Molecular characterization and expression variation of the odorant receptor co-receptor in the Formosan subterranean termite

Paula Castillo, Claudia Husseneder, Qian Sun*

Department of Entomology, Louisiana State University Agricultural Center, Baton Rouge, Louisiana, United States of America

* qsun@agcenter.lsu.edu

Abstract

Subterranean termites live in underground colonies with a division of labor among castes (i.e., queens and kings, workers, and soldiers). The function of social colonies relies on sophisticated chemical communication. Olfaction, the sense of smell from food, pathogens, and colony members, plays an important role in their social life. Olfactory plasticity in insects can be induced by long- and short-term environmental perturbations, allowing adaptive responses to the chemical environment according to their physiological and behavioral state. However, there is a paucity of information on the molecular basis of olfaction in termites. In this study, we identified an ortholog encoding the odorant receptor co-receptor (Orco) in the Formosan subterranean termite, Coptotermes formosanus, and examined its expression variation across developmental stages and in response to social conditions. We found that C. formosanus Orco showed conserved sequence and structure compared with other insects. Spatial and temporal analyses showed that the Orco gene was primarily expressed in the antennae, and it was expressed in eggs and all postembryonic developmental stages. The antennal expression of Orco was upregulated in alates (winged reproductives) compared with workers and soldiers. Further, the expression of Orco decreased in workers after starvation for seven days, but it was not affected by the absence of soldiers or different group sizes. Our study reveals the molecular characteristics of Orco in a termite, and the results suggest a link between olfactory sensitivity and nutritional status. Further studies are warranted to better understand the role of Orco in olfactory plasticity and behavioral response.

Introduction

Chemical communication is essential for the regulation of fundamental behaviors in social insects [1]. In termites (order Blattodea), the division of labor in a colony is maintained through a caste system consisting of reproductives (queens and kings), workers, and soldiers. In subterranean termites (family Rhinotermitidae), colonies are typically founded by a pair of dispersal alates (i.e., winged reproductives), which shed their wings, build an underground
nest chamber, and rear the first cohort of brood as the primary queen and king [2]. Newly
developed workers then take over the tasks of brood care, tunnel in soil to search for food, and
perform hygienic activities [3, 4]. Soldiers defend the colony through aggressive behavior
toward competitors and predators [5]. The elaborate caste system results from developmental
plasticity, which is mainly mediated by the social environment. After hatching from the eggs,
the larvae follow either an apterous pathway to differentiate into workers and soldiers, or an
imaginal pathway to develop into nymphs and eventually alates [6]. Except for the alates, sub-
terranean termites have poor visual ability and heavily rely on chemicals to organize social
activities and detect environmental changes [7]. Pheromones also play key roles in mediating
caste differentiation in termite colonies [8–10].

In insects, perception of olfactory cues is primarily performed by the antennae, which carry
numerous and diverse sensilla [11]. Olfactory sensilla are characterized by a multiporous
surface that allows volatile chemicals from the environment to enter the sensillar lymph. Den-
drites of olfactory sensory neurons (OSNs) extend to these hair-like sensilla, and odorant
receptors (ORs) are expressed in the dendritic membranes [12]. Through activities of OSNs,
odorant signals are transduced into electrical impulses, which are integrated in the antennal
lobes and may further project to higher brain centers, such as mushroom bodies, to trigger a
behavioral response [12, 13]. The ORs have undergone rapid evolution in insects, facilitating
their adaptation to a wide range of ecological niches [12, 14]. While each species possesses a
distinct OR repertoire, most species express only one odorant receptor co-receptor (Orco),
which is highly conserved across insects [12]. Subunits of Orco form a tetramer arranged
around a central pore and are bound together by a small cytoplasmic anchor domain [15].
While ORs confer odorant specificity, they form Orco-OR heterotetramers that compose the
odorant-gated ion channels. Therefore, Orco is required for localization of ORs to dendritic
membranes and their proper function [15]. Most OSNs express only one unique member of its
OR family along with the ubiquitous chaperone Orco [16–18].

The function of Orco has been investigated in many solitary and a few social insects, revealing
its role in the regulation of essential behavior and neurodevelopment. For example, muta-
tion of Orco gene through CRISPR-Cas9 disrupted larval feeding and adult mating behavior in
the domestic silk moth (Bombyx mori) [19] and foraging behavior in the hawkmoth (Manduca
sexta) [20]. Silencing of Orco through RNA interference (RNAi) impaired mating behavior in
the olive fruit fly (Bactrocera oleae) [21] and responses to aggregation pheromone and food
seeking behavior in the white-spotted flower chafer beetle (Protaetia brevitarsis) [22]. In addi-
tion, expression of Orco is required to avoid degeneration of OSNs in the maxillary palps in
the vinegar fly (Drosophila melanogaster) [23]. Orco is also essential for maintaining social
behavior and the development of antennal lobes in the clonal raider ant (Ooceraea biroi) [24],
the Jerdon’s jumping ant (Harpegnathos saltator) [25], and the honey bee (Apis mellifera) [26].
In Blattodea, Orco has been functionally characterized in the German cockroach (Blatella ger-
manica) [27], and in the worker caste of two termite species, a fungus-growing termite (Odont-
totermes formosanus) [28] and a subterranean termite (Reticulitermes chinensis) [29]. In B.
germanica, the silencing of Orco increased their response time to the sex pheromone blatella-
quinone and impaired their food-seeking behavior [27]. In termites, the knockdown of Orco
altered nestmate discrimination in O. formosanus [28] and affected trail-following and loco-
motion behavior in both O. formosanus and R. chinensis [29]. As numerous studies examine
the function of Orco, the expression of Orco may be one of the indicators of the olfactory sensi-
tivity and activity in olfaction-related neurogenesis. However, its expression variation during
caste development is not examined in social insects.

