Transcriptomic basis and evolution of ant nurse-larval social regulatory interactions

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Abstract

Development is often strongly regulated by interactions among close relatives, but the underlying molecular mechanisms are largely unknown. In eusocial insects, interactions between caregiving worker nurses and larvae regulate larval development and resultant adult phenotypes. Here, we begin to characterize the social interactome regulating ant larval development by collecting and sequencing the transcriptomes of interacting nurses and larvae across time. We find that the majority of nurse and larval transcriptomes exhibit parallel expression dynamics across larval development. We leverage this widespread nurse-larva gene co-expression to infer putative social gene regulatory networks acting between nurses and larvae. Genes with the strongest inferred social effects tend to be peripheral elements of within-tissue regulatory networks and are often known to encode secreted proteins. This includes interesting candidates such as the nurse-expressed *giant-lens*, which may influence larval epidermal growth factor signaling, a pathway known to influence various aspects of insect development. Finally, we find that genes with the strongest signatures of social regulation tend to experience relaxed selective constraint and are evolutionarily young. Overall, our study provides a first glimpse into the molecular and evolutionary features of the social mechanisms that regulate all aspects of social life.

Author Summary

Social interactions are fundamental to all forms of life, from single-celled bacteria to complex plants and animals. Despite their obvious importance, little is known about the molecular causes and consequences of social interactions. In this paper, we study the molecular basis of nurse-larva social interactions that regulate larval development in the pharaoh ant *Monomorium pharaonis*. We infer the effects of social interactions on gene expression from samples of nurses and larvae collected in the act of interaction across a developmental time series. Gene expression appears to be closely tied to these interactions, such that we can identify genes expressed in nurses with putative regulatory effects on larval gene expression.

Genes which we infer to have strong social regulatory effects tend to have weak regulatory effects within individuals, and highly social genes tend to experience relatively weaker natural selection in comparison to less social genes. This study represents a novel approach and foundation upon which future studies at the intersection of genetics, behavior, and evolution can build.

Introduction

Social interactions play a prominent role in the lives of nearly all organisms [1] and strongly affect trait expression as well as fitness [2–4]. Social interactions in the context of development (e.g. parental care) often strongly regulate developmental trajectories and resultant adult phenotypes, for example via transferred compounds such as milk in mammals [5,6], milk-like secretions in arthropods [7,8], and other forms of nutritional provisioning [9,10]. In many taxa including certain birds, mammals, and insects, care for offspring and the regulation of offspring development has shifted at least in part from parents to adult siblings, who perform alloparental care [11]. In eusocial insect societies, sterile nurse workers regulate the development of their larval siblings by modulating the quantity and quality of nourishment larvae receive [12–14], as well as through the direct transfer of growth-regulating hormones and proteins [15,16]. At the same time, larvae influence nurse provisioning behavior via pheromones [17–20] and begging behavior [21,22].

In general, traits such as caregiving behavior that are defined or influenced by social interactions are the property of the genomes of multiple interacting social partners [2,14]. This has implications for both the mechanistic (e.g., molecular) underpinnings of development and trait expression as well as the genetic basis of trait variation at the population level -- i.e. how allelic variation in the genomes of interacting social partners affects trait variation [2,14]. Furthermore, because social traits are expressed in one individual but impact the fitness of other individuals, social behavior and socially-influenced traits experience distinct forms of selection, including kin selection and social selection [23,24]. Altogether, these distinct genetic features and patterns of selection are often thought to lead to distinct evolutionary features, such as rapid evolutionary dynamics in comparison to other traits [25–27]. In eusocial insects,

77 previous studies show that variation in larval developmental trajectories and ultimate adult phenotypes
78 (including reproductive caste, body size, etc.) depends on the combination of larval and nurse genotypes
79 [28–34]. However, the identity of specific genes and molecular pathways that are functionally involved in
80 the expression of social interactions (e.g., genes underlying nurse and larval traits affecting nurse-larva
81 interactions) and the patterns of molecular evolution for these genes have remained less well studied
82 [15,16,35,36].

83 Transcriptomic studies are often used to identify sets of genes underlying the expression of
84 particular traits by performing RNA-sequencing on individuals that vary in the expression of such traits.
85 For example, in social insects, recent studies have compared the transcriptomes of workers that perform
86 nursing versus foraging tasks [37–39], or nurses feeding larvae of different stages or castes [35,40].
87 However, given the phenotypic co-regulation known to occur between interacting social partners (here,
88 nurses and larvae), it is likely that genes expressed in one social partner affect the expression of genes in
89 the other social partner, and vise-versa, such that interacting social partners are connected by "social"
90 gene regulatory networks [14,32,41,42]. Thus, identifying the genes important for *social interactions* such
91 as nurse-larva interactions is only possible by studying the transcriptomic dynamics of both interacting
92 social partners across a time series of interactions.

93 To understand the transcriptomic basis of host-symbiont interactions, recent studies have
94 reconstructed gene regulatory networks acting between hosts and symbionts by collecting and profiling
95 the transcriptomes of each social partner across a time series of interactions [43–47]. Here, we use
96 analogous methodology to study transcriptomic signatures of nurse-larva interactions in the pharaoh ant,
97 *Monomorium pharaonis*. We sample a developmental time series of larvae as well as the nurses that feed
98 each larval stage in this series, collecting individuals at the moment of interaction in order to identify
99 genes involved in the expression of nurse-larva interactions, as well as genes affected by these
100 interactions (i.e. the full "social interactome" [14]). Pharaoh ant nurses tend to specialize on feeding
101 young versus old larvae, and nurses feeding young versus old larvae show different transcriptomic
102 profiles [40]. Larval transcriptomic profiles also change over development [48,49]. Given these results,

103 we predicted that we would observe concerted changes in broad-scale gene expression in larvae and their
104 nurses across larval development (Fig 1), reflective of the functional importance of nurse-larva
105 interactions. Based on our dual RNA-seq data, we infer social gene regulatory networks acting between
106 nurses and larvae to identify candidate genes predicted to have important social regulatory effects.
107 Finally, we combine our measures of social regulatory effects with available population genomic data
108 [48] to characterize the patterns of molecular evolution of genes underlying nurse-larva interactions.
109

110 **Results**

111 *Transcriptome-wide signatures of nurse-larva co-expression across larval development*

112 To elucidate transcriptomic signatures of nurse-larva interactions, we performed RNA-sequencing on
113 worker-destined larvae across five developmental stages and nurses that fed larvae of each developmental
114 stage (termed "stage-specific" nurses; see S1 Fig for sampling scheme, S1 Table for list of samples),
115 building upon a previously published dataset focused on caste development in *M. pharaonis* [48]. We
116 hypothesized that if genes expressed in larvae regulate the expression of genes in nurse and vice versa, we
117 would observe correlated expression profiles across larval development in larvae and nurses (Fig 1). As a
118 biological control, we collected "random nurses" that we observed feeding any stage of larvae in the
119 colony, and hence would not be expected to show correlated expression dynamics with larvae across the
120 five larval developmental stages. We also collected reproductive-destined larvae, but unless clearly stated
121 otherwise, all analyses were performed on only worker-destined larvae. We collected ten individuals of
122 each sample type to pool into one sample, and we sequenced whole bodies of larvae but separated nurse
123 heads and abdomens prior to sequencing.

