Ant nurse workers exhibit behavioral and transcriptomic specialization on larval stage but not caste

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Abstract

Division of labor within and between the worker and queen castes is thought to underlie the tremendous success of social insects. Colonies might benefit if subsets of nurse workers specialize in caring for larvae of a certain stage or caste, given that nutritional requirements depend on larval stage and caste. We used short- (<1 hr) and long-term (ten days) behavioral observations together with transcriptomic analysis to determine whether nurses of the pharaoh ant *Monomorium pharaonis* exhibit such behavioral and/or physiological specialization. We found that nurses were behaviorally specialized based on larval instar but not on larval caste. This specialization was widespread, with 56% of nurses in the short-term and between 22-27% in the long-term showing significant specialization. We also found transcriptomic signatures of nurse specialization on larval stage but not caste. Genes associated with nurse specialization included vitellogenin and mrjp2, which have previously been implicated in the transfer of nutrition from nurse to larvae and the regulation of larval development and caste, as well as other genes coding for secreted proteins, which may also be passed from nurses to larvae via trophallaxis. Altogether, our results provide the first evidence in any social insect for a division of labor among nurse workers based on larval stage.

Keywords: eusociality, division of labor, brood care, behavioral specialization, gene expression, ant nurse

Introduction

Division of labor, one of the defining characteristics of eusociality, is believed to be the primary reason for the tremendous success of social insects [1-3]. Increased worker efficiency within colonies is thought to be the main colony-level benefit of division of labor. Behavioral specialists, through learning, are expected to be more efficient than generalists [2, 4, 5], but see [6, 7]. Indeed, social insect behavioral specialists demonstrate increased efficiency in nest emigration [8], nest excavation [9], undertaking [10, 11], and response to sucrose [12].

Within this system of division of labor, workers specialize on tasks including brood care, foraging, and nest defense while queens specialize on reproduction [2, 3, 13]. In many species, worker specialization depends on age, with younger workers generally performing tasks inside the nest (e.g. brood care) and older workers performing tasks outside the nest (e.g. foraging) [2, 4, 13, 14]. Worker specialization may also depend on body size and shape, as many species exhibit morphologically distinct worker sub-castes that perform different roles within the colony [2, 13]. Worker variation in behavioral specialization can also occur independently of age and morphology [15, 16]. This interindividual variability can be the result of genetic diversity among workers [17], environmental differences during early development [18, 19], variation in adult nutritional state [20-22], prior experience [23], and the social environment [24].

Cooperative brood care, which includes feeding, grooming, and carrying brood, is one of the most important suites of tasks performed by adult workers [2, 3]. Different larvae have different nutritional requirements depending upon their caste and developmental stage [25]. For

example, young larvae of many ant species are fed exclusively liquid food via nurse-larva trophallaxis while older larvae are also fed solid protein [26-28]. Furthermore, late-instar larvae require more frequent and longer feedings than early-instar larvae [25, 29].

The caste fate of developing larvae in social insects is socially regulated by nurse workers, often based on the quantity and quality of nutrition provided to larvae [30-34]. In ants, adult queens tend to have higher fat and protein content relative to workers, and it is usually assumed that queen-destined larvae are fed different quantities and qualities of food compared to worker-destined larvae [31, 35-37]. Furthermore, recent research in the ant *Camponotus floridanus* found that nurse workers transfer juvenile hormone, microRNAs, hydrocarbons, various peptides, and other compounds during feeding [38], providing a potential further mechanism for nurses to provide stage- and caste-specific nutrition to larvae that may regulate larval development. Recent research in honey bees (*Apis mellifera*) suggests that nurse workers do show both behavioral and physiological specialization [39, 40] on larval caste. However, these studies did not test for specialization on larval instar and, to the best of our knowledge, no previous study has investigated the potential for nurse specialization on caste or larval stage in ants.

In this study, we asked whether individual pharaoh ant (*Monomorium pharaonis*) nurse workers exhibited behavioral specialization on different larval stages or castes, as measured on both short (< 1 hr) and long (10 days) timescales. To complement our behavioral observations, we also tested whether nurse workers show physiological specialization, as reflected by their transcriptomic profiles in both their heads and abdomens (i.e. gasters) based on stage and caste of larvae fed.

Methods

(a) Background and overall design

We created experimental *Monomorium pharaonis* colonies used in this study by mixing multiple, genetically similar stock colonies. We fed the colonies twice per week with an agar-based synthetic diet [41], frozen crickets, and mealworms. We maintained all colonies at 27 ± 1 °C, 50% relative humidity, and a 12:12 LD cycle. We conducted all nursing observations manually using a dissecting microscope and red light. To keep the temperature constant during behavioral observations, we kept the colonies on a heating pad set to 27 °C.

M. pharaonis larvae have three instars [42] that are distinguishable by body size, body shape, hair abundance, and hair morphology [43]. Although reproductive-destined larvae (males and gynes) cannot be distinguished from worker-destined larvae as eggs or 1st instar larvae, they can be readily distinguished after the 1st instar [43, 44]. Since colonies usually only produce new gynes and males in the absence of fertile queens [45, 46], we set up queen-absent colonies, which rear both worker- and reproductive-destined larvae, when testing for specialization on larval caste. For both the behavioral observations and transcriptomic analyses, we classified the larvae into five stages based on size and hair morphology: 1st instar, 2nd instar, and small, medium, and large 3rd instar (see [37, 43] for details). However, for all behavioral analyses, we grouped all observations on 3rd instars to increase the sample size.