The olfactory system displays sex dimorphism in many insects and caste polyphenism in
some social species, reflecting their differential olfactory capacity and behavioral repertoire

Competing interests: The authors have declared that no competing interests exist.
In social Hymenoptera (ants, bees, and wasps), most social behavior is displayed by females (workers and queens), and males are produced for mating purpose only. Correspondingly, these sexes/castes exhibit variation in terms of peripheral sensory structures and chemosensory gene profiles [30, 31]. For instance, the hydrocarbon-sensitive basiconic sensilla, as well as the 9-exon subfamily of ORs, which detects cuticular hydrocarbons for nestmate recognition, are only found in female ants of *O. biroi* [31]. Different from social Hymenoptera, hemimetabolous termite castes are comprised of both females and males [6]. Our previous study in the Formosan subterranean termite, *Coptotermes formosanus*, showed that the composition of antennal sensilla varied between reproductive and non-reproductive castes, but not between female and male alates [32]. In the Japanese subterranean termite, *R. speratus*, caste-specific chemosensory gene expression profiles are reported [33]. It is unknown, however, if the expression of Orco varies during the plastic development of caste system in termites.

Olfactory plasticity is widely observed in animals, which enables them to modify behavioral responses according to physiological state such as age, feeding, and mating status [34]. Long-term environmental conditions and the immediate sensory environment can influence olfactory plasticity [35]. Olfaction can be tuned through changes in chemosensory gene expression, neuromodulators, and endocrinological mechanisms [34]. For example, starvation increases olfactory sensitivity and enhances food seeking behavior in insects, worms, rodents, and humans [36–38]. Changes in chemosensory gene expression were induced by starvation in the oriental fruit fly, *Bactrocera dorsalis* [39], and African cotton leafworm, *Spodoptera littoralis* [40]. In *B. dorsalis*, expression of Orco was upregulated by a sexual attractant, methyl eugenol [41]. In termites, social environment is important for both caste development and immediate behavioral response [6, 7], but the expression variation of Orco in response to environmental conditions has not been investigated. The function of social colonies is maintained by collective activities through pheromonal regulation and nestmate recognition. A number of pheromones with behavioral and/or physiological activities have been identified in termites. For example, soldiers produce a pheromone that modulates worker behavior and worker-soldier differentiation [10], workers release a trail pheromone to recruit nestmates for foraging [42], and the queen produces a pheromone that suppresses nestmate fertility and/or promotes their tending behavior [8, 9]. In addition, cuticular hydrocarbons are important for nestmate and caste recognition [7]. So far, the conserved Orco has been identified in five termite species: two subterranean termites, *R. speratus* [33] and *R. chinensis* [29], a dampwood termite *Zootermopsis nevadensis* [43], a drywood termite *Cryptotermes secundus* [44], and a fungus-growing termite *O. formosanus* [28, 29]. However, the role Orco plays in olfactory plasticity remains unclear.

The overall goal of this study was to characterize the Orco ortholog and examine its expression variation in *C. formosanus*, which is a cosmopolitan invasive species. Since antennae are the primary olfactory organs, and the division of labor in social insects is often associated with olfactory capabilities, we hypothesized that Orco expression is tissue-specific and varies across termite castes. In addition, as social behaviors in termites are largely mediated through chemical cues, we further hypothesized that the expression of Orco changes in response to social environment. To test the hypotheses, we (i) cloned and sequenced the full-length cDNA of Orco in *C. formosanus*, (ii) analyzed Orco protein structure in *C. formosanus* and its phylogenetic relationship with other insects, (iii) examined the spatial (tissue) and temporal (developmental stage) distribution of *C. formosanus Orco*, and (iv) investigated the expression profiles of *C. formosanus Orco* under three sets of varying social conditions, including starvation, soldier percentage, and group size.
Materials and methods

Insects

Three colonies of *C. formosanus* workers and soldiers were collected at Brechtel park in New Orleans, Louisiana (29°54’32”N, 90°00’32”W). These colonies were kept at 25 ± 1°C in clear acrylic containers (38.48 × 45.72 × 22.86 cm), provided with an approximately 4.0 cm layer of organic soil at the bottom and moistened pine wood logs as the food source. The relative humidity (RH) in each container was monitored weekly, and water was added to maintain 80–99% RH. Workers, soldiers, pre-soldiers, and nymphs were obtained from these colonies. Female and male alates were collected using ultraviolet light traps (BioQuip, USA) during the swarm season (May and June) from three populations in Baton Rouge, Louisiana (population A: 30°22’14”N, 91°06’39”W; population B: 30°24’33.3”N, 91°06’18.5”W; population C: 30°22’12.2”N, 91°06’16.8”W). For RNA sample preparation, the alates were utilized within 16 h upon collection from the field. Heterosexual pairs of alates were allowed to form incipient colonies in Petri dishes (5.0 cm in diameter, Thermo Fisher Scientific, Waltham, MA USA) provisioned with moist filter paper and pine wood chips. These incipient colonies were kept at 27 ± 1°C and 80–99% RH in complete darkness for two months, and eggs and larvae were collected from three colonies.

Cloning and sequencing of Orco

Total RNA from pools of 200 antennae from 100 workers, female, or male alates were extracted using TRIzol reagent (Invitrogen, Waltham, MA, USA) following the manufacturer’s protocol. Then, all samples were treated with DNAse I to remove traces of genomic DNA, utilizing the TURBO DNase kit (Invitrogen), following the manufacturer’s protocol. The quantification of the purified total RNA was performed on a NanoDrop™ One spectrophotometer (Thermo Fisher Scientific). To obtain the full-length transcript, RACE-ready cDNA was synthesized with the GeneRacer Kit (Invitrogen) following the manufacturer’s protocol. Gene specific primers (GSPs) for 5’ and 3’ cDNA ends were designed upon conserved regions of *Orco* gene across different termite species (S1 Table), utilizing the NCBI primer designer online tool PrimerBLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The touchdown PCR reactions were carried out with the Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen), using the following cycling conditions: initial denaturation at 94 °C for 2 min, followed by 15 cycles at 94 °C for 15 s (denaturation), annealing temperature touchdown from 78 °C to 68 °C for 45 s, and 72 °C for 1 min (extension); then, 20 cycles at 94 °C for 15 s, 66 °C for 45 s, and 68 °C for 1 min. A final extension step was added at 68 °C for 2 min. The PCR products were purified from an agarose gel, using the Zymoclean Gel DNA Recovery kit (Zymo Research, Irvine, CA, USA), and cloned into the pCR®-4-TOPO® vector (Invitrogen), followed by transformation in the One Shot® TOP10 chemically competent *E. coli* cells, according to the manufacturer’s protocol. Transformants were obtained after selection with ampicillin, and the plasmid isolation was achieved by using the QIAprep® Spin Miniprep Kit (Qiagen, Germantown, MD, USA). Then, the sequences in both directions were obtained through Sanger sequencing utilizing the forward and reverse M13 primers at the Genomics Facility of Louisiana State University.

