no alteration in their non-aggressive responses to NMs. This lack of misdirected aggression toward NMs as well as the failure to correctly attack nNMs in ants treated with these highly selective Orco/OR modulators demonstrates that, in *C. floridanus*, aggression is specifically mediated by the OR-dependent detection of specific and unambiguous odor cue signatures from nNM foes rather than the general absence or incorrect processing of familiar signatures of NM friends. Furthermore, in order to assess whether the VUAA-mediated disruption of OR-signaling reduces aggression within the narrow social context of NM/nNM recognition or alternatively acts to broadly inhibit aggressive behaviors, we conducted parallel bioassays that utilized mechanical rather than chemical stimuli to evoke aggression. Here, using a modified aggression bioassay based on previous methods described in (Guerrieri & d'Ettorre, 2008) and (Gospocic et al., 2017), individual ants were challenged with a chemically neutral mechanical stimulus (i.e. a clean Von Frey filament) and subsequently scored for biting responses as well as wide opening

of the mandibles as indicators of aggression. Importantly, inasmuch as there was no significant difference in aggression among the various treatment groups (Fig. S1) we can conclude that VUAA-treatments do not generally inhibit aggressive responses in *C. floridanus* but instead specifically impacts workers' ability to discriminate NMs from nNMs and aggressively respond to the latter.

In order to further control for potentially confounding variables in response to these volatilization treatments, the activity of a single ant was recorded immediately following a 10-minute acclimation period. These trials consisted of a continuous 9-minute bioassay separated into three 3-minute segments: during the first segment, the ants were exposed to a continuous flow of untreated air ('Acclimation'); for the second segment, the ants were exposed to a continuous flow of volatilized VUAA0, VUANT1, or VUAA4 or untreated air in the case of the blank control using the same parameters established for the volatilization aggression bioassay ('Treatment'); and lastly, during the third segment, the ants were again exposed to a continuous flow of untreated air ('Recovery'). A Y-junction connected to the compressed air tank alternated between the empty test tube during the Acclimation and Recovery phases and the treatment or blank tube during the Treatment phase. An examination of overall mobility parameters revealed no significant interaction effect when comparing control ants and ants treated with either an Orco agonist or antagonist before, during, or after exposure to each treatment (Fig. 2C-E).