(b) Short-term observations

To determine whether nurses exhibit short-term specialization based on larval instar or caste, we observed colonies until we saw a nurse feed a larva of any instar or caste and followed that nurse for as long as possible (max = 67 minutes). We recorded each time the nurse fed a

larva, as well as the stage and caste of the larva, using the event logging software "BORIS" [47]. We defined feeding behavior as a stereotypical behavioral interaction between the nurse worker and larva in which the mouthparts of the nurse and larva were in contact for at least three seconds. We defined both the transfer of solid food particles and liquid food via trophallaxis from nurse to larva as feeding behavior and did not distinguish between these two nursing behaviors.

We conducted observations on both queen-present and queen-absent colonies to determine whether nurses specialized on larval instar (using queen-present colonies) or caste (using queen-absent colonies). For the queen-absent colonies, we began observations two weeks after queen removal. At this time, all larvae had matured to the 3rd instar stage which allowed us to morphologically distinguish between worker- and reproductive-destined larvae. We restricted subsequent analysis to nurses we observed feeding at least three separate larvae.

(c) Long-term observations

Next, we attempted to test whether individually-marked nurses in queenless colonies express long-term specialization (across ten days). We wanted to track nurses for at least ten days because this time scale includes the entire amount of time that *M. pharaonis* workers tend to perform nursing behavior [14]. To track nurses over time, we anesthetized individuals with carbon dioxide and marked heads and abdomens of workers with a dot of paint using Sharpie extra-fine point, oil-based paint pens [6, 22, 48]. In each of five colonies, we uniquely painted 63 focal individuals with paint dots on their heads and abdomens using combinations of eight colors. To control for potential behavioral effects of the paint, we painted all remaining adult workers in the colonies with black dots on their heads and abdomens. To control for possible

effects of nurse age on potential behavioral specialization, we collected the 63 focal individuals as approximately one day old callow workers, allowing us to follow an age-matched cohort. The paint marks remained on the ants for the full ten observation days. Although we observed the painted ants attempting to groom the paint off themselves and others, the paint did not seem to affect worker mortality and the workers still performed the full range of brood care behaviors.

We constructed the five queen-less colonies with 400 workers and 2.5 mL of brood (i.e. approximately 500 eggs, larvae, and pupae of different stages). In two of these colonies, we recorded all nursing, larval grooming, and larval carrying behavior conducted by any of the 63 focal individuals in each of the colonies. In the other three colonies, we focused only on nursing behavior because nursing events were rarer than the other behaviors. Unfortunately, these colonies failed to produce reproductive-destined larvae, likely due to minor regular disturbances associated with long-term observation. Therefore, we were unable to test for long-term specialization on larval caste and focused only on specialization on larval instar. We defined grooming as an interaction between worker mandibles and a larva for a minimum of three seconds. As in the short-term observations, if the worker's mandibles interacted with the larva's mandibles for at least three seconds, the behavior was classified as nursing. We defined carrying as a worker lifting a larva with her mandibles and transporting the larva to another location. We observed all colonies for three hours per day for ten consecutive days. We restricted subsequent analysis to individuals we observed feeding, grooming, or carrying at least three separate larvae.

(d) Statistical analysis of behavioral specialization

We performed all statistical analyses in R version 3.4.1 [49]. For both short and long-term observations, we used the R package lme4 [50] to fit generalized linear mixed models

(GLMMs) for larval identity with the identity of the nurse as a random effect and observer, colony identity, and nurse age as fixed effects when appropriate. To test for nurse specialization on larval instar, we grouped 1st and 2nd instar larvae as "young" larvae and all 3rd instar larvae as "old" larvae. This grouping is biologically meaningful as 1st and 2nd instar larvae are fed solely a liquid diet while 3rd instar larvae are also fed solid food [26-28]. We analyzed all models using binomial distributions with Laplace approximations. We evaluated the significance of both fixed and random effects using likelihood ratio (LR) tests. LR tests are appropriate for evaluating the significance of random effects in binomial models when the models contain fewer than three random effects [51]. A significant random effect of nurse identity in these models indicates that there is variation among individual nurses for degree of behavioral specialization, providing initial evidence for behavioral specialization within colonies.

We used binomial tests to ask whether each individual significantly specialized on young versus old or reproductive versus worker larvae based on recorded observations.We restricted analysis to nurses with at least six observations because this is the minimum number of observations that could potentially identify significant ($P < 0.05$) specialization with a binomial test (e.g., if a nurse fed one age class six times and fed the other age class zero times). We estimated the expected frequency (i.e. "probability of success" in the binomial test) of interacting with larvae of one stage/caste relative to another stage/caste based on the observed proportion of interactions for the two stages/castes (e.g., the number of observed interactions between nurses and 1st instar larvae relative to 3rd instar larvae). In order to first determine whether any individuals could be confidently classified as specialists, we first used binomial tests with a type I error rate corrected for multiple comparisons. Given that some individuals were confidently identified as specialists with these conservative criteria, we estimated the overall proportion of

specialist versus non-specialist nurses in our study colonies using a type I error rate of 0.05 for each binomial test run separately for each individual nurse.