Structural simulation and phylogenetic analysis

The *Orco* sequence in *C. formosanus* was compared with other insect species using BLAST (https://blast.ncbi.nlm.nih.gov/). Identification of the full open reading frame (ORF) and translation into protein was performed with the on-line tool ORFfinder (https://www.ncbi.
Identification of conserved protein domains was carried out using the Simple Modular Architecture Research Tool (SMART) (http://smart.embl.de/) and was confirmed with InterPro scan (https://www.ebi.ac.uk/interpro/). A 2D structure of the putative C. formosanus Orco protein was predicted with the TMHMM 2.0 online tool (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0), and the graphical representation was generated with TMRPres2D (http://bioinformatics.biol.uoa.gr/TMRPres2D/) following the developer’s recommendations [45]. The 3D structure was predicted using the SWISS-MODEL server (https://swissmodel.expasy.org/) and the Cryo-EM structure of Orco from Apocrypta bakeri as a template [15]. Prediction of molecular mass and isoelectric point of the putative Orco protein were estimated with the pl/Mw tool in Expasy (https://www.expasy.org/). Amino acid sequences of Orco from different insect species were downloaded from the NCBI database (S2 Table). Then, protein alignment was performed using the MUSCLE module in the Geneious primer software (Biomatters, Inc, San Diego, CA, USA), which was further utilized for phylogenetic reconstruction through IQ-Tree (http://www.iqtree.org/) using the maximum-likelihood method. Node support values were obtained from 1000 bootstrap replications. The graphic representation of the phylogenetic tree was obtained and adjusted using the iTOL online platform (https://itol.embl.de/). The sequence of the pea aphid (Acyrthosiphon pisum) was manually selected as the root for the phylogenetic tree.

Spatial and temporal expression of Orco

To determine the spatial expression of Orco, its expression levels in antennae, head (without antennae), legs, and carcass (remaining body part) were analyzed in workers, soldiers, and female and male alates collected from three colonies or populations (n = 3 per tissue type). These termites were processed for sample collection within 16 hours upon collection. Termites were anesthetized on ice prior to dissection. To obtain sufficient RNA, 200 antennae were pooled from 100 individuals, and 600 legs were pooled from 100 individuals, along with the heads of 30 individuals and the carcasses of 10. The heads and carcasses of soldiers were not analyzed due to the interference of frontal gland secretion with RNA isolation. For the temporal expression, different developmental stages and castes, i.e., eggs, larvae, workers, pre-soldiers, nymphs, female and male alates, were analyzed. Specifically, pools of 25 to 45 eggs, 7 to 11 larvae (first and second instars), or a whole body of each caste (third instar or older) from three colonies or populations were used (n = 3 per developmental stage/caste).

Tissues or whole bodies were collected directly in TRIzol (Invitrogen). Total RNA isolation and treatment with DNase was performed as described in section 2.2. The cDNA was synthesized with the SuperScript III first-strand synthesis kit (Invitrogen) from 330 ng of total RNA from each sample, following the manufacturer’s protocol. The relative quantification of Orco gene expression was achieved through quantitative real-time PCR (qRT-PCR) using 1 μL of diluted cDNA (4.125 ng/μL) and the PowerUp ™ SYBR™ Green Master Mix (Applied Biosystems) on a QuantStudio 3 real-time PCR system (Thermo Fisher Scientific). A final concentration of 300 nM of the primers listed in S1 Table were utilized for Orco and each reference gene. The cycling conditions were: 2 min at 50 °C for UDG activation; 2 min at 95 °C hot start activation; then, 40 cycles of 15 s at 95 °C (denaturation), and 1 min at 60 °C (annealing/extension). Finally, the melting curve analysis to confirm the specificity of the reaction was performed with an initial denaturation step of 15 s at 95 °C, followed by a heating temperature ramp from 60 °C to 95 °C with an increase at 0.05 °C/s. ROX was utilized as the passive reporter.

Four reference genes, the ribosomal protein S18 (rps18), ribosomal protein L32 (rpl32), elongation factor 1-α (ef1-α), and the structural protein β-Actin were assessed for their stability
across different gene expression analyses in all tested conditions using the BestKeeper Excel-based tool [46]. The selection criteria of the best reference genes by this method consider the following factors: 1) genes with a standard deviation of raw Ct values higher than 1.0 are considered inconsistent, and 2) genes with expression 3-fold over or under their geometric mean Ct should be discarded. Primers for all reference genes were taken from Du, et al. [47] (S3 Table), and primer specificity was determined by the melting curve analysis. Efficiency for each qRT-PCR reaction was determined utilizing the raw amplification data and the LinRegPCR software [48, 49]. The baseline corrected data was used to calculate the relative expression levels of Orco using the 2^\(-\Delta\Delta\text{Ct}\) method described by Livak and Schmittgen [50]. One stable reference gene, rps18, was selected for normalization of the data in these calculations.

**Expression of Orco in response to different social environments**

Three assays were performed to evaluate the expression of Orco in response to starvation, the presence of soldiers, and different group sizes. In all three assays, termites were maintained in Petri dishes (35 mm in diameter) lined with moist filter papers as the food source, and the termites were kept at 27 ± 1°C, 80–99% RH, in complete darkness. For the starvation assay, groups of 50 termites (45 workers and five soldiers) were starved for 0, 5, or 7 days. Three replicates were conducted per colony, and three colonies were used (n = 9 per treatment). For the influence of soldiers, groups of 45 workers were maintained with the addition of five soldiers (10%) or without soldiers (0%) for seven days. Six replicates (two each from three colonies) were performed. For the test of group size, workers were kept in Petri dishes for seven days as individuals, groups of 15, or groups of 100. Each group size was tested with six replicates (two each from three colonies). For each sample, 20 antennae from 10 workers were dissected and collected directly in TRIzol (Invitrogen), and total RNA isolation and gene expression analysis were performed as described in section 2.4. rps18 was used as the reference gene for qRT PCR analysis (S3 Table).