124 We grouped genes into co-expression profiles or "modules" using an algorithm designed to
125 characterize gene co-expression dynamics across a short time series [50], known as Short Time-Series
126 Expression Mining (STEM) [51]. Each module represents a standardized pre-defined expression profile,
127 consisting of five values that each represent the $\log_2$ fold-change between the given developmental stage
128 and the initial (L1) stage (see S2 Fig; this results in a total of 81 possible modules). We sorted genes into

129 the module that most closely represented their expression profile by Pearson correlation. We identified
130 modules containing a greater than expected number of genes, where we formed null expectations using
131 permutation tests across developmental stages [50]. We identified such significantly-enriched modules
132 separately for larvae, stage-specific nurse heads, stage-specific nurse abdomens, random nurse heads, and
133 random nurse abdomens. We focused on both parallel (i.e. positive regulation or activation) and anti-
134 parallel (i.e. inhibitory) correlated expression patterns by identifying significantly-enriched modules that
135 were shared in both larvae and nurses (parallel), as well as significantly-enriched modules for which the
136 inverse of the module was identified as significantly-enriched in the social partner (anti-parallel).
137 Larvae and stage-specific nurses shared many significantly-enriched modules (S2 Table). These
138 shared modules contained the majority of genes expressed in nurses (65% of genes in stage-specific nurse
139 heads and 76% in abdomens). A substantial proportion of the larval transcriptome was also shared with
140 stage-specific nurse heads (22% of larval genes) and abdomens (60% of larval genes). Overall there was a
141 widespread signature of correlated transcriptional patterns between stage-specific nurses and larvae across
142 larval development (Fig 2A-D). These coordinated dynamics were dominated by parallel associations in
143 nurse abdomens (possibly reflecting shared metabolic pathways) but anti-parallel associations in nurse
144 heads (possibly reflecting the social regulation of larval growth). In contrast to stage-specific nurses,
145 random nurses (our biological control) shared few significantly-enriched modules with larvae (S2 Table),
146 and modules shared between random nurses and larvae contained significantly fewer genes than modules
147 shared between stage-specific nurses and larvae (Fig 2E; Wilcoxon test, $P < 0.001$ for all comparisons).
148 Specifically, 2% of genes expressed in random nurse heads and 13% of genes expressed in random nurse
149 abdomens were in modules shared with larvae; 3% of genes expressed in larvae were in modules shared
150 with random nurse heads, and 2% of genes expressed in larvae were in modules shared with random nurse
151 abdomens.
152
153 *Identification of genes putatively involved in social interactions*

154 Given that we observed transcriptome-wide patterns consistent with nurse-larva transcriptional co-
155 regulation across larval development, we next identified the genes that might be driving these patterns
156 (see S3 Fig). We performed differential expression analysis to identify genes that varied in larval
157 expression according to larval developmental stage, as well as genes that varied in nurse expression
158 according to the developmental stage of larvae they fed. We identified 8125 differentially expressed
159 genes (DEGs) in larvae (78% of 10446 total genes). We identified 2057 and 1408 DEGs in stage-specific
160 nurse heads and abdomens, respectively, compared to 599 and 520 DEGs in random nurse heads and
161 abdomens, respectively. We removed genes differentially expressed in both stage-specific and random
162 nurses (N = 272 DEGs in heads, N = 140 DEGs in abdomens), which might differ among our colony
163 replicates due to random colony-specific effects that were not consistently associated with social
164 regulation of larval development. After this removal, we retained the top 1000 DEGs, sorted by P-value,
165 for each sample type other than random nurses (larvae, stage-specific nurse heads, stage-specific nurse
166 abdomens) for social gene regulatory network reconstruction, reasoning that these genes were the most
167 likely to be involved in the regulation of larval development.
168
169 *Reconstruction of social gene regulatory networks*
170 To infer putative gene-by-gene social regulatory relationships between nurses and larvae, we
171 reconstructed gene regulatory networks acting within and between nurses and larvae (S3 Fig). The output
172 of regulatory network reconstruction is a matrix of connection strengths, which indicate the regulatory
173 effect (positive or negative) one gene has on another, separated according to the tissue the gene is
174 expressed in. To identify the most highly connected (i.e. centrally located, upstream) genes of regulatory
175 networks, we calculated within-tissue connectivity and social connectivity by averaging the strength of
176 connections across each connection a gene made, differentiating between within-tissue (nurse-nurse or
177 larva-larva) and social connections (nurse-larva) (Fig 1B). On average, within-tissue connectivity was
178 higher than social connectivity (Wilcoxon test; $P < 0.001$ in all tissues), and within-tissue connectivity
179 was negatively correlated with social connectivity in each tissue (S4 Fig). The top enriched gene ontology

180 terms based on social connectivity in nurses were entirely dominated by metabolism (S3,S4 Tables; see
181 also S5 Table for the top 20 genes by nurse social connectivity).
182
183 *Secreted proteins and social gene regulation*
184 While based on our data it is not possible to distinguish between genes that code for protein products that
185 are actually exchanged between nurses and larvae versus genes that affect behavior or physiology within
186 organisms (Fig 1A), proteins that are known to be cellularly secreted represent promising candidates for
187 the social regulation of larval development [40]. We downloaded the list of proteins that are known to be
188 cellularly secreted from FlyBase [52] and used a previously-generated orthology map to identify ant
189 orthologs of secreted proteins [40]. Genes coding for proteins with orthologs that are cellularly secreted in
190 *Drosophila melanogaster* had higher social connectivity than genes coding for non-secreted orthologs in
191 nurse heads (Fig 3A; Wilcoxon test; P = 0.025), though not for nurse abdomens (P = 0.067).
192 For the most part, we have focused on broad patterns of nurse-larva gene coregulation. In this
193 paragraph, we will highlight the potential social role of one of the genes with the highest social
194 connectivity within nurse heads, *giant-lens* (S6 Table; *giant-lens* is the 7th highest gene coding for
195 secreted proteins by social connectivity in nurse heads). *Giant-lens* is an inhibitor of epidermal growth
196 factor receptor (EGFR) signaling [53], and g*iant-lens* expression in nurse heads was negatively associated
197 with the expression of the homolog of *eps8*, human EGFR substrate 8 in larvae, most prominently seen in
198 the spike in nurse *giant-lens* expression accompanied by a drop in larval *eps8* expression at the end of
199 larval development (Fig 3B). *Giant-lens* was also used in regulatory network reconstruction in larvae (i.e.
200 it was one of the top 1000 DEGs), and *giant-lens* expression in larvae drops steadily throughout
201 development (S5 Fig; in contrast to the pattern of *giant-lens* expression in nurse heads). Interestingly,
202 *eps8* does not exhibit a similar peak and drop in expression level in reproductive-destined larvae in
203 comparison to worker-destined larvae (S6 Fig). It is important to note that these patterns were not seen for
204 all genes in the EGFR pathway, and the results presented here cannot be taken as concrete evidence of

