



## Ant nurse workers exhibit behavioural and transcriptomic signatures of specialization on larval stage

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Division of labour 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 further in caring for larvae of a certain stage or caste, given that larval nutritional requirements depend on stage and caste. We used short-term (<1 h) and long-term (10 days) behavioural observations to determine whether nurses of the pharaoh ant, *Monomorium pharaonis*, exhibit such specialization. We found evidence for behavioural specialization among nurses based on larval instar but not larval caste. This specialization was widespread, with 56% of nurses in the short-term and 22–27% in the long-term showing significant specialization. Additionally, we identified ~200 genes that were differentially expressed in nurse head and abdominal tissues between nurses feeding young versus old larvae. These included 18 genes predicted to code for secreted proteins, which may be passed from nurses to larvae via trophallaxis, as well as *vitellogenin* and *major royal jelly protein-1*, which have previously been implicated in the transfer of nutrition from nurse to larvae and the regulation of larval development and caste in social insects. Altogether, our results provide the first evidence in any social insect for a division of labour among nurse workers based on larval stage, and our study begins to elucidate the molecular mechanisms underlying this specialization.

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Division of labour, one of the defining characteristics of eusociality, is believed to be the primary reason for the tremendous success of social insects (Oster & Wilson, 1978; Wilson, 1971, 1987). Within this system of division of labour, queens specialize on reproduction while workers specialize on tasks including brood care, foraging and nest defence (Beshers & Fewell, 2001; Oster & Wilson, 1978; Wilson, 1987). Increased worker efficiency within colonies is thought to be the main colony-level benefit of division of labour. Behavioural specialists, through learning or physiological differences, are expected to be more efficient than generalists (Oster & Wilson, 1978; Robinson, 1992; Wahl, 2002), but see Dornhaus (2008) and Muscedere, Willey, and Traniello (2009). Indeed, social insect behavioural specialists demonstrate increased efficiency in nest emigration (Langridge, Sendova-Franks, & Franks, 2008), nest excavation (Jeanson et al., 2008), undertaking (Julian & Cahan, 1999; Trumbo & Robinson, 1997) and response to sucrose (Perez, Rolland, Giurfa, & d'Ettorre, 2013).

Worker specialization is widespread and is driven by a diversity of factors and proximate mechanisms. 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) (Beshers & Fewell, 2001; Mikheyev & Linksvayer, 2015; Oster & Wilson, 1978; Robinson, 1992). Alternatively, worker tasks can be allocated based on body size and shape, as many species exhibit morphologically distinct worker subcastes that perform different roles within the colony (Beshers & Fewell, 2001; Oster & Wilson, 1978). Worker variation in behavioural specialization can also occur independently of age and morphology (Gordon, 1989; Jeanson & Weidenmuller, 2014). This interindividual variability can be the result of genetic diversity among workers (Oldroyd & Fewell, 2007), environmental differences during early development (Tautz, Maier, Groh, Rossler, & Brockmann, 2003; Weidenmuller, Mayr, Kleineidam, & Roces, 2009), variation in adult nutritional state (Ament et al., 2011; Blanchard, Orledge, Reynolds, & Franks, 2000; Charbonneau et al., 2017), prior experience (Theraulaz, Bonabeau, & Deneubourg, 1998) and the social environment (Webster & Ward, 2011).

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Cooperative brood care, which includes feeding, grooming and carrying brood, is one of the most important suites of tasks performed by adult workers (Oster & Wilson, 1978; Wilson, 1987). Different larvae have different nutritional requirements depending upon their caste and developmental stage (Cassill & Tschinkel, 1996; Lim, Chong, Chong, & Lee, 2005). For example, young fire ant, *Solenopsis invicta*, larvae are fed exclusively liquid food via nurse–larva trophallaxis while older larvae are also fed solid protein (Cassill, Butler, Vinson, & Wheeler, 2005; Petralia, Sorensen, & Vinson, 1980; Tschinkel, 1988). Furthermore, old larvae require more frequent and longer feedings than young larvae (Cassill & Tschinkel, 1996, 1999).

The caste fate of developing larvae in social insects is socially regulated by nurse workers (Linksvayer, 2015; Linksvayer et al., 2011; Vojvodic et al., 2015), often based on the quantity and quality of nutrition provided to larvae (Hunt & Nalepa, 1994; Tribble & Kronauer, 2017; Wheeler, 1986). 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 (Amor et al., 2016; Hunt & Nalepa, 1994; Smith & Suarez, 2010; Warner, Kovaka, & Linksvayer, 2016). Furthermore, recent research in the Florida carpenter ant, *Camponotus floridanus*, found that nurse workers transfer juvenile hormone, microRNAs, hydrocarbons, various peptides and other compounds to larvae during feeding (LeBoeuf et al., 2016), 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 exhibit both behavioural and transcriptomic specialization on larval caste (He et al., 2014; Vojvodic et al., 2015). However, these studies did not test for specialization on larval stage 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 tested whether individual pharaoh ant, *Monomorium pharaonis*, nurse workers exhibit behavioural specialization on different larval stages or castes, as measured on both short (<1 h) and long (10 days) timescales. We estimated how widespread such specialization is and the contribution of specialists to colony-level brood care. Building on our behavioural results, we used an existing transcriptomic data set (Warner, Mikheyev, & Linksvayer, 2017) to identify genes with expression patterns that may be associated with nurse specialization. Overall, we sought to elucidate whether nurse specialization exists in ants, how it contributes to colony-level brood care and what gene expression patterns might be associated with such specialization.