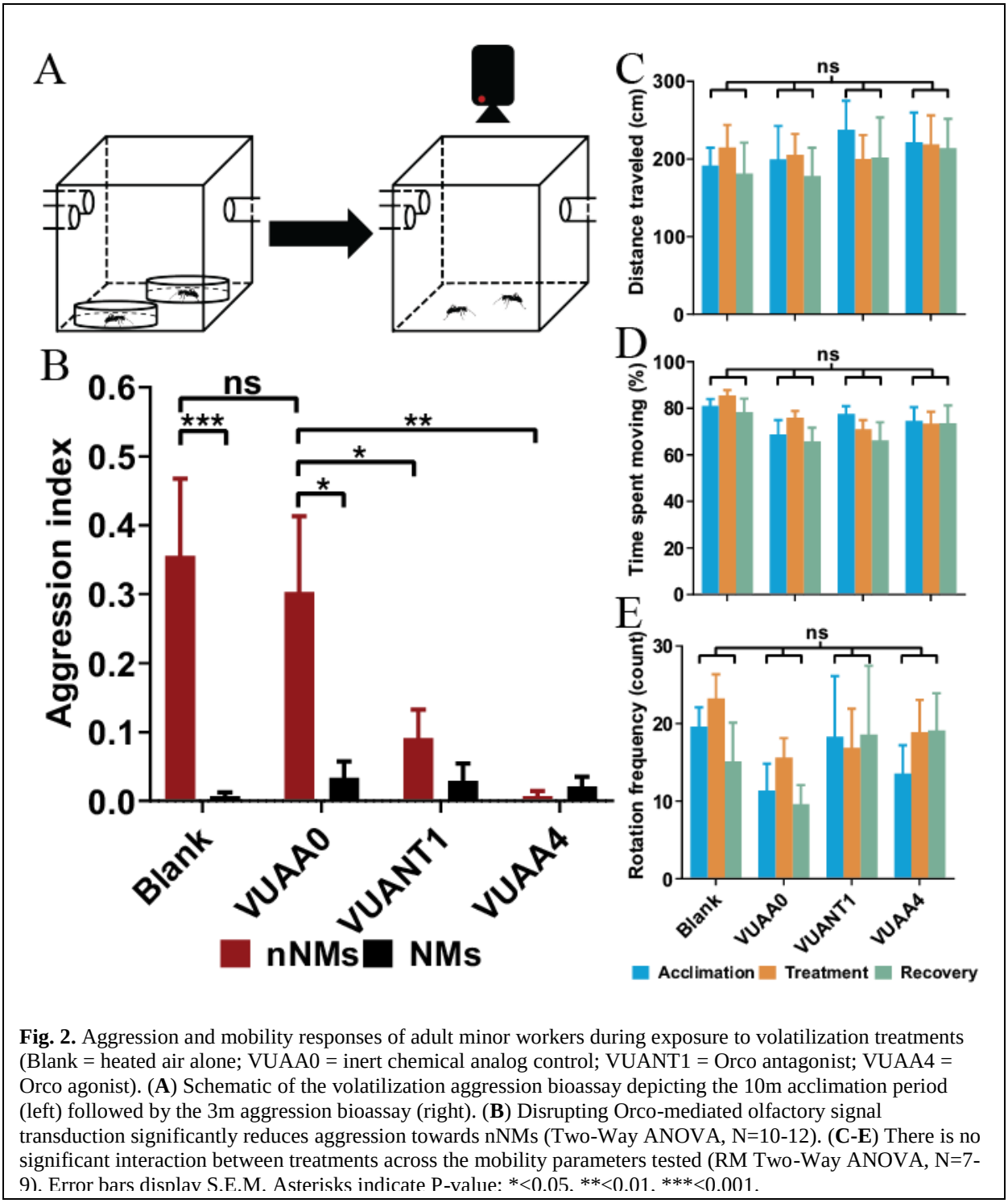

Fig. 2. Aggression and mobility responses of adult minor workers during exposure to volatilization treatments (Blank = heated air alone; VUAA0 = inert chemical analog control; VUANT1 = Orco antagonist; VUAA4 = Orco agonist). (**A**) Schematic of the volatilization aggression bioassay depicting the 10m acclimation period (left) followed by the 3m aggression bioassay (right). (**B**) Disrupting Orco-mediated olfactory signal transduction significantly reduces aggression towards nNMs (Two-Way ANOVA, N=10-12). (**C-E**) There is no significant interaction between treatments across the mobility parameters tested (RM Two-Way ANOVA, N=7-9). Error bars display S.E.M. Asterisks indicate P-value: *<0.05, **<0.01, ***<0.001.

Discussion

In ants and other eusocial insects, NM recognition depends on the ability to discriminate between self and non-self where the recognition of non-self—in this instance nNMs—often leads

to aggression (reviewed in (Sturgis & Gordon, 2012)). These aggressive responses are mediated by the detection of subtle differences in the CHC profiles that demarcate individual colonies (Guerrieri et al., 2009; Leonhardt et al., 2007; Morel et al., 1988; Neupert et al., 2018). Here, we demonstrate that the lack of any odor signal or the presence of ambiguous odor cues that are expected after treatment with an Orco antagonist or agonist, respectively, are both equally insufficient to elicit aggression between nNMs. The observation that an Orco antagonist decreases aggression between nNMs is broadly consistent with a simple U-present rejection model and supports the view that ants are not actively recognizing friends (Guerrieri et al., 2009; van Zweden & d'Ettorre, 2010). However, the curious finding that an Orco agonist would also decrease aggression between nNMs rather than increase aggression between NMs suggests that the simple presence of foreign or otherwise ambiguous cues are also insufficient to elicit aggression. Rather, these studies support a model in which an unambiguous triggering stimulus must be precisely detected in order to evoke aggression (Fig. 3). As such, we propose that the recognition mechanism in *C. floridanus* occurs via a lock-and-key mechanism whereby the specific parameters of the foreign chemical label key, defined by the combinatorial presence and/or absence of salient odor cues, must be precisely detected by an OR-mediated lock. Under this assumption, ants may identify nNMs