205 EGFR regulation via social processes. Nonetheless, the mechanism illustrated here represents a tangible
206 example of how nurse-larva interactions could function at the molecular level.
207
208 *Molecular evolution of social gene regulatory networks*
209 To investigate the selective pressures shaping social regulatory networks, we used population genomic
210 data from 22 resequenced *M. pharaonis* workers, using one sequenced *M. chinense* worker as an outgroup
211 [48]. Using polymorphism and divergence data, we estimated gene-specific values of selective constraint,
212 which represents the intensity of purifying selection that genes experience [54]. To identify genes
213 disproportionately recruited to the core of social regulatory networks, we calculated "sociality index" as
214 the difference between social connectivity and within-tissue connectivity for each gene. Sociality index
215 was negatively correlated to selective constraint due to a positive correlation between within-tissue
216 connectivity and constraint and a negative correlation between social connectivity and constraint (Fig 4A-
217 C). Additionally, genes differed in sociality index according to their estimated evolutionary age, with
218 ancient genes exhibiting lower sociality indices than genes in younger age categories (Fig 4D). Finally,
219 while evolutionary age and evolutionary rate appear to be somewhat empirically confounded [55],
220 selective constraint and evolutionary age were each independently associated with sociality index, based
221 on a model including both variables as well as tissue (GLM; LRT; evolutionary age: $\chi^2 = 21.536$, $P <$
222 0.001; selective constraint: $\chi^2 = 22.191$, $P < 0.001$).
223
224 **Discussion**
225 In organisms with extended offspring care, developmental programs are controlled in part by socially-
226 acting gene regulatory networks that operate between caregivers and developing offspring [14,42]. In this
227 study, we sequenced the transcriptomes of ant nurses and larvae as they interacted across larval
228 development to assess the effects of social interactions on gene expression dynamics. We found that large
229 sets of genes (i.e. modules) expressed in ant larvae and their caregiving adult nurses show correlated

230 changes in expression across development (Fig 2). The majority of nurse and larval transcriptomes was
231 represented in these correlated modules, suggesting that the tight phenotypic co-regulation characterizing
232 nurse-larva interactions over the course of larval development is also reflected at the molecular level.
233 To characterize the overall network and evolutionary patterns of genes involved in nurse-larva
234 interactions, we reverse engineered nurse-larva gene regulatory networks and calculated the "social
235 connectivity" for each gene, defined as the sum of inferred social regulatory effects on all genes
236 expressed in social partners. We found that genes with high social connectivity tended to have low
237 within-individual connectivity (S4 Fig; where within-individual connectivity is defined as the sum of
238 inferred regulatory effects acting within a given tissue). Nurse-expressed genes with higher sociality
239 indices (i.e disproportionately higher social connectivity than within-individual connectivity) tended to be
240 evolutionarily young and rapidly evolving due to relaxed selective constraint (Fig 4). Genes with high
241 social connectivity were enriched for a number of Gene Ontology (GO) categories associated with
242 metabolism (S3,S4 Tables), consistent with the idea that molecular pathways associated with metabolism
243 are involved in the expression of social behavior [56,57]. Previously, many of the proteins found to be
244 widely present in social insect trophallactic fluid transferred from nurses to larvae were involved in sugar
245 metabolism (e.g. Glucose Dehydrogenase, several types of sugar processing proteins) [15]. Along the
246 same lines, many of the genes with with high social connectivity in our study are also annotated with
247 terms associated with sugar metabolism (S5 Table; e.g. Glycerol-3-phosphate dehydrogenase, Glucose
248 dehydrogenase FAD quinone, Pyruvate dehydrogenase). Finally, we found that genes encoding for
249 orthologs of cellularly-secreted proteins in *Drosophila melanogaster* (possibly important for intercellular
250 signaling) tended to exhibit higher levels of social connectivity than their non-secreted counterparts (Fig
251 3A).
252 One gene that stands out in terms of being cellularly secreted and exhibiting a relatively high
253 social connectivity is *giant-lens*, which inhibits EGFR signaling [53]. EGFR signaling affects eye and
254 wing development [58] as well as body size in *D. melanogaster* [59], caste development in the honey bee
255 *Apis mellifera* [59,60] via the transfer of royalactin from nurses to larvae [59], and worker body size

variation in the ant *Camponotus floridanus* [61]. Further experimental work is necessary to ascertain whether *giant-lens* is actually orally secreted by nurses and transferred to larvae, but gene expression dynamics are consistent with the social transfer of *giant-lens* from nurses to larvae, followed by the inhibition of EGFR signaling at the end of larval development in worker-destined larvae (Fig 3B). Importantly, this inhibition is not seen in reproductive-destined larvae (S6 Fig). While caste in *M. pharaonis* is socially regulated in the first larval stage [49], social inhibition of EGFR signaling could play a role in the regulation of worker body size [61] or secondary caste phenotypes such as wings [62,63].

In terms of broad evolutionary patterns, our study complements previous results suggesting genes with worker-biased expression tend to be rapidly evolving, evolutionarily young, and loosely connected in regulatory networks in comparison to genes with queen-biased expression [38,48,64–66]. Because pharaoh ant workers are obligately sterile, their traits are shaped indirectly by kin selection, based on how they affect the reproductive success of fertile relatives (i.e. queens and males) [23,67]. As a result, all-else-equal, genes associated with worker traits are expected to evolve under relaxed selection relative to genes associated with queen traits [68,69].

In general, the suite of genic characteristics commonly associated with worker-biased genes (rapidly evolving, evolutionarily young, loosely connected) are all consistent with relaxed selection acting on genes associated with workers [49]. Here, we show that within the worker caste, genes that appear to be functionally involved in the expression of social behavior (i.e. nursing) experience relaxed selective constraint relative to genes important for within-worker processes. Therefore, the combination of kin selection as well rapid evolution thought to be characteristic of social traits [25] likely act in concert to shape the labile evolutionary patterns commonly associated with worker-biased genes. Finally, it has also been suggested that plastic phenotypes such as caste recruit genes which were evolving under relaxed selection prior to the evolution of such plastic phenotypes [70–72]. Our results could also be consistent with this hypothesis, though the population genomic patterns we observe show that relaxed selective constraint is ongoing.

282 In this study, we sought to reconstruct regulatory networks acting between nurses and larvae,
283 beginning with the assumption that nurse gene expression changes as a function of the larval stage fed.
284 This is more likely to be the case when nurses are specialized on feeding particular larval stages.
285 According to a previous study, about 50% of feeding events are performed by specialists (though note
286 specialization is likely a continuous trait, and the 50% figure is the result of a binomial test) [40].
287 Therefore, we expect our stage-specific nurse samples to comprise about 50% specialists. We also expect
288 random nurse samples to contain 50% specialist nurses, but, crucially, the specialists should be relatively
289 evenly divided among larval stages since random nurses were collected regardless of which larval stage
290 they were observed feeding. Because our stage-specific nurse samples did not consist of 100% specialists,
291 we expect that the signal of nurse-larva co-expression in our analysis is effectively diluted. In order to
292 maximize the signal of nurse-larval co-expression dynamics, future studies would ideally focus entirely
293 on specialists, as well as on tissues such as brains and the specific exocrine glands [73] known to be
294 important for social behavior and communication. Despite these limitations, we were still able to observe
295 transcriptomic signatures consistent with the social regulation of larval development.
296

297 **Conclusions**

298 In this study, we uncovered putative transcriptomic signatures of social regulation and identified distinct
299 evolutionary features of genes that underlie "social physiology", the communication between individuals
300 that regulates division of labor within social insect colonies [74,75]. Because we simultaneously collected
301 nurses and larvae over a time series of interactions, we were able to elucidate the putative molecular
302 underpinnings of nurse-larval social interactions. This is a promising approach that could be readily
303 extended to study the molecular underpinnings of all forms of social regulation in social insect colonies,
304 including regulation of foraging, regulation of reproduction, etc.. Furthermore, by adapting the
305 methodology presented here (i.e. simultaneous collection over the course of interactions followed by
306 sequencing), the molecular mechanisms and evolutionary features of genes underlying a diverse array of
307 social interactions, including courtship behavior, dominance hierarchy formation, and regulation of