## METHODS

### Background and Overall Design

All colonies used in this study were reared in the laboratory and were derived from stock colonies that have been systematically interbred for the past 10 years. We fed the colonies twice per week with an agar-based synthetic diet (Dussutour & Simpson, 2008) and mealworms, and we maintained all colonies at  $27 \pm 1$  °C and 50% relative humidity on a 12:12 h light:dark cycle. We conducted all behavioural observations manually using a dissecting microscope and red light. To keep the temperature constant during behavioural observations, we kept the colonies on a heating pad set to 27 °C.

*Monomorium pharaonis* larvae have three instars (Alvares, Bueno, & Fowler, 1993), which are distinguishable by body size, body shape, hair abundance and hair morphology (Berndt & Kremer, 1986). Although reproductive-destined larvae (males and gynes) cannot be distinguished from worker-destined larvae as

eggs or first-instar larvae, they can be readily distinguished after the first instar (Berndt & Kremer, 1986; Edwards, 1991). Since colonies usually only produce new gynes and males in the absence of fertile queens (Edwards, 1987; Peacock, Sudd, & Baxter, 1955), we set up queen-absent colonies, which rear both worker- and reproductive-destined larvae, when testing for specialization on larval caste. For both the behavioural observations and transcriptomic analyses, we initially classified the larvae into five stages based on size and hair morphology: first instar, second instar and small, medium and large third instar (for details, see Berndt & Kremer, 1986; Warner et al., 2016). However, for subsequent behavioural analyses, we only considered larval instar.

### Short-term Observations

First we conducted short-term observations of unmarked workers in both queen-present ( $N = 8$ ) and queen-absent ( $N = 3$ ) colonies to determine whether nurses exhibited short-term specialization based on larval instar (using queen-present colonies) or larval caste (using queen-absent colonies). We observed colonies until we saw a worker feed a larva of any instar or caste, and then we continuously observed that nurse worker for as long as possible (maximum = 67 min). 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' (Friard & Gamba, 2016). We defined feeding behaviour as a stereotypical behavioural interaction between the nurse worker and larva in which the mouthparts of the nurse and larva were in contact for at least 3 s. We defined both the transfer of solid food particles and liquid food via trophallaxis from nurse to larva as feeding behaviour and did not distinguish between these two feeding behaviours. We restricted subsequent analysis to nurses that we observed feeding at least three times.

### Long-term Observations

Next, we attempted to test whether individually marked nurses in queenless colonies express long-term specialization (across 10 days). We wanted to track nurses for at least 10 days because this timescale includes the entire amount of time that *M. pharaonis* workers tend to perform nursing behaviours (Mikheyev & Linksvayer, 2015). In each of five colonies, we collected a cohort of 63 1-day-old callow workers and we uniquely painted each of these focal individuals with paint dots on their heads and abdomens using combinations of eight colours. Specifically, we lightly anaesthetized them with carbon dioxide and marked their heads and abdomens with a dot of paint using Sharpie extra-fine point, oil-based paint pens (Charbonneau et al., 2017; Dornhaus, 2008; Dornhaus, Holley, Pook, Worswick, & Franks, 2008). To control for potential behavioural effects of the paint, we painted all remaining adult workers in the colonies with black dots on their heads and abdomens. Because all 63 focal individuals in each colony were age-matched, we were able to control for possible effects of nurse age on potential behavioural specialization.

We constructed queen-absent colonies with 400 workers and 2.5 ml of brood (i.e. approximately 500 eggs, larvae and pupae of different stages; Warner et al., 2016; Warner, Lipponen, & Linksvayer, 2018) and recorded all observed feeding, grooming or carrying behaviours performed by all focal individually marked workers. We initially used queen-absent colonies because such colonies normally raise new queens and we wanted to test for longer-term specialization for caste. However, given that we observed no short-term specialization for caste, and our colonies ended up not producing sexual brood, we only considered potential long-term specialization based on larval stage. We defined feeding as described above, when an individually marked worker's

mandibles interacted with a larva's mandibles for at least 3 s. We defined grooming as an interaction between worker mandibles and a larva for a minimum of 3 s. We defined carrying as a worker lifting a larva with her mandibles and transporting the larva to another location. We analysed feeding, grooming and carrying behaviour separately. We observed all colonies for 3 h per day for 10 consecutive days and restricted subsequent analysis to individuals we observed feeding, grooming or carrying at least three times.