(e) Gene expression analysis

Previously, we performed RNA-sequencing on larvae of various developmental stages and nurses feeding such larvae [52]. Warner et al. [52] focused on the evolution of caste-biased genes across development; here we utilized the published data to compare transcriptomes of nurses feeding different larval stages and castes. In the next paragraph, we briefly summarize the sample collection protocol. For details on RNA extraction, library preparation and sequencing, and mapping of reads to generate expected gene-level counts, refer to [52].

Thirty colonies were established and assigned to one of five developmental stages [52], corresponding to the five larval developmental stages described above (see also figure S1). For half of the colonies, queens were removed to stimulate the production of reproductive (male- and queen-destined) larvae, such that the study contained three replicate queen-absent and three replicate queen-present colonies for each developmental stage. Each colony was sampled at a single time point. The study was performed longitudinally such that a cohort of larvae was tracked across development and sampled as appropriate (i.e. L1 colonies were sampled after ~three days, L2 after ~six days, and so on). Nurses were collected when observed through a dissecting microscope feeding larvae of the appropriate developmental stage. After all nurses were collected, larvae of the appropriate stage were collected. Nurses feeding worker-destined larvae were collected from queen-present and queen-absent colonies, while nurses feeding reproductive-destined larvae were collected from solely queen-absent colonies. For each sample, ten individuals were collected and pooled. RNA was extracted from whole bodies of larvae,

while heads and abdomens (i.e. gasters) were processed separately for nurses. We combined data from queen-absent and queen-present colonies, as we previously detected 0 DEGs between nurses based on queen presence (Warner et al., unpublished data).

To broadly investigate whether worker nurse expression profiles varied according to the developmental stage of larvae they fed, we first compared the average expression profile (averaged across all replicates of a given stage) of each stage by calculating pairwise Pearson correlations, in which each nurse stage was represented by a vector of average expression for each gene. This analysis, and the following differential expression analysis, included all genes after filtering out lowly expressed genes (FPKM < 1 in ½ samples).

Next, we utilized the package EdgeR [53] to identify differentially expressed genes. We constructed a glm-like model, including larval stage fed, replicate and queen presence as additive effects to identify genes differentially expressed between young and old nurses (1st instar versus large 3rd instar; separately for head and abdomens). We chose to focus our inquiry on nurses feeding very young versus very old larvae to maximize the potential for differential expression based on the age of larvae fed; intermediate comparisons showed intermediate numbers of differentially expressed genes (results not shown). We identified genes differentially expressed between nurses feeding worker- and reproductive-destined larvae across all stages using a model with replicate, stage, and larval caste. We identified differentially expressed genes as those with FDR < 0.05. We calculated GO term enrichment of differentially expressed genes using the R package GOstats, with a cut-off P-value of 0.05 [54].

To test whether genes found to be differentially expressed between nurses tended to be secreted in the model *Drosophila melanogaster*, we compiled a list of genes annotated as coding for secreted proteins according to the online tool GLAD [55]. From this list, we identified

secreted proteins with orthologs in *M. pharaonis* using a recently created orthology map between *M. pharaonis*, *Apis mellifera*, and *D. melanogaster* (Warner et al, unpublished data). We estimated the association between a gene's likelihood to be differentially expressed and secreted, removing all genes for which a *D. melanogaster* ortholog was not detected. We generated plots using the R packages ggplot2 [56] and VennDiagram [57].

Results

(a) Short-term specialization on larval stage

We observed 52 nurses feed at least three larvae (mean = 8.8 feeding events) and we included these nurses in the GLMMs. The random effect of individual was significant, suggesting nurse specialization on either young or old larvae (table 1). Additionally, the effect of observer was significant (table 1). This observer effect was likely due to our observation scheme because we made an attempt to balance the number of old and young recorded short-term nursing events. To test for specialization of individual nurses, we used an expected proportion of old larvae relative to young plus old larvae of 0.781 (the proportion of old larvae fed across all individuals in long-term nursing observations) for binomial tests. When using a type I error rate corrected for multiple comparisons, which should produce a conservative estimate of the frequency of specialists across the whole study, we classified about 56% (18/32) of nurses as specialists (bonferroni-adjusted $P < 0.05$). When using a type I error rate of 0.05, which should yield an unbiased estimate of the frequency of specialists versus generalists within colonies, we again classified about 56% (18/32) of nurses as specialists and these specialists performed about 65% (242/375) of the observed feedings (figure 1).

(b) Short-term specialization on caste

We observed 22 nurses feed at least three larvae (mean = 5.64 feeding events). Of those 22 nurses, 18 fed both worker- and reproductive-destined larvae while the remaining four fed only reproductive-destined larvae. The random effect of individual in the GLMM was not significant (table 1). In the binomial tests, we included the ten nurses we observed feed at least six larvae and used an expected proportion of reproductive-destined larvae of 0.534. When correcting for multiple comparisons, we classified zero nurses as specialists. When using a type I error rate of 0.05, we classified 10% (1/10) of nurses as specialists and this specialist performed about 6% (9/142) of the observed feedings (figure 1).