**Statistical analyses**

All statistical analyses were carried out using the R software version 4.1.2 (The R Foundation, Vienna, Austria) [51]. To check the homogeneity of variances and normality of data distributions, Levene’s test and Shapiro–Wilk test were performed, respectively. As data did not meet the assumptions of parametric tests (P < 0.05 for Levene’s test or Shapiro–Wilk test), Kruskal–Wallis followed by Dunn’s tests were performed to analyze the expression levels of Orco for its spatial and temporal distribution, and in response to different social environments. An alpha level of 0.05 was chosen for all tests performed. All gene expression data were plotted utilizing GraphPad Prism version 9.3.1 (San Diego, California, USA).

**Results**

**Bioinformatic analysis of Orco**

Only one isoform of Orco was identified in *C. formosanus* with an ORF of 1,419 bp in length (GenBank accession number OL845867). It encoded a predicted protein of 472 amino acids (aa) (Fig 1) with a predicted molecular mass of 53.15 kDa and an isoelectric point (pI) of 7.21. The full-length cDNA included a 214 bp 5’ untranslated region (UTR) and an 878 bp 3’ UTR with a poly (A) tail.

Multiple amino acid sequence alignments showed that Orco was highly conserved across termite species (Fig 1). The putative Orco sequence in *C. formosanus* shared 94.92% identity with Orco in the Chinese subterranean termite *R. chinensis* (Rhinotermitidae); 94.70% with the
Japanese subterranean termite *R. speratus* (Rhinotermitidae); 91.74% with the fungus-growing termite *O. formosanus* (Termitidae); 91.95% with the dampwood termite *Z. nevadensis* (Termitidae); 89.19% with the drywood termite *C. secundus* (Kalotermitidae); and 82.42% with the German cockroach *B. germanica* (Ectobiidae), which is an omnivorous and group-living species.

The structural analysis of the putative Orco protein utilizing the TMHMM online tool revealed a short intracellular domain (amino acids 1 to 41) at the N-terminus (probability < 0.2), followed by 7 predicted transmembrane regions that belong to the 7tm_6 domain (Tm1-7) family (probability > 0.85); which are characteristics of odorant receptors (Fig 1). A schematic representation of the 2D structure of Orco with predicted transmembrane, cytoplasmic, and extracellular regions is shown in Fig 2A. The in silico simulation for *C. formosanus* Orco with SWISS-MODEL rendered a 3D model that shared 63.93% sequence identity with *A. bakery* Orco protein. In this model, the transmembrane regions folded as alpha-helices are shown in Fig 2B for a single Orco subunit. The subunits of Orco were predicted to form a homotetramer, with each transmembrane region assisting in the formation of a channel pore in the center (Fig 2C).

The phylogenetic analysis showed that all known Orco sequences in termites formed a monophyletic clade, which is closely related to *Blattella germanica* Orco (Fig 3). Based on the phylogenetic reconstruction, the Orco proteins of Blattodea are evolutionary closer to those in Orthoptera than in Hemiptera, Hymenoptera, Coleoptera, Lepidoptera, and Diptera (Fig 3).

**Spatial and temporal expression of Orco**

The expression levels of Orco were analyzed in tissues (antennae, head, legs, and carcass) from different castes for the spatial distribution pattern, and in whole bodies of different castes or
developmental stages for temporal distribution. The results showed that Orco was predominantly expressed in the antennae of all castes, and it was found at very low levels in other tissues (Fig 4A). Orco expression levels were not significantly different between the antennae of female and male alates ($P = 0.2856$, Kruskal-Wallis followed by Dunn’s test), or between worker and soldier antennae ($P = 0.2140$, Kruskal-Wallis followed by Dunn’s test). However,
significantly higher expressions were found in the antennae of alates than in workers and soldiers (Fig 4A, $P < 0.05$, Kruskal-Wallis followed by Dunn’s test). A similar expression pattern was observed in the heads of all castes, where the Orco expression levels in alates were significantly higher than in workers (Fig 4A; worker-female alate: $P = 0.0056$; worker-male alate: $P = 0.0480$; Kruskal-Wallis followed by Dunn’s test). In the legs, the expression of Orco was similar in workers, female and male alates; however, the level of expression in the soldier legs was significantly lower than in female and male alates (Fig 4A, $P = 0.0087$, Kruskal-Wallis followed by Dunn’s test). For the carcass, Orco expression in female alates was significantly higher than that in workers (Fig 4A, $P = 0.0056$, Kruskal-Wallis followed by Dunn’s test).

The temporal expression analysis showed that Orco was expressed in all castes as well as the early developmental stages, such as eggs and larvae. Orco expression levels did not differ significantly between the whole bodies of workers and alates (female and male) (Fig 4B, $P > 0.05$, Kruskal-Wallis followed by Dunn’s test). Eggs and nymphs showed the lowest expressions of Orco, which were significantly lower than workers and alates of both sexes (Fig 4B; eggs-worker: $P = 0.0126$; eggs-female alate: $P = 0.0089$; egg-male alate: $P = 0.0019$; nymph-worker: $P = 0.0106$; nymph-female alate: $P = 0.0075$; nymphs-male alate: $P = 0.0015$; Kruskal-Wallis followed by Dunn’s test). The expression levels of Orco in larvae were not significantly different from workers, pre-soldiers, or alates of both sexes (Fig 4B, $P > 0.05$, Kruskal-Wallis followed by Dunn’s test).

### Expression of Orco in response to different social environments

To investigate if Orco expression in worker antennae is affected by social conditions, termites were exposed to different levels of food deprivation, soldier percentage, and group size. In all three assays, termite mortality did not exceed 10% (S1 Fig). The results showed that the expression of Orco declined with increasing starvation period (Fig 5A). Workers starved for seven days had significantly lower expression of Orco than the ones starved for one day or not starved (Fig 5A, 0 day-1 day: $P = 0.2380$; 0 day-7 days: $P = 0.0001$; 1 day-7 days: $P = 0.0012$; Kruskal-Wallis followed by Dunn’s test).
The presence or absence of soldiers did not significantly affect the expression of Orco in the antennae of workers (Fig 5B; $P = 0.1093$ by Kruskal-Wallis test). Similarly, there were no significant differences in Orco expression among workers that were isolated as individuals or in groups of 15 or 100 ($P = 0.8809$, Kruskal-Wallis test).