#### Statistical Analysis of Behavioural Specialization

Previous studies have often looked for evidence of behavioural specialization by identifying individuals that were statistical outliers among all individuals in colonies, in terms of performing a behaviour (e.g. undertaking behaviour; Diez, Le Borgne, Lejeune, & Detrain, 2013; Julian & Cahan, 1999) more times than expected based on a Poisson distribution. We asked whether individuals repeatedly performed nursing behaviours towards one of two larval categories (for caste: worker- destined versus reproductive-destined larvae; for stage: young versus old). Importantly, our approach provides an unbiased means to test whether every individual displays a significant bias (i.e. 'specialization') in nursing based on larval stage or larval caste, and also to quantify the proportion of all individuals that display such specialization.

We performed all statistical analyses in R version 3.4.1 (R Core Team, 2014). For both short- and long-term observations, we first used binomial generalized linear mixed models (GLMMs) to ask whether individual nurses differed significantly in their degree of specialization on larval stage or caste. To test for nurse specialization on larval instar, we grouped first- and second-instar larvae as 'young' larvae and all third-instar larvae as 'old' larvae. This grouping is biologically meaningful as young fire ant larvae are fed solely a liquid diet while old larvae are also fed solid food (Cassill et al., 2005; Petralia et al., 1980; Tschinkel, 1988). During our observations, we similarly observed *M. pharaonis* nurses feed first- and second-instar larvae only a liquid diet but third-instar larvae both liquid and solid food. Specifically, we fitted GLMMs with the R package 'lme4' (Bates, Mächler, Bolker, & Walker, 2015) for the proportion of fed larvae that were young versus old, with the identity of the nurse as a random effect and colony identity and nurse age as fixed effects when appropriate. Similarly, to test for nurse specialization on larval caste, we fitted GLMMs for the proportion of fed larvae that were reproductive- versus worker-destined larvae. 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 (Bolker et al., 2009). A significant random effect of nurse identity in these models indicates that there is variation among individual nurses for degree of behavioural specialization, providing initial evidence for behavioural specialization within colonies.

Next, given that we found evidence for behavioural specialization (see Results), we used binomial tests to ask whether each individual significantly specialized on young versus old larvae, 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. We estimated the expected frequency (i.e. 'probability of success' in the binomial test) of interacting with larvae of one stage or caste relative to another stage or caste based on the observed proportion of interactions for the two stages or castes (e.g. the number of observed interactions between nurses and first-instar larvae relative to third-instar larvae). To determine whether any of the individual nurses we observed could be

confidently classified as specialists, we first used binomial tests with a type I error rate corrected for multiple comparisons across all tested individuals. Given that some individuals were confidently identified as specialists with these conservative criteria, we next estimated the overall proportion of specialist versus nonspecialist nurses in our study colonies using a type I error rate of 0.05 for each binomial test run separately for each individual nurse. This test provides an unbiased approach to determine, one at a time, whether each individual displayed significant specialization or not.

#### Gene Expression Analysis

Warner et al. (2017) performed RNA sequencing on a developmental time series of the five *M. pharaonis* larval stages as well as nurses collected in the act of feeding each of these larval stages. This previous study focused on identifying caste-biased genes across development and studying patterns of molecular evolution of these genes. In the current study, we take advantage of the fact that nurse samples used in Warner et al. (2017) were collected in the act of feeding one of the five larval stages, and we use the Warner et al. (2017) data set to compare transcriptomes of nurses feeding different larval stages. We chose to focus on nurses feeding very young versus very old larvae to maximize our power to detect differential expression based on the stage of larvae fed. Specifically, we used 11 samples of tissues from nurses collected in the act of feeding first-instar larvae (5 head samples, 6 abdomen samples) and 10 samples of nurses collected in the act of feeding large third-instar larvae (5 head, 5 abdomen) to identify genes differentially expressed between nurses feeding larvae at the extreme young and old end of the developmental trajectory. Warner et al. (2017) immediately flash-froze the collected nurses in liquid nitrogen. See Supplementary material for a brief summary of the sample collection procedure; for details of sample collection, RNA extraction, library preparation, sequencing and estimation of per-locus expression, see Warner et al. (2017).

After removing lowly expressed genes (fragments per kilobase of transcript per million mapped reads, FPKM  $< 1$  in half of the samples), we used the package EdgeR (Robinson, McCarthy, & Smyth, 2010) for differential expression analysis. We constructed a GLM-like model, including larval stage fed, replicate and queen presence as additive effects to identify genes differentially expressed between nurses feeding young versus old larvae (first instars versus large third instars; separately for head and abdomen). We calculated gene ontology (GO) term enrichment of differentially expressed genes using the R package GOstats, with a cutoff  $P$  value of 0.05 (Falcon & Gentleman, 2007).

To test whether genes found to be differentially expressed between nurses tended to code for secreted proteins in *Drosophila melanogaster*, we compiled a list of genes annotated as coding for secreted proteins according to the online tool GLAD (Hu, Comjean, Perkins, Perrimon, & Mohr, 2015). From this list, we identified secreted proteins with orthologues in *M. pharaonis* using a recently created orthology map between *M. pharaonis*, *A. mellifera* and *D. melanogaster* (orthology map included as Supplementary Data). We estimated the association between a gene's likelihood to be differentially expressed and secreted, removing all genes for which a *D. melanogaster* orthologue was not detected. We generated plots using the R package 'ggplot2' (Wickham, 2009).