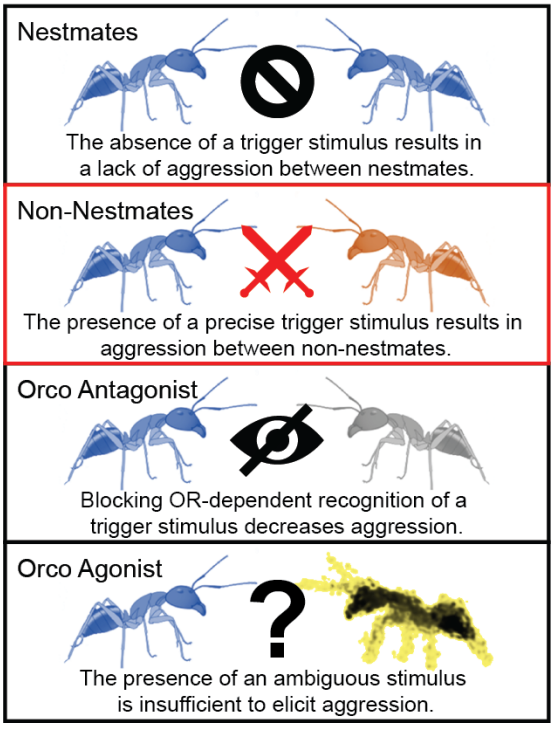

Fig. 3. Model of nNM recognition and aggression. Precise OR-dependent recognition of nNM cues leads to aggression against foes (red); however, blocking OR-dependent recognition of NM/nNM cues does not lead to aggression nor does the presence of an ambiguous chemical cue.

in two different ways which are not necessarily mutually exclusive: 1. unfamiliar nNM labels are compared to a familiar NM template with bounded thresholds wherein the label must be sufficiently different from the template but not so different as to be ambiguous; or 2. unfamiliar nNM labels are compared to intruder templates that represent odor profiles which should be rejected from the colony and a certain level of precision between the label and template is required to elicit aggression.

Furthermore, these data suggest that, when faced with some level of uncertainty, *C. floridanus* workers default towards acceptance rather than rejection. Over and above the benefits of conserving energy by avoiding potentially unnecessary aggression, for ants that spend the majority of their life cycles within colonies where they are more likely to encounter NMs than nNMs, this strategy may also reduce acceptance errors and therefore increase overall colony fitness (Reeve, 1989). It will be interesting to determine whether similar processes occur across worker behavioral task groups that may spend more time outside the nest (i.e. scouts and foragers) or whether different recognition methods have evolved across castes and/or species.

Here we show that Orco/OR-mediated signaling is necessary for the active detection and precise processing of a discrete stimulus that triggers aggression towards nNMs in *C. floridanus*. These results are consistent with previous literature suggesting that, at least in the context of aggression-mediated discrimination, NM recognition may be more appropriately described as nNM recognition (Guerrieri et al., 2009; van Zweden & d'Ettorre, 2010). While the roles of individual ant ORs or even specific subsets of ORs in aggression-mediated NM/nNM recognition remain to be elucidated, the combinatorial interactions that are expected even among specialized ORs (Pask et al., 2017; Slone et al., 2017), the plasticity of the potentially numerous neuronal templates (Leonhardt et al., 2007; Neupert et al., 2018) and the similarly diverse and plastic

289 labels (Kaib et al., 2000; Nascimento, Tannure-Nascimento, Dantas, Turatti, & Lopes, 2013; R.
290 K. Vander Meer, Saliwanchik, & Lavine, 1989; Wagner et al., 1998) as well as the observation
291 that even repeated stimulation with colony odors produced variable response patterns in the
292 antennal lobe (A. Brandstaetter et al., 2011), are likely to make those studies extremely
293 challenging.

294 Nevertheless, by excluding other signaling pathways and modalities, and directly
295 demonstrating that precise and unambiguous OR-based signaling is necessary for ants to
296 distinguish foe from friend, our findings represent a significant advance to link the longstanding
297 interest in social insect behavior with more recent studies detailing the evolutionary complexity
298 of the insect olfactory system (Hölldobler & Wilson, 1990; Zhou et al., 2015; Zhou et al., 2012).
299 Moreover, in addition to the basic biology we have examined, these studies provide a proof
300 concept for the use of Orco allosteric modulators to disentangle the role of OR-mediated
301 olfaction in behavior in otherwise genetically intractable systems. The development of these and
302 other molecular techniques will provide important tools as we continue to refine our
303 understanding of the molecular mechanisms governing recognition in eusocial systems. Taken
304 together, these results highlight the importance of the OR family in mediating the precise
305 signaling paradigms that drive social behaviors in ant taxa. It is tempting to speculate that similar
306 processes may mediate aggressive responses in other animal systems.