308 biofilm production could all be investigated. Overall, this study provides a foundation upon which future
309 research can build to elucidate the genetic underpinnings and evolution of interacting phenotypes.
310

311 **Methods**

312 This study builds on previous work investigating genomic signatures of kin selection in which we
313 characterized transcriptomic profiles from adult queens and workers, as well as queen- and worker-
314 destined larvae [48]. While stage-specific nurses were used in the previous analysis, the knowledge of the
315 developmental stage of larvae they fed was not, as they were simply treated as adult workers. This study
316 also complements the past dataset with new data from random nurses, which were collected concurrently
317 with previous samples.
318

319 *Study Design*

320 To construct experimental colonies, we began by creating a homogenous mixture of approximately fifteen
321 large source colonies of the ant *Monomorium pharaonis*. From this mixture, we created thirty total
322 replicate experimental colonies of approximately equal sizes (~300-400 workers, ~300-400 larvae). We
323 removed queens from ½ the study colonies to promote the production of reproductive-destined larvae.
324 Reproductive caste is determined in *M. pharaonis* by the end of the first larval instar, likely in the egg
325 stage [76], and queen presence promotes culling of reproductive-destined L1 larvae. Removing queens
326 halts this culling, but it is unknown which colony members actually perform such culling [76]. While we
327 initially expected the presence of queens to impact the gene expression profiles of nurses, we detected 0
328 DEGs (FDR < 0.1) between queen-present and queen-absent colonies for every sample type. This could
329 indicate that nurses don't perform culling and that worker developmental trajectories (and nutritional
330 needs) are not appreciably different between queen-present and queen-absent colonies. Because queen
331 presence did not substantially impact gene expression, in this study we pooled samples across queen-
332 present and queen-absent colonies for all analyses.

333 We pre-assigned colonies to one of five larval developmental stages (labeled L1-L5, where L1
334 and L2 refer to 1st-instar and 2nd-instar larvae and L3, L4, and L5 refer to small, medium, and large 3rd-
335 instar larvae [77]). We identified larval stage through a combination of hair morphology and body size.
336 L1 larvae are nearly hairless, L2 larvae have straight hairs and are twice the length of L1 larvae, and L3-
337 L5 larvae have dense, branched hairs [78]. We separated 3rd-instar larvae into three separate stages based
338 on body size [77] because the vast majority of larval growth occurs during these stages. We sampled
339 individuals (larvae as well as nurses) across larval development time: beginning at the L1 stage, we
340 sampled colonies assigned to each subsequent stage at intervals of 3-4 days, by the time the youngest
341 larvae in colonies lacking queens were of the assigned developmental stage (note that in colonies lacking
342 queens, no new eggs are laid so the age class of the youngest individuals progressively ages). We sampled
343 each colony once, according to the developmental stage we had previously assigned the colony (e.g. for
344 colonies that we labeled 'L4', we waited until it was time to sample L4 larvae and nurses and sampled
345 individuals from that colony at that time). From each colony, we sampled stage-specific nurses and
346 worker-destined larvae, as well as random nurses from colonies with queens and reproductive-destined
347 larvae from colonies without queens (starting at the L2 stage, because at L1 caste cannot be distinguished
348 [76,77]. Reproductive-destined larvae include both males and queens (which cannot be readily
349 distinguished), though samples are expected to be largely made up of queen-destined individuals given
350 the typically skewed sex ratio of *M. pharaonis* [48]. See S1 Table for full sample list.
351 For each time point in each assigned colony, we collected stage-specific nurses, nurses feeding
352 larvae of the specified developmental stage (L1, L2, etc). Concurrently, we collected random nurses,
353 nurses we observed feeding a larva of any developmental stage. Rather than paint-marking nurses, we
354 collected them with forceps as soon as we saw them feeding larvae. We collected random nurses as soon
355 as we observed them feeding a larva of any developmental stage in the course of visually scanning the
356 colony. We did not make an attempt to systematically collect nurses from different areas of the nest but
357 did so haphazardly, such that the distribution of larval stages fed resembled overall colony demography.
358 Nurses feed L1 and L2 larvae exclusively via trophallaxis (i.e. liquid exchange of fluid), while nurses

359 feed L3-L5 larvae both via trophallaxis and by placing solid food in larval mouthparts [79]. To get a
360 representative sample of all types of nurses, we did not distinguish between nurses feeding liquid and
361 solid food, though all L3-L5 samples contained a mixture of the two. After collecting nurses, we
362 anaesthetized the colony using carbon dioxide and collected larvae of the specified developmental stage.
363 All samples were flash-frozen in liquid nitrogen immediately upon sample collection. Note that workers
364 in *M. pharaonis* are monomorphic [80].
365 We performed mRNA-sequencing on all samples concurrently using Illumina HiSeq 2000 at
366 Okinawa Institute of Science and Technology Sequencing Center. Reads were mapped to the NCBI
367 version 2.0 *M. pharaonis* assembly [38], and we used RSEM [81] to estimate counts per locus and
368 fragments per kilobase mapped (FPKM) for each locus. For further details on RNA extraction and library
369 preparation, see [48].
370
371 *Transcriptome-wide signatures of nurse-larva co-expression across larval development*
372 We used an algorithm that categorizes genes based on their expression dynamics over time into a number
373 of modules represented by pre-defined expression profiles [50]; see S2 Fig for workflow). To create
374 modules, we started at 0 and either doubled, halved, or kept the expression level the same at each
375 subsequent stage, resulting in 81 possible modules (3*3*3*3 = 81; four stages after L1). To generate
376 gene-specific expression profiles based on real results, we calculated the average $\log_2$ fold change in
377 expression (FPKM) of the gene at each developmental stage compared to the initial expression level at
378 stage L1. We then assigned each gene to the closest module by Pearson correlation between gene
379 expression profile and module expression profile [50]. To identify significantly-enriched modules, we
380 generated null distributions of the number of genes present in each module (based on permutation of
381 expression over time), and retained modules with a significantly greater than expected number of genes
382 based on these null distributions (FDR < 0.05 after Bonferroni multiple correction [50]).
383
384 *Identification of genes putatively involved in social interactions*

385 We used the package EdgeR [82] to construct models including larval developmental stage and replicate
386 and performed differential expression analysis for each sample type separately. We retained genes
387 differentially expressed according to a nominal P-value of less than 0.05 (i.e. no false discovery
388 correction), as the purpose of this step was simply to identify genes that could be involved in interactions
389 that shape larval development (rather than spurious interactions arising from replicate-specific effects).
390 See S1 Dataset for a list of all stage-specific nurse and larval differentially expressed genes.
391
392 *Social regulatory network reconstruction*
393 We normalized expression for each gene using the inverse hyperbolic sine transformation of FPKM. As
394 input to the algorithm, we constructed "meta-samples" by combining expression data within the same
395 replicate and time point from nurses and larvae and labeling genes according to the tissue they were
396 expressed in, along the lines of host-symbiont studies [43,45]. We utilized the program GENIE3 [83,84]
397 to construct two types of networks: those acting between larvae and nurse heads, and those acting
398 between larvae and nurse abdomens.
399 GENIE3 uses a random forest method to reconstruct regulatory connections between genes, in
400 which a separate random forest model is constructed to predict the expression of each gene, with the
401 expression of all other genes as predictor variables. The output of GENIE3 is a matrix of pairwise
402 directional regulatory effects, where the regulatory effect of gene *i* on gene *j* is estimated as the feature
403 importance of the expression of gene *i* for the random forest model predicting the expression of gene *j*
404 (i.e. regulatory effect is how important the expression of gene *i* is for determining the expression of gene
405 *j*). These regulatory effects (or strengths) include both positive and negative as well as non-linear effects,
406 though these different effect types are not distinguished.
407 As a side note, a version of GENIE3 that was developed for time series data, dynGENIE3 [85],
408 does exist. However, we opted to utilize the original GENIE3 algorithm because we reasoned that the
409 temporal spacing of developmental stages was likely too sparse for regulatory network reconstruction to
410 incorporate time (note also that the co-expression algorithm we used, STEM, was explicitly designed for

411 short time series such as ours). While our method therefore does not explicitly incorporate temporal
412 dynamics, we purposefully biased our results to emphasize larval development over differences between
413 replicates by only utilizing genes differentially expressed across larval development (or based on larval
414 stage fed in the case of nurses).