## RESULTS

### Short-term Specialization on Larval Stage

We observed 52 nurses feed at least three times (mean = 8.8 feeding events) and we included these nurses in the GLMMs. The

random effect of nurse identity was significant, suggesting that nurses tended to specialize on feeding either young or old larvae (Table 1). Next, to classify each individual nurse as showing significant specialization or not (i.e. to classify nurses as specialists or nonspecialists), we used binomial tests with an expected proportion of old larvae relative to young plus old larvae of 0.781 (the observed proportion of old larvae fed across all individuals in long-term feeding observations). We used the observed proportion from long-term observations, as opposed to short-term observations, because we specifically attempted to balance the number of recorded feeding events involving old and young larvae (in terms of total number of observations, not per individual) during short-term observations. Therefore, the observed short-term proportions are not an accurate representation of the naturally occurring proportions. We included the 32 nurses we observed feed at least six times. 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  $P$ ). 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. These specialists performed about 65% (242/375) of the observed feedings.

#### Short-term Specialization on Caste

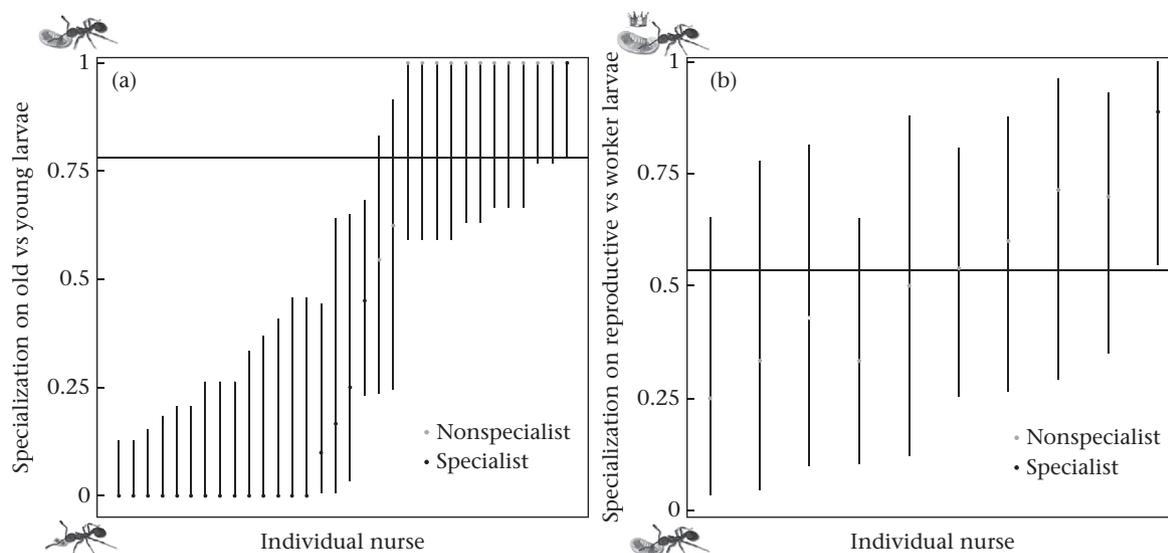
We observed 22 nurses feed at least three times (mean = 5.64 feeding events). The random effect of nurse identity in the GLMM was not significant (Table 1), indicating that nurses did not specialize on larval caste. In the binomial tests, we included the 10 nurses we observed feed at least six times and we 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, which performed about 6% (9/142) of the observed feedings (Fig. 1).