(c) Long-term nursing specialization on larval stage

We observed at least three nursing events for 40 nurses (mean = 12.9 nursing events). The effect of individual and the identity of both the colony and observer were significant on larval stage (table 1). The age of the nurse was not significant (table 1). In the binomial tests, we included the 30 nurses we observed feed at least six larvae and used an expected proportion of old larvae of 0.781. When correcting for multiple comparisons, we classified 20% (6/30) of nurses as specialists on larval stage. When using an uncorrected type I error rate of 0.05, we classified about 27% (8/30) of nurses as specialists and these specialists performed about 42% (201/480) of the observed feedings (figure 2). Specialists performed significantly more feedings than non-specialists (Mann-Whitney-Wilcoxon test; W= 19.5, P = 0.0013).

(d) Long-term grooming specialization on larval stage

We observed 32 individuals grooming larvae at least three times (mean = 33.9 grooming events). The effect of individual and the identity of the colony were significant (table 1). The age of the nurse and the identity of the observer were not significant (table 1). In the binomial tests, we included the 24 nurses we observed groom at least six larvae and used an expected proportion of old larvae of 0.581. When correcting for multiple comparisons, we classified about 13% (3/24) of nurses as specialists. When using an uncorrected type I error rate of 0.05, we classified 25% (6/24) of nurses as specialists and these specialists performed about 39% (406/1053) of the observed groomings (figure 2). The number of groomings performed by specialists and non-specialists was not significantly different (W = 29, P = 0.1021).

(e) Long-term carrying specialization on larval instar

We observed 17 individuals carrying a larva at least three times (mean = 13.4 carrying observations). The effect of individual and the identity of the colony were significant (table 1). The age of nurse and the identity of the observer were not significant (table 1). In the binomial tests, we included the nine nurses we observed carrying at least six different larvae and used an expected ratio of old to young of 0.107. When correcting for multiple comparisons, we classified zero nurses as specialists. When using an uncorrected type I error rate of 0.05, we classified about 22% (2/9) of nurses as specialists and these specialists performed about 12% (24/197) of the carrying observations (figure 2). The number of groomings performed by specialists and non-specialists was not significantly different (W = 13, P = 0.100).

(f) Transcriptomic analysis

Nurse gene expression profiles generally diverged over time; that is, nurses feeding larvae of similar developmental stages exhibited qualitatively more similar expression profiles (figure 3). To identify genes associated with nurse specialization based on larval developmental stage, we chose to focus on genes differentially expressed between nurses feeding 1st instar and large 3rd instar larvae, as those samples span the extremes of larval age and exhibited the most drastic differences in profile (figure 3). We identified 209 and 173 differentially expressed genes (DEGs) between nurses feeding young (i.e. 1st instar) and old (i.e. large 3rd instar) worker larvae in heads and abdomens respectively (figure 4*a*,*b*). In both heads and abdomens, we identified more up-regulated genes in young nurses compared to old (two-sided binomial, null hypothesis of 50% upregulated in young nurses; heads: N = 209, P < 0.001; abdomens: N = 173, P < 0.001). There was a positive association between genes up-regulated in young nurse heads and abdomens (Figure 4*c*; χ^2 = 312, df = 1, P < 0.001), as well as between genes up-regulated in old nurse heads and abdomens (figure 4*c*; χ^2 = 260, df = 1, P < 0.001), indicating that some genes associated with nurse specialization are differentially expressed throughout nurse bodies.

For genes associated with each nurse type, gene ontology was largely dominated by metabolism-related categories (table S1). However, genes up-regulated in young nurse heads were also associated with isoprenoid (a type of hydrocarbon) processing, and genes up-regulated in young nurse abdomens were associated with transport and localization.

Finally, genes that were differentially expressed in nurses based on larval stage were more likely to be secreted in *Drosophila melanogaster* (χ^2 = 29.1, df = 1, P < 0.001; 18 secreted DGEs out of 148 total DGEs with orthologs in *D. melanogaster*. 178 genes have secreted orthologs in *D. melanogaster*, out of 5391 genes in the analysis). Nearly all of these secreted

DEGs were upregulated in young nurses (14/14 in heads, 9/10 in abdomens, see table S2 for complete list of DEGs based on larval stage fed).

In contrast to our results for nurse specialization on different worker larval stages, we detected very few genes differentially expressed between nurses feeding alternate worker- versus reproductive-destined larvae (10 DEGs in heads and 0 DEGs in abdomens). However, it is notable that all 10 genes were upregulated in nurses feeding reproductive larvae. These genes included three genes (Cytochrome P450 4g15 [2 copies], Cytochrome P450 6k1) in the Cytochrome p450 complex and two genes (fatty acyl-CoA reductase I, fatty acid synthase) involved in lipid production, according to NCBI annotation.

Discussion

The tremendous ecological success of social insects is thought to be primarily due to efficient division of labor within colonies. Here we provide to the best of our knowledge the first evidence for the existence of a division of labor within nurse workers based on larval instar. We found evidence for behavioral specialization in the short-term (less than an hour) for nursing and in the long-term (over 10 days) for nursing, grooming, and the carrying of larvae. Furthermore, we found evidence of nurse physiological specialization on feeding different larval instars, as nurses feeding larvae of similar developmental stages exhibited more similar expression profiles. Finally, we found no evidence for behavioral or physiological specialization based on larval caste.