**Discussion**

**Sequence and bioinformatics analysis of Orco**

In this study, we identified and characterized an Orco ortholog in C. formosanus. This gene encodes a protein with similar length and sequence identity with orthologs from five other termite species. These species represent different termite families and ecological niches, including subterranean (Rhinotermitidae), dampwood (Termopsidae), drywood (Kalotermitidae), and fungus-growing (Termitidae) termites [28, 29, 33, 43, 44]. Our results confirm that Orco is highly conserved across termites. Consistent with previous studies in other insects [14], only one Orco is found in C. formosanus. The predicted structure of C. formosanus Orco protein is highly similar to that in R. chinensis and O. formosanus [28, 29], with seven transmembrane regions that are characteristics of the 7tm_6 protein family, and an extracellular C-terminal end. However, different from the two mentioned termite species, the prediction of C. formosanus Orco structure provides a low statistical support for the existence of an additional transmembrane region before the 7tm_6 (probability < 0.2). The 7tm_6 region and extracellular C-terminus are characteristics of odorant receptors [52, 53]. With 63.93% amino acid sequence identity, the predicted 3D structure of C. formosanus Orco exhibited similar membrane topology with that in the fig wasp A. bakeri characterized by cryo-electron microscopy [15].

Our phylogenetic reconstruction supports the high conservation of Orco across insect taxa, and Orco proteins of Blattodea are evolutionary closer to Orthoptera than other orders. While the divergent OR family confers odorant specificity and co-varies with the chemical ecology of
insects, Orco is considered as one of the most conserved genes in insects in terms of sequence, protein structure, and function [52, 54].

**Spatial and temporal expression of Orco**

The gene expression analysis revealed that *C. formosanus* Orco is primarily expressed in the antennae (Fig 4A), the main olfactory organ for the expression of most ORs in insects [12]. It is worth noting that the antennal expression of Orco is significantly higher in alates than workers and soldiers (Fig 4A). This result is consistent with our previous morphological analysis that alates of both sexes possess longer antennae and higher numbers of antennal sensilla than the non-reproductive castes [32]. The discrepancy implies differential olfactory sensitivity between reproductive and non-reproductive individuals in *C. formosanus*. Compared with other individuals, alates have an expanded behavioral repertoire, which may rely on perception of a wider range of olfactory cues. Dispersal alates are exposed to the open environment, and they must detect the sex pheromone from mates and a suitable nest site for successful colony foundation [55, 56]. During the incipient stage of a colony, chemical signals are expected to mediate brood care and nestmate recognition by the young queens and kings [57, 58]. Workers and soldiers, by contrast, live in enclosed underground nests and perform collective behavior related to foraging, colony hygiene, and defense [5, 6]. The increase in Orco expression in alates is likely associated with the need for dispersal and mating. Antennal expression of Orco did not differ between workers and soldiers, despite the behavioral differences in the two castes. Additional olfactory mechanisms, such as ORs and neural circuits, warrant further investigation to understand the division of labor in termites.

In *C. formosanus*, Orco is expressed in all developmental stages, including eggs, larvae, presoldiers, and nymphs at varying levels (Fig 4B). Low expression of Orco in eggs and larvae has also been reported in holometabolous insects, such as the oriental fruit fly (*Bactrocera dorsalis*) [41] and the common green bottle fly (*Lucilia sericata*) [59]. Due to the tissue-specific distribution of Orco and body size variation among the developing individuals, the expression level of Orco in whole-body samples does not necessarily reflect olfactory sensitivity. The expression of Orco during early development, such as eggs and larvae, may be important for proper development of olfactory processing neurons in the brain. The expression of Orco is required for the development of antennal lobe glomeruli in several species of social Hymenoptera, including the honey bee (*A. mellifera*) [26] and two ants (*O. biroi* and *H. saltator*) [24, 25]. In *Drosophila*, Orco is fundamental for proper trafficking and structural localization of ORs in the outer ciliated dendrites of OSNs [18], which is fundamental for proper activity and responsiveness of these neurons to odorants. In *C. formosanus* eggs and larvae, Orco expression is possibly needed for proper structural arrangement of ORs in the developing OSNs. However, this hypothesis is yet to be tested.

In this study, RNA samples from the head, carcass, and whole body of soldiers were not obtained due to technical issues caused by defensive chemicals stored in their frontal gland. In *C. formosanus* soldiers, the frontal gland extends from head to abdomen, containing abundant lignoceric acid, hexacosanoic acid, and other lipids in aqueous mucopolysacharides [60, 61]. Additionally, the sticky fluid is enclosed in very thin epithelium (8–16 μm) [62], which makes removal of the intact gland from soldiers technically challenging.

**Expression of Orco in response to different social environments**

In addition to environmental conditions during postembryonic development, olfactory plasticity in insects can also be modulated by the immediate sensory environment. The plastic changes allow insects to respond to the chemical surroundings according to their physiological
and behavioral state [35]. In C. formosanus workers, starvation for seven days downregulated \textit{Orco} expression in the antennae, while this effect was not detected for one-day starvation (Fig 5A). Food availability is an important environmental factor that mediates foraging behavior in subterranean termites. Compared to conditions when different food sizes were provided, food deprivation was observed to promote exploratory tunneling behavior in C. formosanus [63], whereas the role olfaction plays in the process remains unclear. Generally, starvation enhances olfactory sensitivity in insects through changes in chemosensory gene expression for odorant perception and neuropeptides for olfactory processing [35, 39, 40, 64, 65]. Consistent with our observations of C. formosanus, Drosophila larvae exhibited a decrease in \textit{Orco} expression due to decreased activity in the insulin signaling pathway after starvation, although starvation enhanced their olfactory behavior toward certain odorants [65]. Nutritional effects on \textit{Orco} and olfactory sensitivity have also been demonstrated in adult \textit{D. melanogaster} [66]. Feeding on a high fat diet for 14 days decreased olfactory sensitivity in the flies, which was correlated with a decreased expression of \textit{Orco}, along with reduced expression of the insulin-like peptide 2 and the insulin receptor, as well as nine upregulated and 21 downregulated odorant binding proteins (OBPs) [66]. These studies suggest that \textit{Orco} is involved in nutrition-dependent olfactory behavior by downstream modulation of insulin signaling in OSNs and through complex interactions with other chemosensory genes. The reduced \textit{Orco} expression in C. formosanus workers does not necessarily indicate decreased olfactory sensitivity after starvation, as ORs, OBPs, and other chemosensory proteins are likely involved in olfactory regulation and may affect the overall sensitivity. The consequences of starvation on olfaction and the regulatory mechanisms await further investigation in termites.