415 We repeated the entire regulatory reconstruction reconstruction process 1000 times and averaged
416 pairwise connection strengths across runs, as the algorithm is non-deterministic. To capture the total
417 effect of each gene on the transcriptome dynamics within tissues, we averaged the regulatory effects each
418 gene had on all other 999 genes expressed in the same tissue ("within-individual connectivity").
419 Similarly, to capture the effect each gene had on the transcriptome of social partners, we averaged
420 regulatory effects each gene had on the 1000 genes expressed in social partners ("social connectivity").
421

422 *Estimation of selective constraint, and evolutionary rate*

423 Previously, we performed whole-genome resequencing on 22 diploid *M. pharaonis* workers as well as
424 one diploid *M. chinense* worker to serve as an outgroup [48]. We estimated selective constraint using
425 MKtest2.0 [86], assuming an equal value of alpha (an estimate of the proportion of nonsynonymous
426 substitutions fixed by positive selection) across all genes. Selective constraint is the estimate of the
427 proportion of nonsynonymous mutations that are strongly deleterious and thereby do not contribute to
428 polymorphism or divergence [86]. Selective constraint is estimated using polymorphism data, so it
429 represents the strength of purifying selection genes experience within the study population [54].
430

431 *Phylostratigraphic Analysis*

432 Phylostrata are hierarchical taxonomic categories, reflecting the most inclusive taxonomic grouping for
433 which an ortholog of the given gene can be found [87–90]. We focused on distinguishing between genes
434 that were evolutionarily "ancient", present in non-insect animals, versus genes present in only insects,
435 hymenopterans, or ants [49]. We constructed a database containing 48 hymenopteran available genomes,
436 10 insect non-hymenopteran genomes, and 10 non-insect animal genomes (S2 Dataset). For outgroup

437 genomes, we focused on well-annotated genomes which spanned as many insect orders and animal phyla
438 as possible. Using this database, we estimated evolutionary age of genes based on the most evolutionarily
439 distant identified BLASTp hit (E-value 10^{-10}).
440
441 *Gene Set Enrichment Analysis*
442 We performed gene set enrichment analysis based on social connectivity for each gene in each tissue
443 separately using the R package topGO [91]. We identified enriched gene ontology terms using
444 Kolmogorov-Smirnov tests ($P < 0.05$).
445
446 *General Analyses*
447 We performed all statistical analyses and generated all plots using R version in R version 3.4.0 [92], aided
448 by the packages "reshape2" [93], "plyr" [94], and "ggplot2" [95].
449
450 *Data Availability*
451 All raw reads are available at DDBJ bioproject PRJDB3164. All source data for generating figures is
452 included as S3 Dataset. All scripts and processed data (e.g. expression matrices, evolutionary measures)
453 are available at https://github.com/warnerm/MonomoriumNurseLarva.
454
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458 hymenopteran genomes for use in phylostratigraphy, and Rohini Singh for comments on the manuscript.
459
460 **References**
461 1. Frank SA. All of life is social. Curr Biol. 2007 Aug 21;17(16):R648–50.
462 2. Moore AJ, Brodie ED III, Wolf JB. Interacting phenotypes and the evolutionary process: I. Direct

and indirect genetic effects of social interactions. Evolution. 1997;51(5):1352–62.

3. Wolf JB, Brodie ED III, Cheverud JM, Moore AJ, Wade MJ. Evolutionary consequences of indirect genetic effects. Trends Ecol Evol. 1998 Feb 1;13(2):64–9.

4. Bleakley BH, Wolf JB, Moore AJ. The quantitative genetics of social behaviour. Social behaviour: genes, ecology and evolution. 2010;29–54.

5. Bijma P. Estimating maternal genetic effects in livestock. J Anim Sci. 2006 Apr;84(4):800–6.

6. Bouwman AC, Bergsma R, Duijvesteijn N, Bijma P. Maternal and social genetic effects on average daily gain of piglets from birth until weaning 1. J Anim Sci. 2010;88(9):2883–92.

7. Stay B, Coop AC. "Milk" secretion for embryogenesis in a viviparous cockroach. Tissue and Cell. 1974;6(4):669–93.

8. Chen Z, Corlett RT, Jiao X, Liu S-J, Charles-Dominique T, Zhang S, et al. Prolonged milk provisioning in a jumping spider. Science. 2018 Nov 30;362(6418):1052–5.

9. Moczek AP. Horn polyphenism in the beetle *Onthophagus taurus*: larval diet quality and plasticity in parental investment determine adult body size and male horn morphology. Behav Ecol. 1998 Jan 1;9(6):636–41.

10. Lindström J. Early development and fitness in birds and mammals. Trends Ecol Evol. 1999 Sep;14(9):343–8.

11. Wilson Edward O. Sociobiology: the new synthesis. Cambridge, MA: Belknap; 1975.

12. Wheeler DE. Developmental and physiological determinants of caste in social Hymenoptera: evolutionary implications. Am Nat. 1986;128(1):13–34.

13. Linksvayer TA, Kaftanoglu O, Akyol E, Blatch S, Amdam GV, Page RE. Larval and nurse worker control of developmental plasticity and the evolution of honey bee queen–worker dimorphism. J Evol Biol. 2011 Sep 1;24(9):1939–48.

14. Linksvayer TA. The molecular and evolutionary genetic implications of being truly social for the social insects. In: Zayed A, Kent CF, editors. Advances in Insect Physiology. Academic Press; 2015. p. 271–92.

15. LeBoeuf AC, Waridel P, Brent CS, Gonçalves AN, Menin L, Ortiz D, et al. Oral transfer of chemical cues, growth proteins and hormones in social insects. eLife Sciences. 2016 Nov 29;5:e20375.

16. LeBoeuf AC, Cohanim AB, Stoffel C, Brent CS. Molecular evolution of juvenile hormone esterase-like proteins in a socially exchanged fluid. Sci Rep. 2018 Dec 13;8:17830.

17. Brian MV. Larval recognition by workers of the ant *Myrmica*. Anim Behav. 1975 Nov;23(4):745–56.

18. Le Conte Y, Sreng L, Poitout SH. Brood pheromone can modulate the feeding behavior of *Apis mellifera* workers (Hymenoptera: Apidae). J Econ Entomol. 1995 Aug 1;88(4):798–804.

19. Slessor KN, Winston ML, Le Conte Y. Pheromone communication in the honeybee (*Apis mellifera* L.). J Chem Ecol. 2005 Nov;31(11):2731–45.

20. Penick CA, Liebig J. A larval “princess pheromone”identifies future ant queens based on their juvenile hormone content. Anim Behav. 2017 June;128:33-40.