#### Long-term Feeding Specialization on Larval Stage

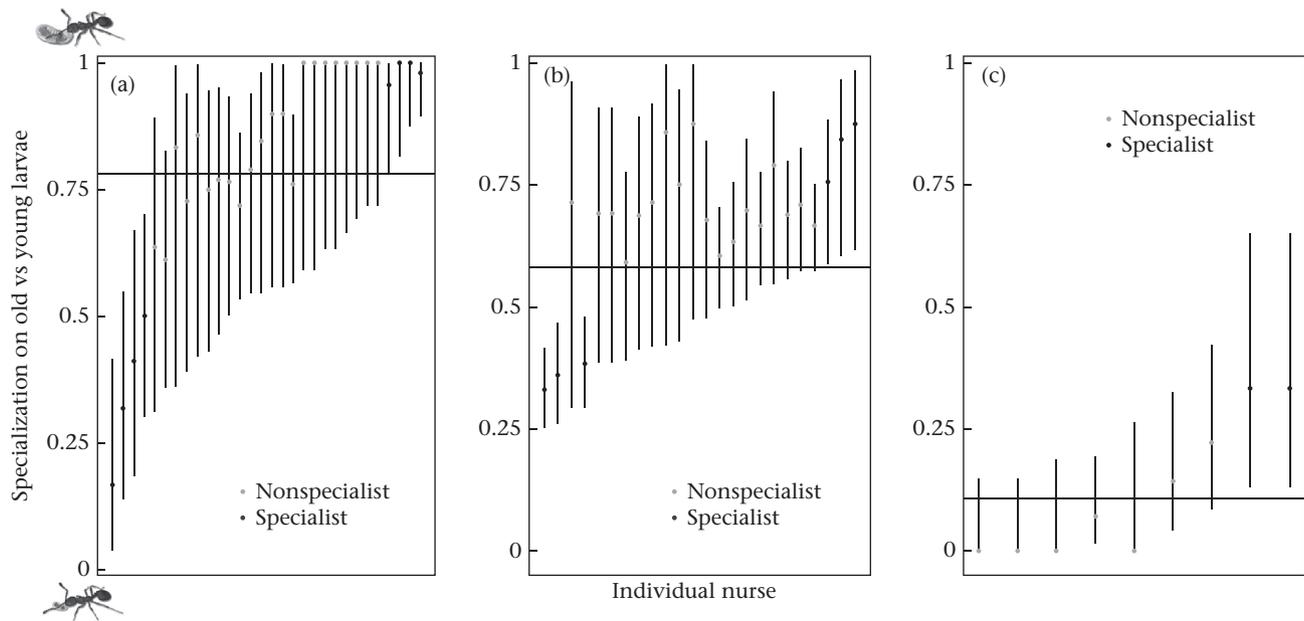
We observed 40 nurses feed at least three times (mean = 12.9 feeding events). Nurses fed old larvae in the majority of observed feeding events (78.1%). The effects of nurse identity and colony identity were significant (Table 1), indicating that nurses tended to specialize on feeding either young or old larvae. The age of the nurse was not significant. In the binomial tests, we included the 30 nurses we observed feed at least six times and we used an expected proportion of old larvae of 0.781. When correcting for multiple comparisons, we classified 20% (6/30) of nurses as being long-term specialists on larval stage. When using an uncorrected type I error rate of 0.05, we classified about 27% (8/30) of nurses as long-term specialists and these long-term specialists performed about 42% (201/480) of the observed feedings (Fig. 2). Long-term specialists performed significantly more feedings than nonspecialists (Mann–Whitney test:  $W = 19.5$ ,  $P = 0.0013$ ).

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

	$\chi^2$	df	$P$
<b>Short-term feeding</b>			
Caste			
Individual nurse	1.430	1	0.232
Stage			
Individual nurse	345.56	1	<0.0001
<b>Long-term feeding</b>			
Individual nurse	36.934	1	<0.0001
Colony	57.750	4	<0.0001
Age	0.018	1	0.892
<b>Long-term grooming</b>			
Individual nurse	21.357	1	<0.0001
Colony	49.576	1	<0.0001
Age	0.022	1	0.882
<b>Long-term carrying</b>			
Individual nurse	4.500	1	0.034
Colony	5.048	1	0.025
Age	0.021	1	0.886



**Figure 1.** Short-term nurse worker specialization on (a) young vs old larvae and (b) worker- vs reproductive-destined larvae. The dots represent the proportions of old larvae (a) or reproductive larvae (b) that each nurse worker fed and the error bars are the 95% confidence intervals from the binomial tests. The horizontal line represents the expected proportion based on overall observed proportion of interactions. In (a), a proportion of 1 means that the nurse worker fed only old larvae while a 0 means that the worker fed only young larvae. In (b), a proportion of 1 means that the nurse worker fed only reproductive-destined larvae while a 0 means that the worker fed only worker-destined larvae.



**Figure 2.** Nurse worker specialization on young vs old larvae for (a) long-term feeding, (b) grooming and (c) carrying. The dots represent the proportions of old larvae that each nurse worker cared for and the error bars are the 95% confidence intervals from the binomial tests. The horizontal line represents the expected proportion based on overall observed proportion of interactions. A proportion of 1 means that the nurse worker cared for only old larvae while a 0 means that the worker cared for only young larvae.

#### Long-term Grooming Specialization on Larval Stage

We observed 32 individuals grooming larvae at least three times (mean = 33.9 grooming events). Nurses groomed old larvae in the majority of observed grooming events (58.1%). The effects of nurse identity and colony identity were significant (Table 1), indicating that nurses tended to specialize on grooming either young or old larvae. The age of the nurse was not significant. In the binomial tests, we included the 24 nurses we observed groom at least six times and we 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 (Fig. 2). The number of groomings performed by specialists and nonspecialists was not significantly different ( $W = 29, P = 0.1021$ ).