Our results showed that \textit{Orco} expression in workers was not affected by the absence of soldiers, suggesting soldier cue does not influence short-term (seven day) olfactory capacity of C. formosanus workers (Fig 5B). In termites, workers and soldiers both constitute the foraging population, and 10% soldiers are generally found in C. formosanus colonies [67]. In R. flavipes, the presence of soldiers provided a social buffering effect, which altered worker behavior and reduced their mortality induced by predator-induced stress in two days [68]. In this study, the absence of soldiers without environmental stress did not influence worker \textit{Orco} expression in seven days, but it needs to be determined whether there is a longer-term effect. In the absence of soldiers, C. formosanus workers differentiate into soldiers through an intermediate pre-soldier stage. Previous studies showed that this transition took 35 days or longer, and the differentiation was triggered by increased juvenile hormone (JH) titers in workers and dependent on group size [69]. Many genes in the JH signaling and insulin pathways are involved in the caste transition [70], but changes in olfaction-related gene expression during this process and their roles in worker-soldier interactions require further investigation.

In social insects, colony functions are achieved through collective activities and interactions among colony members, and isolation from the social group affects individual fitness [24]. In C. formosanus, group size is a social factor regulating the self-organized tunneling activity during foraging, and tunnel construction is positively correlated with the density and flow rate of individuals through nestmate interactions, presumably based on olfaction and/or mechanical stimuli [71, 72]. Our study in C. formosanus showed that isolated workers did not differ in their antennal expression of \textit{Orco} compared with those in groups of 15 or 100 (Fig 5C). Similarly, in the silky ant, \textit{Formica fusca}, \textit{Orco} was not differentially expressed between isolated larvae and those exposed to social cues, while many other chemosensory genes, such as ORs and OBPs, were upregulated upon social stimulation [73]. Taken together, while interactions with soldiers and nestmate workers are both important for the social life of termites, social cues indicating soldier presence and group size did not alter \textit{Orco} expression in C. formosanus workers. As \textit{Orco} is crucial for all aspects of olfactory activity, its stable expression might be
important for maintaining the basic olfactory function in termites, which enables rapid behavioral response to the dynamic social environment through the changes of other odorant-specific chemosensory genes. Olfactory plasticity via changes in Orco expression is likely associated with physiological changes as induced by starvation and during caste differentiation. Future studies of additional mechanisms, such as ORs and neurotransmitters, are necessary to better understand the short-term fine tuning of olfaction-related behavior in termites.

Supporting information
S1 Table. Primers used in this study.
(DOCX)
S2 Table. Accession numbers of the Orco gene from different insect species utilized for phylogenetic analysis.
(DOCX)
S3 Table. Descriptive statistical results for the reference genes evaluated with BestKeeper.
(DOCX)
S1 Fig. Termite survivorship in gene expression analyses.
(DOCX)
S1 Data. Original data used in this study.
(XLSX)

Acknowledgments
We thank Justice Rougeau for assistance with sample preparation for the gene expression analyses, Junyan Chen for assistance with phylogenetic analysis, Arjun Khadka and Steven Richardson for their help with termite collection, and Joseph McCarthy for editing the manuscript.

Author Contributions
Conceptualization: Paula Castillo, Qian Sun.
Data curation: Paula Castillo.
Formal analysis: Paula Castillo.
Funding acquisition: Qian Sun.
Investigation: Paula Castillo.
Methodology: Paula Castillo, Claudia Husseneder, Qian Sun.
Resources: Qian Sun.
Supervision: Qian Sun.
Validation: Paula Castillo, Qian Sun.
Visualization: Paula Castillo, Qian Sun.
Writing – original draft: Paula Castillo.
Writing – review & editing: Claudia Husseneder, Qian Sun.
References

1. Blum M. Pheromonal bases of social manifestations in insects. In: Birch M, editor. Pheromones. Amsterdam: North-Holland Publishing; 1974. 190–9.

2. Bignell DE, Roisin Y, Lo N. Biology of termites: a modern synthesis. Springer, Heidelberg; 2010.

3. Su N-Y, Puche H. Tunneling activity of subterranean termites (Isoptera: Rhinotermitidae) in sand with moisture gradients. J. Econ. Entomol. 2003; 96(1):88–93. https://doi.org/10.1093/jee/96.1.88 PMID: 12650349

4. Sun Q, Haynes KF, Zhou X. Dynamic changes in death cues modulate risks and rewards of corpse management in a social insect. Funct. Ecol. 2017; 31:697–706.

5. Tian L, Zhou X. The soldiers in societies: defense, regulation, and evolution. Int. J. Biol. Sci. 2014; 10:296–308. https://doi.org/10.7150/ijbs.6847 PMID: 24644427

6. Korb J, Hartfelder K. Life history and development—a framework for understanding developmental plasticity in lower termites. Int. J. Biol. Sci. 2014; 10:296–308. https://doi.org/10.7150/ijbs.6847 PMID: 18979593

7. Bagnères A-G, Hanus R. Communication and social regulation in termites. Social Recognition in Invertebrates: Springer; 2015. pp. 193–248.