Nurses specialized on either old (3rd instar) or young (1st and 2nd instar) larvae and this specialization was consistent across nursing, grooming, and carrying behavior as the effect of individual was significant in GLMMs for all behaviors. In the short-term, we classified 56% of

nurses as specialists and in the long-term, we classified 27%, 25%, and 22% of workers as specialists on nursing, grooming, and carrying respectively. The specialization of nurse workers on old or young larvae might be explained by specialization on trophallaxis (i.e. feeding liquids) or feeding solid food particles since young larvae are fed only a liquid diet while old larvae are also fed solid protein [26-28]. If so, the nurses specialized for trophallaxis may play a disproportionately large role in regulating larval development since trophallactic fluid contains not only nutrition but also juvenile hormone, microRNAs, hydrocarbons, various peptides, and other compounds [38].

In support of our behavioral observations, we found that nurses demonstrated physiological specialization as evidenced by differences in gene expression profile according to larval stage fed. This specialization was most pronounced between nurses feeding 1st and large 3rd instar larvae, with most genes upregulated in the tissues of 1st instar nurses. In theory, the differentially expressed genes we detected in nurse tissues could directly affect larval development if the proteins were secreted by nurses and transferred to larvae via trophallaxis [33]. Intriguingly, genes with *D. melanogaster* orthologs that are known to be secreted were overrepresented among genes associated with nurse specialization. Genes upregulated in 1st-instar larvae included genes such as vitellogenin (vg-2) [58] and a member of the major royal jelly protein family (MJRP-1) [59], both of which have been implicated in the production and transfer of proteinaceous food to honey bee larvae, which then shapes larval development and caste fate [60] (figure 4a). Interestingly, two odorant binding proteins (OBP) were also differentially expressed in nurse abdomens (figure 4*a*). These OBPs likely play a role in communication between nurses and larvae [61].

Contrary to findings in honey bees [39, 40], we found no behavioral or transcriptomic evidence for nurse specialization on larval caste. This lack of specialization is somewhat surprising, given that worker- and reproductive-destined larvae likely have different nutritional needs [31, 35-37]. In honey bees, caste determination occurs relatively late in development and over a period of time, as queen-worker inter-castes can be produced by experimental manipulation of diet late in development [32, 62, 63]. Therefore, honey bee nurses are likely essential to fine-tune caste dimorphism [32]. The precise mechanism of caste determination is currently unknown in *M. pharaonis*, but caste is socially regulated early in development, during the 1st larval instar [37], possibly via culling of reproductive-destined larvae by nurse workers [58]. Interestingly, in both measured tissues (heads and abdomens), we identified more genes upregulated in 1st-instar versus large 3rd-instar nurses. These genes might be involved in caste regulation that occurs before the caste of larvae can be morphologically distinguished by human observers [42, 43].

Conclusion

This study describes a previously undocumented form of division of labor within ant nurse workers: specialization based on larval instar. We found evidence for this specialization in three different brood care behaviors and in the transcriptomic profiles of nurse workers. Contrary to findings in honey bees, we found no evidence for specialization of nurse workers on larval caste. Further research is necessary to characterize the implications of nurse specialization, elucidate the detailed molecular and physiological underpinnings, and to determine how widespread specialization is across ants and other social insects.

Ethics

We kept lab stocks of *Monomorium pharaonis* colonies following conditions of USDA permits P526P-14-04241 and P526P-17-02328.

Data accessibility

Data supporting this paper are included as supplemental files.

Authors' Contributions

JTW helped design and carry out all behavioral observations and analyses and wrote the manuscript. MRW performed transcriptomic analyses and helped write the manuscript. AK and BJC helped design and carry out the behavioral observations. TAL conceived of and helped design the project, helped with all data analyses, and helped write the manuscript. All authors gave final approval for publication.

Competing Interests

We have no competing interests.

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Table and figure captions

Table 1. Summary of effects of factors on short- and long-term nurse behavior on likelihood ratio tests of GLMMs

Figure 1. Short-term nurse worker specialization on young vs old larvae (A) and worker- vs reproductive-destined larvae (B). The dots represent the proportions of old larvae (A) or reproductive larvae (B) that each nurse worker fed and the error bars are the confidence intervals from the binomial tests. The horizontal line represents the expected proportion based on overall observed proportion of interactions. The black dots represent nurse workers with proportions significantly different than the expected proportions when using a type I error rate of 0.05 (specialists). The gray dots represent nurse workers with proportions that do not differ from the expected proportion (non-specialists). In plot A, a proportion of 1 means the nurse worker fed only old larvae while a 0 means the worker fed only young larvae. In plot B, a proportion of 1 means the nurse worker fed only reproductive-destined larvae while a 0 means the worker fed only worker-destined larvae.

Figure 2. Nurse worker specialization on young vs old larvae for nursing (A), grooming (B), and carrying (C). The dots represent the proportions of old larvae that each nurse worker cared for and the error bars are the confidence intervals from the binomial tests. The horizontal line represents the expected proportion based on overall observed proportion of interactions. The black dots represent nurse workers with proportions significantly different than the expected proportions when using a type I error rate of 0.05. The gray dots represent nurse workers with proportions that do not differ from the expected proportion. A proportion of 1.0 means the nurse worker cared for only old larvae while a 0 means the worker cared for only young larvae.