21. Creemers B, Billen J, Gobin B. Larval begging behaviour in the ant *Myrmica rubra*. Ethol Ecol Evol. 2003 Jul 1;15(3):261–72.

22. Kaptein N, Billen J, Gobin B. Larval begging for food enhances reproductive options in the ponerine ant *Gnamptogenys striatula*. Anim Behav. 2005;69(2):293–9.

23. Hamilton WD. The genetical evolution of social behaviour. I. J Theor Biol. 1964 Jul;7(1):1–16.

24. Wolf JB, Brodie ED III, Moore AJ. Interacting phenotypes and the evolutionary process. II. Selection resulting from social interactions. Am Nat. 1999;153(3):254–66.

25. West-Eberhard MJ. Sexual selection, social competition, and speciation. Q Rev Biol. 1983;58(2):155–83.

26. McGlothlin JW, Moore AJ, Wolf JB, Brodie ED III. Interacting phenotypes and the evolutionary process. III. Social evolution. Evolution. 2010 Sep;64(9):2558–74.

27. Bailey NW, Marie-Orleach L, Moore AJ, Simmons L. Indirect genetic effects in behavioral ecology: does behavior play a special role in evolution? Behav Ecol. 2018 Jan 13;29(1):1–11.

28. Osborne KE, Oldroyd BP. Possible causes of reproductive dominance during emergency queen rearing by honeybees. Anim Behav. 1999 Aug;58(2):267–72.

29. Beekman M, Oldroyd BP. Effects of cross-feeding anarchistic and wild type honey bees: anarchistic workers are not queen-like. Naturwissenschaften. 2003 Apr;90(4):189–92.

30. Linksvayer TA. Direct, maternal, and sibsocial genetic effects on individual and colony traits in an ant. Evolution. 2006 Dec;60(12):2552–61.

31. Linksvayer TA. Ant species differences determined by epistasis between brood and worker genomes. PLoS One. 2007 Oct 3;2(10):e994.

32. Linksvayer TA, Fondrk MK, Page RE Jr. Honeybee social regulatory networks are shaped by colony-level selection. Am Nat. 2009 Mar;173(3):E99–107.

33. Teseo S, Châline N, Jaisson P, Kronauer DJC. Epistasis between adults and larvae underlies caste fate and fitness in a clonal ant. Nat Commun. 2014;5:3363.

34. Villalta I, Blight O, Angulo E, Cerdá X, Boulay R. Early developmental processes limit socially mediated phenotypic plasticity in an ant. Behav Ecol Sociobiol. 2016 Feb 1;70(2):285–91.

35. Vojvodic S, Johnson BR, Harpur BA, Kent CF, Zayed A, Anderson KE, et al. The transcriptomic and evolutionary signature of social interactions regulating honey bee caste development. Ecol Evol. 2015;5(21):4795–807.

36. Benowitz KM, McKinney EC, Cunningham CB, Moore AJ. Predictable gene expression related to behavioral variation in parenting. Behav Ecol;ary179;doi:10.1093/beheco/ary179.

37. Manfredini F, Lucas C, Nicolas M, Keller L, Shoemaker D, Grozinger CM. Molecular and social regulation of worker division of labour in fire ants. Mol Ecol. 2014 Feb;23(3):660–72.

535 38. Mikheyev AS, Linksvayer TA. Genes associated with ant social behavior show distinct
536 transcriptional and evolutionary patterns. Elife. 2015 Jan 26;4:e04775.

537 39. Kohlmeier P, Alleman AR, Libbrecht R, Foitzik S, Feldmeyer B. Gene expression is more strongly
538 associated with behavioural specialisation than with age or fertility in ant workers. Mol Ecol. 2018
539 Dec 7;doi:10.1111/mec.14971.

540 40. Walsh JT, Warner MR, Kase A, Cushing BJ, Linksvayer TA. Ant nurse workers exhibit behavioural
541 and transcriptomic signatures of specialization on larval stage. Anim Behav. 2018 Jul 1;141:161–9.

542 41. Bloch G, Grozinger CM. Social molecular pathways and the evolution of bee societies. Philos Trans
543 R Soc Lond B Biol Sci. 2011 Jul 27;366(1574):2155–70.

544 42. Linksvayer TA, Fewell JH, Gadau J, Laubichler MD. Developmental evolution in social insects:
545 regulatory networks from genes to societies. J Exp Zool B Mol Dev Evol. 2012 May;318(3):159–69.

546 43. Tierney L, Linde J, Müller S, Brunke S, Molina JC, Hube B, et al. An interspecies regulatory
547 network inferred from simultaneous RNA-seq of *Candida albicans* invading innate immune cells.
548 Front Microbiol. 2012 Mar 12;3:85.

549 44. Westermann AJ, Gorski SA, Vogel J. Dual RNA-seq of pathogen and host. Nat Rev Microbiol. 2012
550 Sep;10(9):618–30.

551 45. Schulze S, Henkel SG, Driesch D, Guthke R, Linde J. Computational prediction of molecular
552 pathogen-host interactions based on dual transcriptome data. Front Microbiol. 2015 Feb 6;6:65.

553 46. Westermann AJ, Förstner KU, Amman F, Barquist L, Chao Y, Schulte LN, et al. Dual RNA-seq
554 unveils noncoding RNA functions in host–pathogen interactions. Nature. 2016 Jan
555 20;529(7587):496–501.

556 47. Burns JA, Zhang H, Hill E, Kim E, Kerney R. Transcriptome analysis illuminates the nature of the
557 intracellular interaction in a vertebrate-algal symbiosis. ELife. 2017;6:e22054.

558 48. Warner MR, Mikheyev AS, Linksvayer TA. Genomic signature of kin selection in an ant with
559 obligately sterile workers. Mol Biol Evol. 2017 Jul 1;34(7):1780–7.

560 49. Warner MR, Qiu L, Holmes MJ, Mikheyev AS. Convergent eusocial evolution is based on a shared
561 reproductive groundplan plus lineage-specific plastic genes. Preprint. Available from:
562 https://www.biorxiv.org/content/early/2018/10/26/454645.abstract
563
564 50. Ernst J, Nau GJ, Bar-Joseph Z. Clustering short time series gene expression data. Bioinformatics.
565 2005 Jun;21 Suppl 1:i159–68.

566 51. Ernst J, Bar-Joseph Z. STEM: a tool for the analysis of short time series gene expression data. BMC
567 Bioinformatics. 2006 Apr 5;7:191.

568 52. Gramates LS, Marygold SJ, Santos G dos, Urbano J-M, Antonazzo G, Matthews BB, et al. FlyBase
569 at 25: looking to the future. Nucleic Acids Res. 2016;gkw1016.

570 53. Schweitzer R, Howes R, Smith R, Shilo BZ, Freeman M. Inhibition of *Drosophila* EGF receptor
571 activation by the secreted protein *Argos*. Nature. 1995 Aug 24;376(6542):699–702.

572 54. Kreitman M, Hudson RR. Inferring the evolutionary histories of the *Adh* and *Adh-dup* loci in
573 *Drosophila melanogaster* from patterns of polymorphism and divergence. Genetics. 1991
574 Mar;127(3):565–82.

575 55. Moyers BA, Zhang J. Evaluating phylostratigraphic evidence for widespread de novo gene birth in
576 genome evolution. Mol Biol Evol. 2016 May;33(5):1245–56.

577 56. Toth AL, Robinson GE. Evo-devo and the evolution of social behavior. Trends Genet. 2007
578 Jul;23(7):334–41.