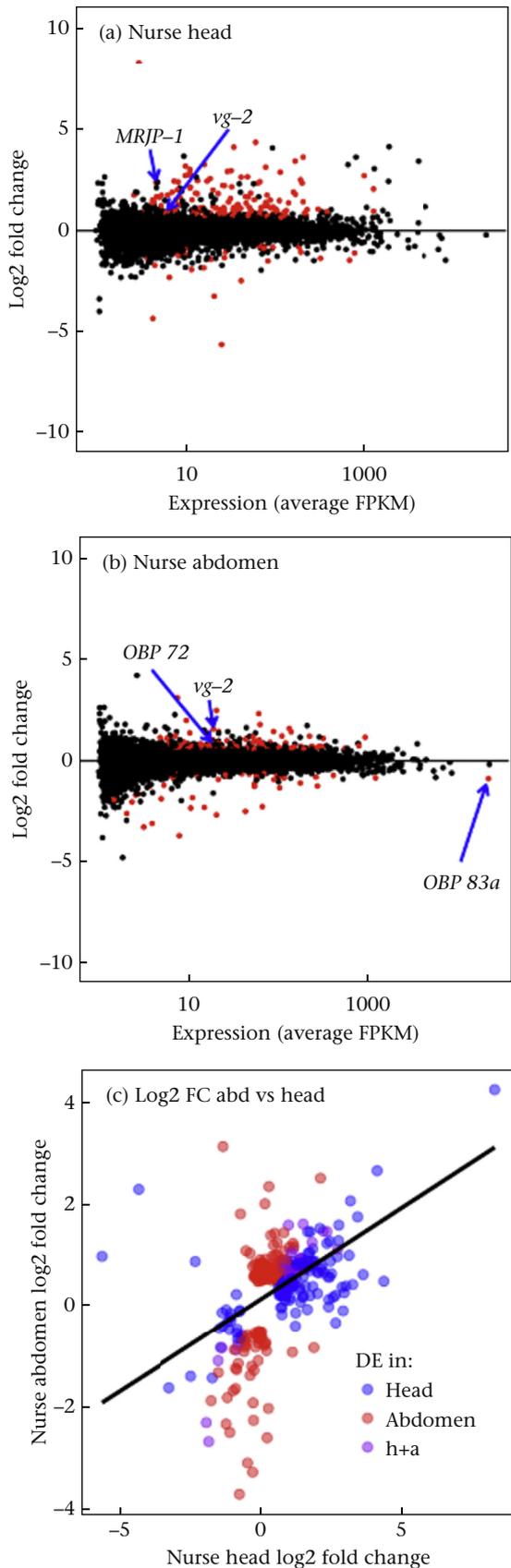
#### Long-term Carrying Specialization on Larval Stage

We observed 17 individuals carrying a larva at least three times (mean = 13.4 carrying observations). Nurses carried young larvae in the majority of observed carrying events (89.3%). The effects of nurse identity and colony identity were significant (Table 1), indicating that nurses tended to specialize on carrying either young or old larvae. The age of nurse was not significant. In the binomial tests, we included the nine nurses we observed carrying a larva at least six times and we used an expected ratio of old to young larvae 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 (Fig. 2). The number of carrying observations performed by specialists and nonspecialists was not significantly different ( $W = 13, P = 0.100$ ).

#### Transcriptomic Analysis

We identified 209 and 173 differentially expressed genes (DEGs) in the heads and abdomens, respectively, of nurses collected while feeding young (i.e. first-instar) versus old (i.e. large third-instar) worker larvae (FDR < 0.05; Fig. 3a and b). In both nurse heads and abdomens, we identified more upregulated genes in nurses feeding young versus old larvae (two-sided binomial, null hypothesis of 50% upregulated in nurses feeding young larvae: heads:  $N = 209, P < 0.001$ ; abdomens:  $N = 173, P < 0.001$ ). Based on contingency table analysis, genes upregulated in heads of nurses feeding young larvae also tended to be upregulated in abdomens of nurses feeding young larvae ( $\chi^2_1 = 312, P < 0.001$ ). Similarly, genes upregulated in the heads of nurses feeding old larvae tended to be upregulated in the abdomens of nurses feeding old larvae ( $\chi^2_1 = 260, P < 0.001$ ). Additionally, there was an overall correlation between expression fold change in nurse heads and abdomens across all differentially expressed genes between nurses feeding young versus old larvae (Fig. 3c). For genes associated with each nurse type, gene ontology was largely dominated by metabolism-related categories (Supplementary Table S1). Genes upregulated in the heads of nurses feeding young larvae were also associated with isoprenoid (a type of hydrocarbon) processing, and genes upregulated in the abdomens of nurses feeding young larvae were associated with transport and localization.

Genes that were differentially expressed in nurses based on larval stage were more likely to code for proteins known to be secreted by cells in *D. melanogaster* ( $\chi^2_1 = 29.1, P < 0.001$ ; 18 DEGs coding for secreted proteins out of 148 total DEGs with orthologues in *D. melanogaster*; 178 genes had orthologues that code for secreted proteins in *D. melanogaster*, out of 5391 genes in the analysis). Nearly all of the DEGs that are predicted to code for secreted proteins were upregulated in nurses feeding young larvae (14/14 in heads, 9/10 in abdomens, see Table S2 for a complete list of DEGs based on larval stage fed).