Materials and Methods

Ant Husbandry

Nine distinct laboratory colonies of *Camponotus floridanus* originating from field collections generously obtained by Dr. J. Liebig (Arizona State University) from the Long Key (D242) and Sugarloaf Key (D601) and Dr. S. Berger (University of Pennsylvania) from the Fiesta Key (C6, K17, K19, K28, K31, K34, and K39) in South Florida, USA. All colonies were independently maintained at 25°C, ambient humidity, with a 12-h light:12-h dark photoperiod. Each colony was provided with Bhatkar diet, crickets, 10% sucrose solution, and distilled water three times per week. Adult minor workers were used for all experiments and were sampled from throughout the colony.

Ablation Aggression Bioassay

Tests were conducted during the ZT diel light cycle between ZT2 and ZT12 at ambient room temperature and humidity and performed using a six-well culture plate with polytetrafluoroethylene-coated well walls (DuPont®). Individual wells of the six-well culture plate served as distinct bioassay arenas for behavioral trials (Fig. S2A). In preparation for experiments, each well (9.6cm^2) of the six-well culture plate was fitted with a removable plastic divider that partitioned the well into two halves. The six-well culture plate and dividers were sterilized using ethanol, air dried, and positioned on top of a light box. Each individual bioassay well utilized two adult minor ants that were selected from either the same home colony (NMs) or two distinct colonies (nNMs). All ants were handled wearing gloves and using sterile, soft-tipped metal forceps and were subsequently discarded after each bioassay to ensure each ant was used only once.

Subject ants were briefly anesthetized with CO_2 before removing their antennal flagella via an incision across the distal portion of the scape using a clean, unused razor blade. Bilaterally ablated ants had both flagella removed while unilaterally ablated ants had only a single (right or left, randomly selected) flagellum removed. Sham treated ants were anesthetized with CO_2, and the razor was gently touched to the antennae without damaging any structures. Subsequent to ablation (or sham) treatment, ants were allowed to recover along with similarly treated NMs for at least 2 hours prior to testing.

Prior to bioassays, two ants (NMs or nNMs) were placed into each well arena, one in either half, and allowed 10 min to acclimate to handling. To document normal ant behavior within each well arena, mobility was recorded using a digital high definition camera (Panasonic® HC-V750) for 3 min (detailed below). The plastic divider within each well arena was subsequently removed and all ant interactions again recorded for 3 min. The order in which the treatments were conducted as well as the colony the ants were selected from for any given trial were randomized using RANDOM.ORG (Randomness and Integrity Services Ltd.).

Volatile Orco Modulator Aggression Bioassay

To facilitate the administration of a continuous flow of air containing volatilized VUAA-class compounds (all custom synthesized as dry solids in-house at Vanderbilt University (P. L. Jones et al., 2011; P. L. Jones et al., 2012; Romaine et al., 2014; Taylor et al., 2012)) into the aggression arena, bioassays were conducted in arenas consisting of modified square plastic boxes with a total area of 85cm^2 (Pioneer Plastics Inc. ®) (Fig. S2A). Conditioned air (78% Nitrogen, 21% Oxygen) was delivered (at a constant 34kpa) from a compressed source (Nashville Gas LLC) to the test arena through a 12x75mm test tube atop a heat block set at 260°C which contained 0.025g of the respective treatment compound (VUAA0, VUANT1, or VUAA4) or an

empty tube (Blank control) via 18G needles inserted into a rubber septum affixed to the top of the test tube before exiting through a dedicated exhaust system. Trials were recorded using a digital high definition camera and scored as described below. Although two plastic tubes were affixed to the arena during the volatilization aggression bioassays, only a single tube was actively delivering the test compound or heated air control (Fig. S2B). In each assay, ants were acclimatized underneath 35mm Petri dish lids (prewashed with ethanol) for 10 minutes after which the lids were then removed (allowing the ants to interact), the airflow started, and the ants were then recorded for the 3-minute test period. All treatment compounds were randomized and coded independently such that the investigator was blinded to the treatment identity. Furthermore, the sequential order in which the compounds were tested as well as the colony the ants were selected from for any given trial was randomized using RANDOM.ORG (Randomness and Integrity Services Ltd.).