579 57. Toth AL, Varala K, Henshaw MT, Rodriguez-Zas SL, Hudson ME, Robinson GE. Brain
580 transcriptomic analysis in paper wasps identifies genes associated with behaviour across social insect
581 lineages. Proc Biol Sci. 2010 Jul 22;277(1691):2139–48.

582 58. Shilo BZ. Signaling by the *Drosophila* epidermal growth factor receptor pathway during
583 development. Exp Cell Res. 2003 Mar 10;284(1):140–9.

584 59. Kamakura M. Royalactin induces queen differentiation in honeybees. Nature. 2011 May
585 26;473(7348):478–83.

586 60. Mutti NS, Dolezal AG, Wolschin F, Mutti JS, Gill KS, Amdam GV. IRS and TOR nutrient-signaling
587 pathways act via juvenile hormone to influence honey bee caste fate. J Exp Biol. 2011 Dec
588 1;214(23):3977–84.

589 61. Alvarado S, Rajakumar R, Abouheif E, Szyf M. Epigenetic variation in the *Egfr* gene generates
590 quantitative variation in a complex trait in ants. Nat Commun. 2015 Mar 11;6:6513.

591 62. Abouheif E, Wray GA. Evolution of the gene network underlying wing polyphenism in ants.
592 Science. 2002 Jul 12;297(5579):249–52.

593 63. Trible W, Kronauer DJC. Caste development and evolution in ants: it's all about size. J Exp Biol.
594 2017 Jan 1;220(Pt 1):53–62.

595 64. Johnson BR, Tsutsui ND. Taxonomically restricted genes are associated with the evolution of
596 sociality in the honey bee. BMC Genomics. 2011 Mar 29;12:164.

597 65. Harpur BA, Kent CF, Molodtsova D, Lebon JMD, Alqarni AS, Owayss AA, et al. Population
598 genomics of the honey bee reveals strong signatures of positive selection on worker traits. Proc Natl
599 Acad Sci U S A. 2014 Feb 18;111(7):2614–9.

600 66. Morandin C, Tin MMY, Abril S, Gómez C, Pontieri L, Schiøtt M, et al. Comparative transcriptomics
601 reveals the conserved building blocks involved in parallel evolution of diverse phenotypic traits in
602 ants. Genome Biol. 2016 Mar 7;17:43.

603 67. Bourke AFG, Franks NR. Social evolution in ants. Princeton University Press; 1995.

604 68. Linksvayer TA, Wade MJ. Genes with social effects are expected to harbor more sequence variation
605 within and between species. Evolution. 2009 Jul;63(7):1685–96.

606 69. Linksvayer TA, Wade MJ. Theoretical predictions for sociogenomic data: the effects of kin selection
607 and sex-limited expression on the evolution of social insect genomes. Front Ecol Evol; 2016
608 June;4:65.

70. Hunt BG, Ometto L, Wurm Y, Shoemaker D, Yi SV, Keller L, et al. Relaxed selection is a precursor to the evolution of phenotypic plasticity. Proc Natl Acad Sci U S A. 2011 Sep 20;108(38):15936–41.

71. Leichty AR, Pfennig DW, Jones CD, Pfennig KS. Relaxed genetic constraint is ancestral to the evolution of phenotypic plasticity. Integr Comp Biol. 2012 Jul;52(1):16–30.

72. Helanterä H, Uller T. Neutral and adaptive explanations for an association between caste-biased gene expression and rate of sequence evolution. Front Genet. 2014 Aug 29;5:297.

73. Eelen D, Børgesen L, Billen J. Functional morphology of the postpharyngeal gland of queens and workers of the ant *Monomorium pharaonis* (L.). Acta Zool. 2006;87(2):101–11.

74. Seeley TD. The Wisdom of the Hive: the social physiology of honey bee colonies. Harvard University Press; 2009. 318 p.

75. Johnson BR, Linksvayer TA. Deconstructing the superorganism: social physiology, groundplans, and sociogenomics. Q Rev Biol. 2010 Mar;85(1):57–79.

76. Warner MR, Lipponen J, Linksvayer TA. Pharaoh ant colonies dynamically regulate reproductive allocation based on colony demography. Behav Ecol Sociobiol. 2018 Mar 1;72(3):31.

77. Warner MR, Kovaka K, Linksvayer TA. Late-instar ant worker larvae play a prominent role in colony-level caste regulation. Insectes Soc. 2016 Nov 1;63(4):575–83.

78. Berndt KP, Kremer G. Larvenmorphologie der Pharaoameise *Monomorium pharaonis* (L.) (Hymenoptera, Formicidae). Zool Anz. 1986;216.

79. Cassill DL, Butler J, Vinson SB, Wheeler DE. Cooperation during prey digestion between workers and larvae in the ant, *Pheidole spadonia*. Insectes Soc. 2005 Nov 1;52(4):339–43.

80. Frumhoff PC, Ward PS. Individual-level selection, colony-level selection, and the association between polygyny and worker monomorphism in ants. Am Nat. 1992 Mar 1;139(3):559–90.

81. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics. 2011 Aug 4;12:323.

82. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 2010 Jan 1;26(1):139–40.

83. Huynh-Thu VA, Irrthum A, Wehenkel L, Geurts P. Inferring regulatory networks from expression data using tree-based methods. PLoS One. 2010 Sep 28;5(9). http://dx.doi.org/10.1371/journal.pone.0012776

84. Marbach D, Costello JC, Küffner R, Vega NM, Prill RJ, Camacho DM, et al. Wisdom of crowds for robust gene network inference. Nat Methods. 2012 Jul 15;9(8):796–804.

85. Huynh-Thu VA, Geurts P. dynGENIE3: dynamical GENIE3 for the inference of gene networks from time series expression data. Sci Rep. 2018 Feb 21;8(1):3384.

86. Welch JJ. Estimating the genomewide rate of adaptive protein evolution in *Drosophila*. Genetics. 2006 Jun;173(2):821–37.

87. Domazet-Lošo T, Tautz D. A phylogenetically based transcriptome age index mirrors ontogenetic

645 divergence patterns. Nature. 2010 Dec 9;468(7325):815–8.

646 88. Quint M, Drost H-G, Gabel A, Ullrich KK, Bönn M, Grosse I. A transcriptomic hourglass in plant
647 embryogenesis. Nature. 2012 Oct 4;490(7418):98–101.

648 89. Drost H-G, Gabel A, Grosse I, Quint M. Evidence for active maintenance of phylotranscriptomic
649 hourglass patterns in animal and plant embryogenesis. Mol Biol Evol. 2015 May;32(5):1221–31.

650 90. Domazet-Lošo T, Brajković J, Tautz D. A phylostratigraphy approach to uncover the genomic
651 history of major adaptations in metazoan lineages. Trends Genet. 2007;23(11):533–9.

652 91. Alexa A, Rahnenfuhrer J. topGO: enrichment analysis for gene ontology. R package version 2.28.0

653 92. R Core Team. R: A language and environment for statistical computing. Vienna, Austria: R
654 Foundation for Statistical Computing; 2017.

655 93. Wickham H. Reshaping data with the reshape package. J Stat Soft. 2007;21(12):1-20.

656 94. Wickham H. The split-apply-combine strategy for data analysis. J Stat Soft. 2011;40(1):1-29.

657 95. Wickham H. ggplot2: elegant graphics for data analysis. New York: Springer-Verlag; 2016.