**Figure 3.** Differential expression between nurses feeding young (first-instar) and old (large third-instar) larvae in (a) nurse heads and (b) nurse abdomens. Genes coloured red are differentially expressed (FDR < 0.05). (c) Correlation of log<sub>2</sub> fold change of differentially expressed genes as measured in nurse abdomens and heads (Spearman

## DISCUSSION

The tremendous ecological success of social insects is thought to be primarily due to efficient division of labour within colonies (Oster & Wilson, 1978; Wilson, 1971, 1987). Here we provide, to the best of our knowledge, the first evidence for the existence of a division of labour within nurse workers based on the instar of larvae they care for. We found evidence for behavioural specialization in the short term (<1 h) and the long term (over 10 days). Of those that specialized, nurses specialized on either old (third-instar) or young (first- and second-instar) larvae, and this specialization was consistent across feeding, grooming and carrying behaviours. In the short term, based on the results of the binomial tests, we classified 56% of nurses as specialists in terms of feeding, and in the long term, we classified 27%, 25% and 22% of workers as specialists in feeding, grooming and carrying, respectively. Specialists are predicted to increase colony efficiency (Oster & Wilson, 1978; Robinson, 1992; Wahl, 2002). Although we cannot say whether specialist nurses increase *M. pharaonis* colony efficiency, our data suggest that specialists do play an important role in the colony as they performed more per-capita feedings than nonspecialists (only 27% of nurses were specialized, but they performed over 42% of the observed feedings).

Nurses specialized on caring for young larvae may play a crucial role in regulating larval development. In both fire ants (Cassill et al., 2005; Petralia et al., 1980; Tschinkel, 1988) and *M. pharaonis* (Lim et al., 2005; J. T. Walsh, M. R. Warner, A. Kase, B. J. Cushing, T. A. Linksvayer, personal observations) young larvae are fed only a liquid diet while old larvae are also fed solid protein. Nurses are likely better able to manipulate the contents of trophallactic fluid than solid food since trophallactic fluid contains not only nutrition but also juvenile hormone, microRNAs, hydrocarbons, various peptides and other compounds (LeBoeuf et al., 2016). On the other hand, solid food may be less prone to manipulation since it is harvested directly from the environment. LeBoeuf et al. (2016) found that supplementing *C. floridanus* workers with juvenile hormone caused the larvae they reared to be larger as adults, suggesting that nurses can regulate worker phenotypes through differences in trophallactic fluid. In some social insect species, nutrition during the early larval stages can influence the caste fate of developing larvae (Asencot & Lensky, 1976; Goetsch, 1937; Haydak, 1943; Metztl, Wheeler, & Abouheif, 2018; Schwander, Lo, Beekman, Oldroyd, & Keller, 2010; Shuel & Dixon, 1960). In *M. pharaonis*, the caste fate of developing larvae is determined by the end of the first instar (Alvares et al., 1993; Berndt & Kremer, 1986; Khila et al., 2010; Warner et al., 2016, 2018). Therefore, although as we discuss further below, we found no nurse specialization on caste in old larvae (i.e. after the point that caste can be morphologically distinguished by human observers), it is conceivable that nurses that specialize on caring for young larvae may regulate the caste fate of these young larvae through differences in trophallactic fluid.

Given that we found evidence for the behavioural specialization of nurses on young versus old larvae, we also tested for differential gene expression in the head and abdominal tissues of nurses feeding young versus old larvae as a first step in identifying transcriptomic signatures of specialization. We expected that differentially expressed genes (DEGs) in these tissues might be

correlation:  $r_s = 0.345$ ,  $P < 0.001$ ). Black line represents trendline of linear model. Genes are coloured by tissue differentially expressed in (FDR < 0.05). In all plots, genes with positive 'log<sub>2</sub> fold change' are upregulated in nurses feeding large first- vs third-instar larvae (i.e. feeding young vs old larvae). FPKM: fragments per kilobase of transcript per million mapped reads; FC: fold change; DE: differential expression; abd: abdomen.

functionally associated with different types of care provided by nurse workers to differently aged larvae. Specifically, genes expressed in brain tissue in the head may be associated with different types of nursing behaviour, and genes expressed in several exocrine glands in the head that are thought to be involved in the production and secretion of compounds fed to larvae (Boonen & Billen, 2016) might actively contribute to the social regulation of larval development (see Vojvodic et al., 2015). Finally, genes expressed in the digestive tract and additional exocrine glands found in abdominal tissue might also be associated with the processing and secretion of nutrition to larvae during nursing.

We identified 209 and 173 DEGs in nurse head and abdominal tissues, respectively, between nurses feeding young versus old worker-destined larvae. Note that this analysis is likely conservative given that our behavioural data indicate that approximately one-half of the individuals used in our gene expression samples are likely to be specialized based on larval age (i.e. nonspecialists included in our sample would weaken the transcriptomic signature of specialists). Interestingly, while the majority of DEGs were tissue specific, there was a positive correlation between log fold expression change from young to old nurses in both heads and abdomens. This indicates that some transcriptomic changes associated with nurse specialization occur consistently throughout nurse bodies.