Aggression Bioassay Scoring

Digital video recordings of all bioassays were viewed post hoc and aggression incidents manually scored for analyses. Trials in which ants did not interact, were disrupted physically during removal of the plastic barrier, or were fatally encumbered at trial onset were discarded from further analyses along with their respective mobility controls in the case of the antennal ablation bioassays. These interactions were scored by three independent, blinded observers in 10 s intervals using a binary scale such that aggression either did or did not occur (a score of 1 or 0, respectively; Movies S1-2). Prior to scoring, each observer was trained to recognize “aggression” as instances in which one or both ants were lunging, biting, or dragging one another. Each 10 s time interval was scored as either containing an instance of aggression or not to establish the proportion of time the ants were engaged in aggressive behavior. An aggression index was

375 calculated by dividing the number of observed acts of aggression by the total number of
376 observed time intervals. The mean aggression index of each video recording across all three
377 independent scores was used for subsequent statistical analysis.

378 **Mobility Control Parameters**

379 Mobility control videos were analyzed using an automated tracking software package
380 (Ethovision® XT v8.5, Noldus Information Technology) to calculate total distance traveled
381 (cm), percentage of time spent moving (%), and the frequency of rotations (count). Time spent
382 moving/not moving was calculated with thresholds of 0.30cm/s (start velocity) and 0.20cm/s
383 (stop velocity) as determined by the EthoVision® XT software with an averaging interval of 1
384 sample. To determine the percent of time spent moving, the time spent moving was divided by
385 the sum of the time spent moving and the time spent not moving to account for instances in
386 which the subject ant was not detected by the software. A single rotation was defined as a
387 cumulative turn angle of 90° over a distance of 1.00cm. Turns in the opposite direction of less
388 than 45° were ignored. The sum of both clockwise and counterclockwise rotations was used to
389 determine rotational frequency. Trials in which the subject ant was not found for at least 95% of
390 the recording were discarded, as were videos in which the ants appeared fatally encumbered at
391 trial onset.

392 **Mechanically Evoked Biting and Mandible Opening Response (BMOR) Bioassay**

393 To determine whether disrupting Orco-mediated olfactory signaling disrupts broadly
394 aggression in a non-social context, individual adult minor workers were briefly anesthetized with
395 CO_2 before being secured with wax in a modified 200µl pipette tip such that the head and
396 antennae were accessible. The ants were allowed to acclimate for 10 minutes before being
397 exposed to a continuous flow of heated air alone or volatilized VU-class compounds as described

above in the Volatile Orco Modulator Aggression Bioassays. A clean, ethanol washed 3.61/0.4g Von Frey hair filament (Baseline® Fold-Up™ Monofilaments Item #12-1741) was then gently brushed along the anterior portion of the ant's head from the ventral to the dorsal side five times. Aggression was scored by six independent, blinded observers on a binary scale such that biting or attempting to bite the filament or wide opening of the mandibles (i.e. the mandibles were opened beyond parallel) either did (score of 1) or did not (score of 0) occur during the duration of the trial (Movie S3). An aggression index was calculated by taking the average score across all observers and used for subsequent statistical analysis. Trials in which the ants had not recovered from the CO_2 before trial onset were discarded.

Statistical Analysis

Statistical analyses were performed using Graphpad Prism v8.0.0 (GraphPad Software, Inc). For the aggression bioassays, a two-way ANOVA was first performed followed by Holm-Sidak's multiple comparisons test to compare NM vs. nNM aggression as well as aggression across antennal treatments. For the antennal ablation mobility controls as well as the BMOR bioassays, a Kruskal-Wallis test was performed followed by Dunn's correction for multiple comparisons. As the volatilization mobility controls had matched samples across different time points, a repeated measures two-way ANOVA with the Geisser-Greenhouse correction for violations of sphericity was performed. The number of replicates for each study were as follows: Ablation Aggression Bioassays: NMs – Sham (9), U.abl (10), B.abl (6); nNMs – Sham (10), U.abl (9), B.abl (6). Mobility Controls (Ablation): Sham (29), U.abl (29), B.abl (24). Volatile Orco Modulator Aggression Bioassays: NMs – Blank (10), VUAA0 (10), VUANT1 (12), VUAA4 (10); nNMs - Blank (12), VUAA0 (11), VUANT1 (10), VUAA4 (12). Volatile Orco Modulator BMOR Bioassay: Blank (11), VUAA0 (10), VUANT1 (10), VUAA4 (10). Mobility

Controls (Volatilization): Blank (8), VUAA0 (8), VUANT1 (7), VUAA4 (9). Information regarding the statistical test performed and the results from these analyses have been detailed in Dataset S1.

Data Availability

All data generated or analyzed during this study are included in this published article (and its supplementary information files) (Datasets S2-6).

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