Figure 3. Nurses observed feeding larvae of similar ages have more correlated transcriptomic profiles than nurses observed feeding larvae of different ages, reflecting physiological specialization of nurses based on the stage of larvae they fed. The strength of correlation decreases across larval development in nurse tissues (A, B) in a qualitatively similar manner to that of larvae (C).The value of the heatmaps is the pairwise pearson correlation between the average expression profile of the given sample [A) nurse head, B) nurse abdomen, C) worker larva] at different developmental stages. In (A) and (B), nurses were sampled feeding the given larval stage (L1, L2, etc), while in (C) worker larvae of the given stage were sampled. L3s, L3m, and L3l refer to small, medium, and large third instar larvae.

Figure 4. Differential expression between nurses feeding young (1st-instar) and old (large 3rd instar) larvae in A) nurse heads and B) nurse abdomens. Genes with positive “log2 fold change” are upregulated in nurses feeding large 3rd vs 1st instar larvae. Genes colored red are differentially expressed (FDR < 0.05). C) Number and overlap of differentially expressed genes (N = 10970 genes in the differential expression analysis).

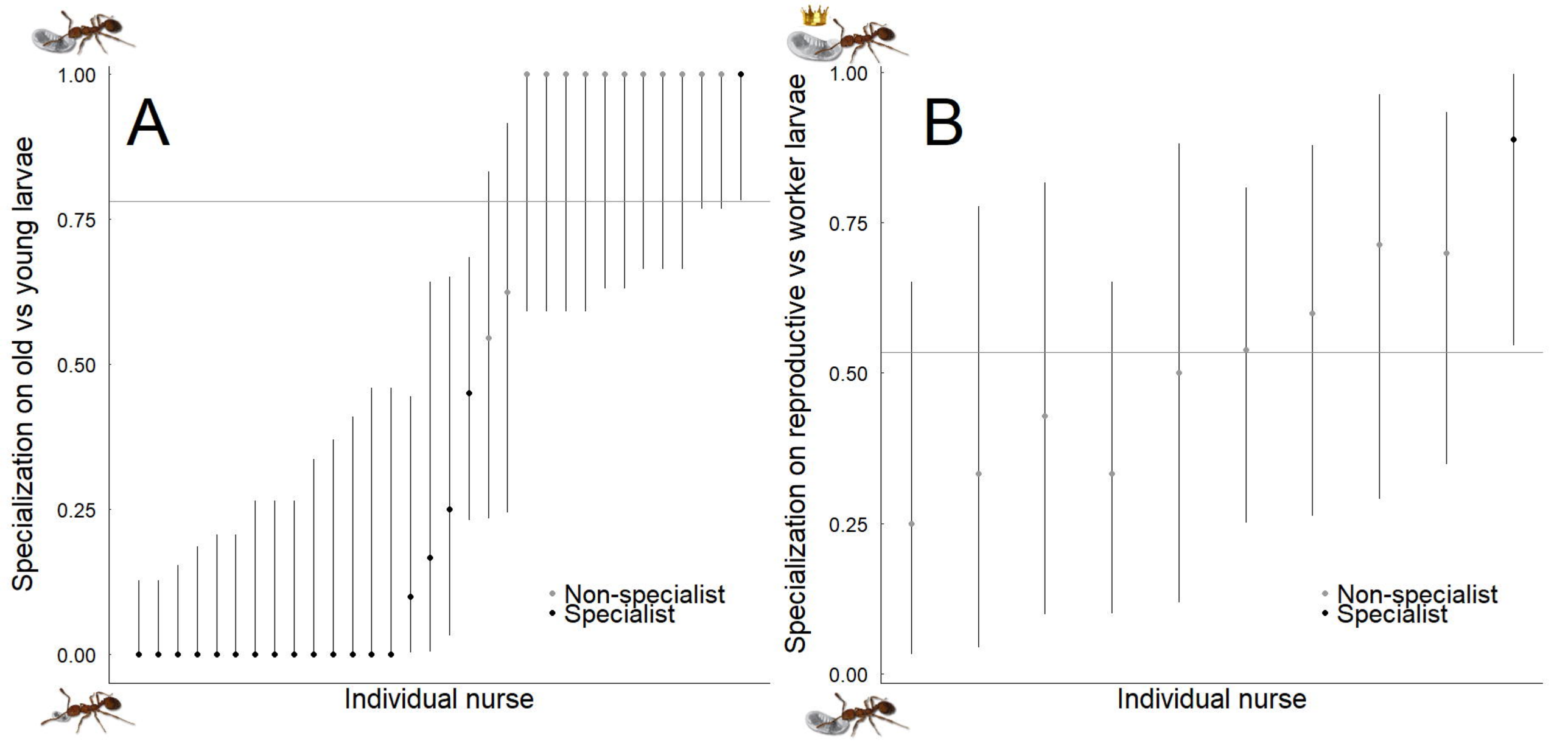
Figure S1. Diagram of sampling scheme for transcriptomic analysis (sample collection performed in [52]). Colonies were created from a mixed source and pre-assigned to larval developmental stages (L1...L3l). Queens were removed from ½ the colonies (top row). Colonies