8. Matsuura K, Himuro C, Yokoi T, Yamamoto Y, Vargo EL, Keller L. Identification of a pheromone regulating caste differentiation in termites. Proc. Natl. Acad. Sci. USA. 2010; 107:12963–8. PMID: 20615972

9. Funaro CF, Böröczky K, Vargo EL, Schal C. Identification of a queen and king recognition pheromone in the subterranean termite Reticulitermes flavipes. Proc. Natl. Acad. Sci. 2018; 115:3888–93. https://doi.org/10.1073/pnas.1721419115 PMID: 29555778

10. Mitaka Y, Mori N, Matsuura K. Multi-functional roles of a soldier-specific volatile as a worker arresting, primer pheromone and an antimicrobial agent in a termite. Proc. Royal Soc. B. 2017; 284:20171134.

11. Zacharuk RY. Ultrastructure and function of insect chemosensilla. Annu. Rev. Entomol. 1980; 25:27–47.

12. Hansson BS, Stensmyr MC. Evolution of Insect Olfaction. Neuron. 2011; 72:698–711. https://doi.org/10.1016/j.neuron.2011.10.003 PMID: 22153368

13. McGuire SE, Le PT, Davis RL. The role of Drosophila mushroom body signaling in olfactory memory. Science. 2001; 293:1330–3. https://doi.org/10.1126/science.1062622 PMID: 11397912

14. Robertson HM. Molecular evolution of the major arthropod chemoreceptor gene families. Annu. Rev. Entomol. 2019; 64:227–42. https://doi.org/10.1146/annurev-ento-020117-043322 PMID: 30312552

15. Butterwick JA, del Mármol J, Kim KH, Kahlson MA, Rogow JA, Walz T, et al. Cryo-EM structure of the insect olfactory receptor Orco. Nature. 2018; 560:447–52. https://doi.org/10.1038/s41586-018-0420-8 PMID: 30111839

16. Benton R, Sachse S, Michnick SW, Vosshall LB. Atypical membrane topology and heteromeric function of Drosophila odorant receptors in vivo. PLoS biology. 2006; 4:e62. https://doi.org/10.1371/journal.pbio.0040020 PMID: 16402875

17. Stengl M, Funk NW. The role of the coreceptor Orco in insect olfactory transduction. J. Comp. Physiol. A. 2013; 199:897–909. https://doi.org/10.1007/s00359-013-0837-3 PMID: 23824225

18. Bahk S, Jones WD. Insect odorant receptor trafficking requires calmodulin. BMC biology. 2016; 14:1–14.

19. Liu Q, Liu W, Zeng B, Wang G, Hao D, Huang Y. Deletion of the Bombyx mori odorant receptor coreceptor (BmOrco) impairs olfactory sensitivity in silkworms. Insect Biochem. Mol. Biol. 2017; 86:58–67. https://doi.org/10.1016/j.ibmb.2017.05.007 PMID: 28577927

20. Fandino RA, Haverkamp A, Bisch-Knaden S, Zhang J, Bucks S, Nguyen TAT, et al. Mutagenesis of odorant coreceptor Orco fully disrupts foraging but not oviposition behaviors in the hawkmoth Manduca sexta. Proc. Natl. Acad. Sci. USA. 2019; 116:15677–85. https://doi.org/10.1073/pnas.1902089116 PMID: 31320583

21. Tsoumani KT, Belavilas-Trovas A, Gregoriou M-E, Mathiopoulos KD. Anosmic flies: what Orco silencing does to olive fruit flies. BMC Genet. 2020; 21:1–10.

22. Zhang X, Liu P, Qin Q, Li M, Meng R, Zhang T. Characterizing the role of Orco gene in detecting aggregation pheromone and food resources in Protaetia brevitarsis Leiwis (Coleoptera: Scarabaeidae). Front. physiol. 2021; 12:649590. https://doi.org/10.3389/fphys.2021.649590 PMID: 33927641

23. Task D, Potter CJ. Rapid degeneration of Drosophila olfactory neurons in Orco mutant maxillary palps. microPubl., biol. 2021; 2021. https://doi.org/10.17912/micropub.biology.000398 PMID: 34007957
24. Trible W, Olivos-Cisneros L, McKenzie SK, Saragosti J, Chang N-C, Matthews BJ, et al. Orco mutagenesis causes loss of antennal lobe glomeruli and impaired social behavior in ants. Cell. 2017; 170:727–35. https://doi.org/10.1016/j.cell.2017.07.001 PMID: 28802042

25. Yan H, Opachaloemphak M, Mancini G, Yang H, Gallitto M, Mlejnek J, et al. An engineered orco mutation produces aberrant social behavior and defective neural development in ants. Cell. 2017; 170:736–47. e739. https://doi.org/10.1016/j.cell.2017.06.051 PMID: 28802043

26. Zhao X, Slone JD, Rokas A, Berger SL, Liebig J, Ray A, et al. Phylogenetic and transcriptomic analysis of chemosensory receptors in a pair of divergent ant species reveals sex-specific signatures of odor coding. PLOS Genet. 2012; 8:e1002930. https://doi.org/10.1371/journal.pgen.1002930 PMID: 22952454

27. Gadenne C, Barrozo RB, Anton S. Plasticity in insect olfaction: to smell or not to smell? Annu. Rev. Entomol. 2016; 61:317–33. https://doi.org/10.1146/annurev-ento-010715-023523 PMID: 26982441

28. Chao MY, Komatsu H, Fukuto HS, Dionne HM, Hart AC. Feeding status and serotonin rapidly and reversibly modulate a Caenorhabditis elegans chemosensory circuit. Proc. Natl. Acad. Sci. USA. 2004; 101:15512–7. https://doi.org/10.1073/pnas.0403369101 PMID: 15492222

29. Tong J, Mannea E, Le N, Sun Q. Comparative antennal morphometry and sensilla organization in the reproductive and non-reproductive castes of the Formosan subterranean termite. Insects. 2021; 12:576. https://doi.org/10.3390/insects12070576 PMID: 34202744

30. Mitaka Y, Kobayashi K, Mikheyev A, Watanabe Y, Matsuura K. Caste-specific and sex-specific expression of chemoreceptor genes in a termite. PLoS One. 2016; 11:e0146125. https://doi.org/10.1371/journal.pone.0146125 PMID: 26760975

31. McKenzie SK, Fetter-Pruneda I, Ruta V, Kronauer DJC. Transcriptomics and neuroanatomy of the clonal raider ant implicate an expanded clade of odorant receptors in chemical communication. Proc. Natl. Acad. Sci. USA. 2016; 113:14091–6. https://doi.org/10.1073/pnas.1608001113 PMID: 27911792