Intriguingly, genes with *D. melanogaster* orthologues that are known to code for proteins that are secreted by cells were over-represented among the DEGs between *M. pharaonis* nurses feeding young versus old larvae. The DEGs we detected in nurse tissues could directly affect larval development if the proteins were secreted by nurses and transferred to larvae via trophallaxis (Linksvayer, 2015). Many of these DEGs, which are predicted to code for secreted proteins, have metabolic functions, suggesting they may play a role in the breakdown of food before it is passed to larvae.

Interestingly, in both head and abdominal tissue, we identified more genes upregulated in nurses feeding first-instar larvae than those feeding third-instar larvae, and all DEGs that code for proteins secreted in *D. melanogaster* were upregulated in nurses feeding first-instar larvae. These genes might be involved in regulating early larval development, or perhaps even regulation of larval caste fate, given that caste determination occurs at least by the end of the first instar (Alvares et al., 1993; Berndt & Kremer, 1986; Khila et al., 2010; Warner et al., 2016, 2018). Genes upregulated in nurses feeding first-instar larvae included genes such as *vitellogenin* (*Vg2*) (Libbrecht et al., 2013) and a member of the major royal jelly protein family (*MRJP-1*) (Schonleben, Sickmann, Mueller, & Reinders, 2007), 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 (Amdam, Norberg, Hagen, & Omholt, 2003) (Fig. 3a). Interestingly, LeBoeuf et al. (2016) found both a *MRJP* homologue and *vitellogenin* in the trophallactic fluid of ant nurses fed to developing larvae. Therefore, it is possible that *M. pharaonis* nurses feeding young larvae are passing on these compounds directly to larvae as a means to regulate larval development.

The DEGs between nurses may also play a role in responding to larval signals. Two odorant-binding proteins (OBP) were differentially expressed in nurse abdomens (Fig. 3a). These OBPs potentially play a role in communication between nurses and larvae (McKenzie, Fetter-Pruneda, Ruta, & Kronauer, 2016; Zhou et al., 2015). Although OBPs are predicted to be primarily expressed in the antennae, previous studies found that OBPs are frequently expressed in nonchemosensory tissues (McKenzie, Oxley, & Kronauer, 2014) and can exhibit various functions beyond olfaction (Dani et al., 2011; Maleszka, Foret, Saint, & Maleszka, 2007; Nomura, Kawasaki, Kubo, & Natori, 1992; Zhang, Wanchoo, Ortiz-

Urquiza, Xia, & Keyhani, 2016). For example, the *Gp-9* gene encodes for the odorant-binding protein SiOBP3 and has been linked to colony organization in the fire ant *S. invicta* (Wang et al., 2013). Expression of *SiOBP3* is found throughout the bodies of workers, gynes and males and is actually lowest in the antennae (Zhang et al., 2016; note that the *M. pharaonis* orthologue of *SiOBP3* was not differentially expressed in this study).

Contrary to findings in honey bees (He et al., 2014; Vojvodic et al., 2015), we found no behavioural evidence for nurse specialization on larval caste. This lack of specialization in *M. pharaonis* is somewhat surprising, given that worker- and reproductive-destined larvae likely have different nutritional needs (Amor et al., 2016; Hunt & Nalepa, 1994; Smith & Suarez, 2010; Warner et al., 2016). However, this difference may be attributable to differences in timing of caste determination. In honey bees, caste determination occurs relatively late in development and over a period of time, as queen–worker intercastes can be produced by experimental manipulation of diet late in development (Dedej, Hartfelder, Aumeier, Rosenkranz, & Engels, 1998; Linksvayer et al., 2011; Wang, Kaftanoglu, Fondrk, & Page, 2014). Therefore, in honey bees, continued nurse–larvae interactions are likely essential to fine-tune caste dimorphism (Linksvayer et al., 2011).

In contrast to honey bees, where each larva develops in an isolated brood cell, worker- and reproductive-destined larvae are not spatially separated in *M. pharaonis*. This lack of separation could also help explain the lack of specialization on larval caste in *M. pharaonis* compared to honey bees. Additionally, many ants (including *M. pharaonis*) spatially arrange their brood such that younger larvae and eggs tend to be in the centre and older larvae and pupae tend to be towards the edge of brood piles (Franks & Sendova-Franks, 1992; Lim & Lee, 2005). This spatial arrangement could potentially contribute to the observed short-term specialization if nurses spend most of their time in one area of the nest and feed larvae close to them. However, we observed nurses frequently moving around the nest during our short-term observations, interacting with other workers or collecting food in between subsequent feedings, so that each individual nurse had the potential to interact with all brood stages.

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. Interestingly, we found significant effects of colony identity for long-term nursing, grooming and carrying. Although outside the scope of this study, it is possible that different colonies exhibit different levels of specialization in either the number of specialists or the proportion of brood care behaviours performed by specialists. Future studies should test for colony-level variation in nurse specialists.

#### Data Accessibility

Data supporting this paper are included as [Supplementary material](#).

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## Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.anbehav.2018.05.015>.

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