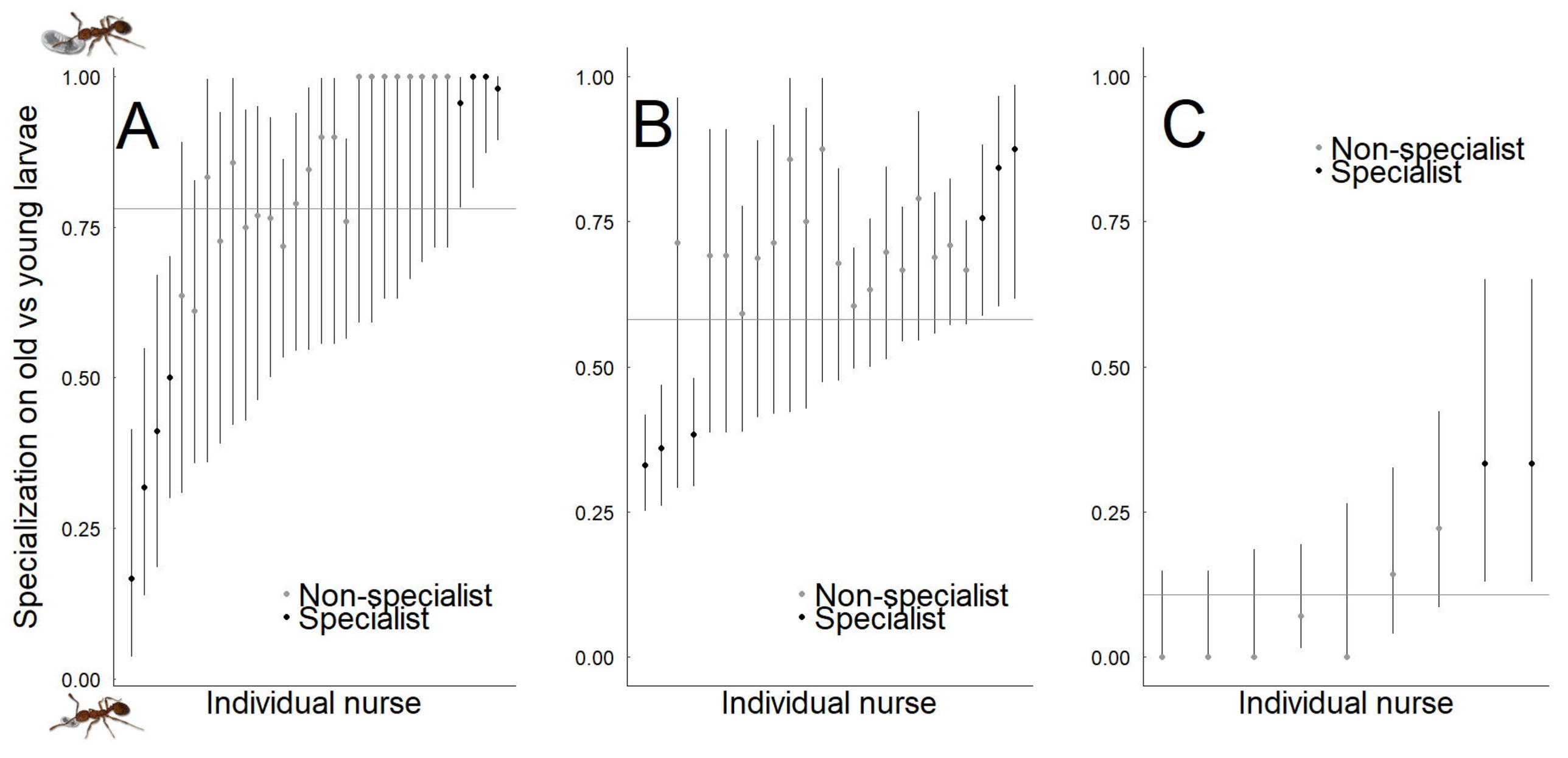
were sampled longitudinally, such that larvae of the designated age were likely to have been eggs at the start of the experiment. Worker-destined larvae [larva (W)] and nurses feeding worker-destined larvae of the designated stage [nurse (W)] were collected from queen-present colonies (bottom row). In addition to worker-destined larvae and nurses feeding worker-destined larvae, reproductive-destined larvae [larva (R)] and nurses feeding reproductive-destined larvae [nurse (R)] were collected from queen-absent colonies (top row). At the first instar (L1), it is not possible to distinguish between worker- and reproductive-destined larvae, so samples are marked "W/R", as larvae could be reproductive- or worker-destined and nurses could have fed either caste as well. L3s, L3m, and L3l refer to small, medium, and large 3rd-instar larvae, respectively. Nurses were collected when witnessed feeding larvae of the designated stage under dissecting microscopes.