32. Castillo P, Le N, Sun Q. Comparative antennal morphometry and sensilla organization in the reproductive and non-reproductive castes of the subterranean termite. Insects. 2020; 11:125. https://doi.org/10.3390/insects11070576 PMID: 34202744

33. Mitaka Y, Kobayashi K, Mikheyev A, Watanabe Y, Matsuura K. Caste-specific and sex-specific expression of chemoreceptor genes in a termite. PLoS One. 2016; 11:e0146125. https://doi.org/10.1371/journal.pone.0146125 PMID: 26760975

34. Gadenne C, Barrozo RB, Anton S. Plasticity in insect olfaction: to smell or not to smell? Annu. Rev. Entomol. 2016; 61:317–33. https://doi.org/10.1146/annurev-ento-010715-023523 PMID: 26982441

35. Anson S, Rössler W. Plasticity and modulation of olfactory circuits in insects. Cell Tissue Res. 2021; 383:149–64. https://doi.org/10.1007/s00441-020-03329-z PMID: 33275182

36. Chao MY, Komatsu H, Fukuto HS, Dionne HM, Hart AC. Feeding status and serotonin rapidly and reversibly modulate a Caenorhabditis elegans chemosensory circuit. Proc. Natl. Acad. Sci. USA. 2004; 101:15512–7. https://doi.org/10.1073/pnas.0403369101 PMID: 15492222

37. Tong J, Mannea E, Le N, Sun Q. Comparative antennal morphometry and sensilla organization in the reproductive and non-reproductive castes of the Formosan subterranean termite. Insects. 2021; 12:576. https://doi.org/10.3390/insects12070576 PMID: 34202744

38. Farhadian SF, Suárez-Fariñas M, Cho CE, Pellegrino M, Vosshall LB. Post-fasting olfactory, transcrip -tional, and feeding responses in Drosophila. Physiol. Behav. 2012; 105:544–53. https://doi.org/10.1016/j.physbeh.2011.09.007 PMID: 21945372

39. Jin S, Zhou X, Gu F, Zhong G, Yi X. Olfactory plasticity: variation in the expression of chemosensory receptors in Bactrocera dorsalis in different physiological states. Front. Physiol. 2017; 8:672. https://doi.org/10.3389/fphys.2017.00672 PMID: 28959208

40. Poivet E, Galliot A, Montagné N, Senin P, Monsemblé C, Legeai F, et al. Transcriptome profiling of starvation in the peripheral chemosensory organs of the crop pest Spodoptera littoralis caterpillars. Insects. 2021; 12:573. https://doi.org/10.3390/insects12070573 PMID: 34201462

41. Zheng W, Zhu C, Peng T, Zhang H. Odorant receptor co-receptor Orco is upregulated by methyl eugenol in male Bactrocera dorsalis (Diptera: Tephritidae). J. Insect. Physiol. 2012; 58:1122–7. https://doi.org/10.1016/j.jinsphys.2012.05.011 PMID: 22634470

42. Tokoro M, Takahashi M, Tsunoda K, Yamaoka R. Isolation and primary structure of trail pheromone of the termite, Coptotermes formosanus Shiraki (Isoptera: Rhinotermitidae). Wood Res: Bull. Wood Res. Inst. Kyoto Univ. 1989; 76:29–38.

43. Terrapon N, Li C, Robertson HM, Ji L, Meng X, Booth W, et al. Molecular traces of alternative social organization in a termite genome. Nat. Commun. 2014; 5:1–12.
63. Hedlund JC, Henderson G. Effect of available food size on search tunnel formation by the Formosan subterranean termite (Isoptera: Rhinotermitidae). J. Econ. Entomol. 1999; 92:610–6.
64. Ko KL, Root CM, Lindsay SA, Zaninovich OA, Shepherd AK, Wasserman SA, et al. Starvation promotes concerted modulation of appetitive olfactory behavior via parallel neuromodulatory circuits. Elife. 2015; 4:e08298. https://doi.org/10.7554/eLife.08298 PMID: 26208339

65. Slankster E, Kollala S, Baria D, Dailey-Krempel B, Jain R, Odell SR, et al. Mechanism underlying starvation-dependent modulation of olfactory behavior in Drosophila larva. Sci. Rep. 2020; 10:1–14.

66. Jung J, Kim D-I, Han G-Y, Kwon HW. The effects of high fat diet-induced stress on olfactory sensitivity, behaviors, and transcriptional profiling in Drosophila melanogaster. Int. J. Mol. Sci. 2018; 19:2855.

67. Haverty ML. The proportion of soldiers in termite colonies: a list and a bibliography. Sociobiology. 1977; 2:199–216.

68. Tian L, Preisser EL, Haynes KF, Zhou X. Social buffering in a eusocial invertebrate: termite soldiers reduce the lethal impact of competitor cues on workers. Ecology. 2017; 98:952–60. https://doi.org/10.1002/ecy.1746 PMID: 28122113

69. Mao L, Henderson G. Group size effect on worker juvenile hormone titers and soldier differentiation in Formosan subterranean termite. J. Insect Physiol. 2010; 56:725–30. https://doi.org/10.1016/j.jinsphys.2009.12.014 PMID: 20045002

70. Miura T, Maekawa K. The making of the defensive caste: Physiology, development, and evolution of the soldier differentiation in termites. Evol. Dev. 2020; 22:425–37. https://doi.org/10.1111/ede.12335 PMID: 32291940

71. Su N-Y, Lee S-H. Tunnel volume regulation and group size of subterranean termites (Isoptera: Rhinotermitidae). Ann. Entomol Soc. 2009; 102:1158–64.

72. Bardunias PM, Su N-Y. Queue size determines the width of tunnels in the Formosan subterranean termite (Isoptera: Rhinotermitidae). J. Insect Behav. 2010; 23:189–204.

73. Pulliainen U, Morandin C, Bos N, Sundström L, Schultzner E. Social environment affects sensory gene expression in ant larvae. Insect Mol. Biol. 2022; 31:1–9. https://doi.org/10.1111/imb.12732 PMID: 34418191