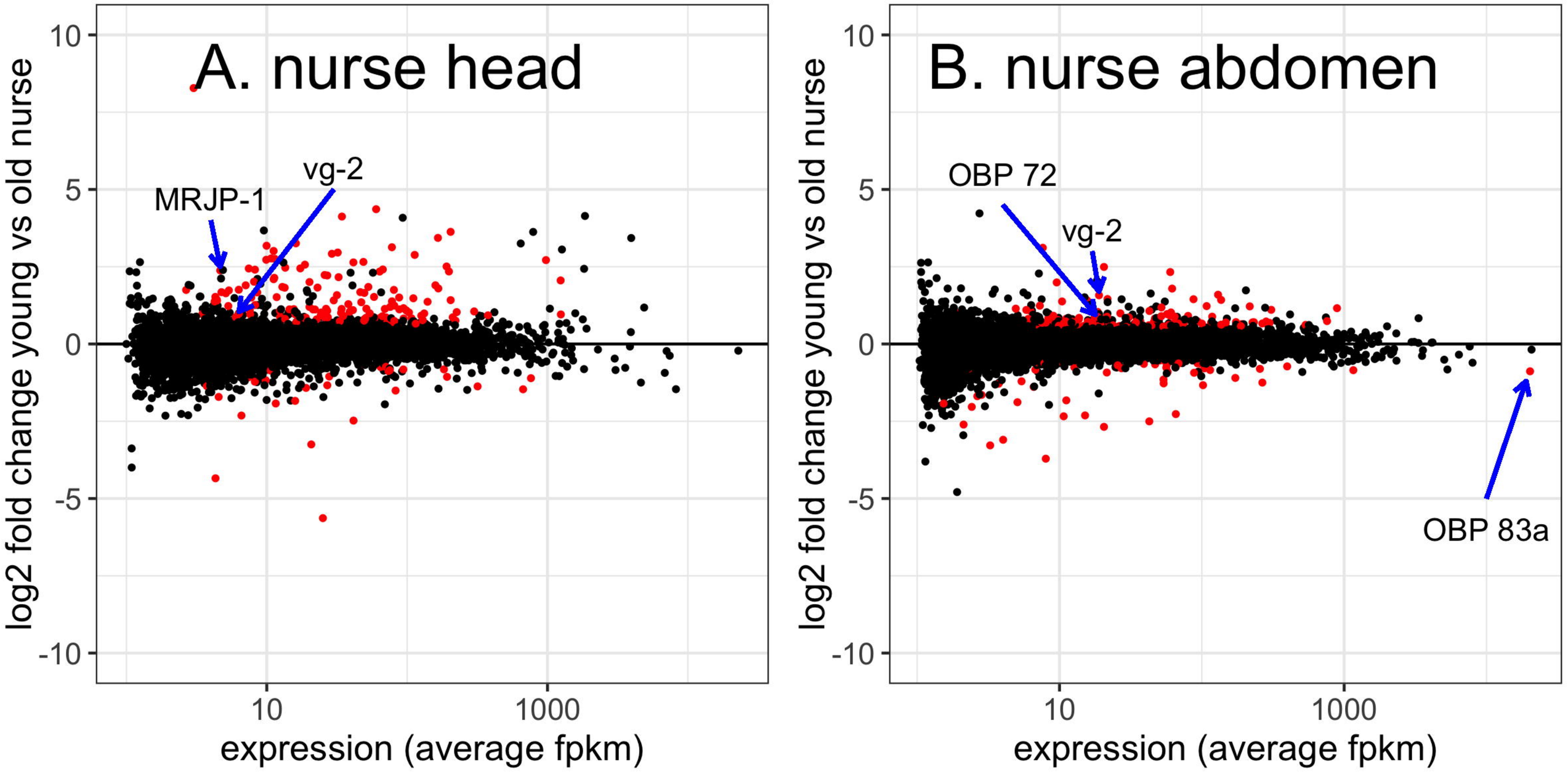
Table S1. Gene ontology terms for differentially expressed genes in nurse heads and abdomens. Top 10 GO terms ($P < 0.05$) are reported per sample type.

Table S2. Complete list of differentially expressed genes. Second and third columns show which direction gene is differentially expressed (upregulated in young or old nurses). Fourth and fifth columns show the NCBI annotation for SwissProt and UniProt databases, respectively. Column marked "Secreted" indicates whether the *Drosophila melanogaster* ortholog is known to be secreted (if an ortholog exists).

	χ^2	df	p
Short-term Nursing			
Caste			
Individual Nurse	1.5539	1	0.2126
Observer	0.7866	1	0.3751
Stage			
Individual Nurse	293.133	2	**<0.0001**
Observer	55.733	2	**<0.0001**
Long-term Nursing			
Individual Nurse	34.140	1	**<0.0001**
Observer	14.716	3	**0.0021**
Colony	50.518	3	**<0.0001**
Age	1.375	1	0.2410
Long-term Grooming			
Individual Nurse	21.1795	1	**<0.0001**
Observer	0.9854	1	0.3209
Colony	48.9282	1	**<0.0001**
Age	0.0148	1	0.9032
Long-term Carrying			
Individual Nurse	4.7370	1	**0.0285**
Observer	1.0820	1	0.2982
Colony	5.2454	1	**0.0220**
Age	0.1634	1	0.6860


A
B
1.00
0.75
0.50
0.25
0.00
Specialization on old vs young larvae
Specialization on reproductive vs worker larvae
Individual nurse
Non-specialist
Specialist


A
B
C
Specialization on old vs young larvae
1.00
0.75
0.50
0.25
0.00
Non-specialist
Specialist
Individual nurse



C. DGEs by larval age fed