658

659 **Figure Captions**

660 **Fig 1. Social regulation of gene expression between ant nurses and larvae.**

661 (A) Cartoon depicting positive gene regulation (i.e. activation) between larvae and nurses, where gene 1 is
662 expressed in nurses and genes 2 and 3 are expressed in larvae. After the expression of gene 1 increases,
663 the expression of gene 2 increases as a result of the social interaction of nursing (depicted in [B]). This
664 can occur if gene 1 itself codes for a protein passed to larvae, if the mRNA transcript is passed directly, or
665 if gene 1 activates the expression of some other gene in nurses, which in turn is passed as mRNA (or
666 codes for a protein that is passed) to larvae. Following the increase in expression of gene 2, the expression
667 of gene 3, which is shown to be activated by gene 2, also increases. While we have depicted a time-lag in
668 this social regulation of gene expression, the time lags are likely too short to observe in our data, as larvae
669 were collected every 3-4 days across development. Therefore, correlated transcriptome dynamics over
670 development (see Fig 2) would reflect mechanisms shown here. (B) Gene regulatory networks act
671 between and within individuals engaged in social interactions. Blue boxes are genes expressed in larvae,
672 and red boxes are genes expressed in nurses. Solid lines depict regulatory interactions within tissues

673 (here, within larvae or within nurses), while dashed lines represent social connections (nurse-larva or vice
674 versa).
675
676 **Fig 2. Nurse and larval transcriptomes show strong signatures of gene co-expression across larval**
677 **development.**
678 Plots (A-D) depict the expression profiles of individual genes (light lines) as expressed in (A) nurse head,
679 and (B) nurse abdomens, as well as (C) larvae, shared with nurse heads, and (D) larvae, shared with nurse
680 abdomens. Dark lines indicate the median expression values of all genes sorted into modules, with pre-
681 defined expression profiles of modules depicted in plot insets. Colors indicate the pre-defined expression
682 profile (i.e. module) that genes have been sorted into. Only the five shared modules containing the most
683 nurse-expressed genes are shown for clarity. Larval expression profiles are divided by the nurse tissue
684 they are shared with, such that (C) depicts larval gene expression shared with nurse heads (A), while (D)
685 depicts larval gene expression shared with nurse abdomens (B). Note that nurse heads and larvae shared
686 inversely-related expression profiles, and that this algorithm does not reveal the direction of regulation as
687 it is simply correlation-based. (E) Stage-specific nurses have more genes than random nurses in modules
688 shared with larvae than do random nurses, reflecting more broad-scale co-expression across development.
689 "Connection type" refers to the tissue that the number of genes was calculated in (i.e. larva → nurse head
690 indicates the number of genes expressed in larvae that are in modules shared with nurse heads), though
691 directionality is not determined in this algorithm. Error bars indicate 95% confidence intervals derived
692 from systematic drop-1 jackknifing of nurse samples. N = 10944 genes total.
693
694 **Fig 3. Genes encoding secreted proteins such as *giant-lens* are important for social gene regulation.**
695 (A) Genes encoding for proteins that are secreted in *Drosophila melanogaster* exhibit higher social
696 connectivity (i.e. more strongly socially regulate larval expression) in nurse heads than genes encoding
697 for non-secreted proteins (P-values from Wilcoxon test). (B) The protein *giant-lens* is one of the genes
698 coding for secreted proteins with the highest social connectivity in nurse heads. Based on our data, *giant-*

699 *lens* expressed in stage-specific nurse heads (red) appears to inhibit the expression of the homolog of
700 human EGFR substrate 8 (*eps8*) expressed in worker-destined larvae (blue). The expression of *giant-lens*
701 in nurses of a given colony was negatively correlated to the expression of *eps8* in larvae of the same
702 sampled colony (rho = -0.270, P < 0.001, N = 25 colony/stage pairings after removing missing samples).
703 Expression at stage *i* is equal to $\log_2(\text{expression}_i/\text{expression}_1)$, i.e. the ratio of expression at the given
704 stage to expression at L1.
705
706 **Fig 4. Highly social genes tend to be less evolutionarily constrained.**
707 Selective constraint, estimated from whole-genome polymorphism data, is (A) positively correlated with
708 within-tissue connectivity (Spearman correlation; head: rho = 0.122, P < 0.001; abdomen: rho = 0.217, P
709 < 0.001), but negatively correlated with (B) social connectivity (head: rho = -0.090, P = 0.009; abdomen:
710 rho = -0.150, P < 0.001) and (C) sociality index (head: rho = -0.132, P < 0.001; abdomen: rho = -0.223, P
711 < 0.001), where sociality index is the difference between social and within-tissue connectivity per gene.
712 Each point in (A-C) indicates a single gene, as expressed in nurse heads or abdomens. Lines are trendlines
713 from linear model. (D) Sociality index differs according to estimated evolutionary age (GLM; LRT; χ^2 =
714 57.357, P < 0.001), as ancient genes tended to have lower sociality indices than all other categories
715 (Tukey's post-hoc test; ancient - insect: P < 0.001, ancient - hymenoptera: P < 0.001, ancient - ant: P <
716 0.001, all other comparisons P > 0.05). Individual points depict average values across nurse heads and
717 abdomens for all genes within each estimated evolutionary age class, indicated by labels on points. Error
718 bars depict 95% confidence intervals from bootstrapping. Numbers in parentheses indicate number of
719 genes in each age class.
720
721 **S1 Fig. Diagram of sampling scheme.**
722 We collected ten worker-destined larvae, ten stage-specific nurses, and ten random nurses from each
723 colony (six colonies per time point, where time points represent larval developmental stages L1, L2, etc).

724 We collected stage-specific nurses when we observed them feeding larvae of the given developmental
725 stage. We collected random nurses when we observed them feeding any stage of larvae.
726
727 **S2 Fig. Identification of significantly-enriched modules shared between larvae and nurses.**
728 Inset tables depict pre-defined expression profiles of modules genes can be assigned to. First, we
729 construct modules using all possible expression profiles (top left bubble). Expression profiles consist of
730 five values, starting at zero, that indicate the $\log_2$ fold-change in expression from the initial value (at stage
731 L1). At each subsequent stage, we either double, halve, or keep the expression level the same. This
732 process is repeated to produce 81 (four stages after L1; 3*3*3*3 = 81) total modules. Next, for each tissue
733 separately (here we depict workflow in larvae with yellow bubbles), we calculate individual gene
734 expression profiles as the $\log_2$ fold-change in expression from the initial value at stage L1 and assign
735 genes to the closest related module by Pearson correlation. Concurrently, we permute the developmental
736 stage labels for each gene and assign the stage-permuted genes to modules (repeated 1000 times). From
737 these stage-permuted results, we calculate the mean number of genes assigned to each module and treat
738 this number as a null expectation (as each expression profile is not equally likely to occur by chance). We
739 then identify significantly-enriched modules using a one-way binomial test (with the calculated mean as
740 the null), with a Bonferroni-corrected false discovery rate of 0.05. This entire process is repeated in a
741 nurse tissue and significantly-enriched modules are found (blue bubble). Finally, we compare
742 significantly-enriched modules between larvae and nurses and retain identical and inverse modules as
743 shared profiles. An example of an inversely related profile is shown in red, where larvae exhibit the
744 enriched module [0, 0, -1, -2, -3] and nurses exhibit the inverse module, [0, 0, 1, 2, 3].
745
746 **S3 Fig. Workflow of preliminary differential expression analysis and gene regulatory network**
747 **reconstruction.**
748 On the left, we identify putatively socially-acting genes through differential expression analysis. First, for
749 nurse heads and abdomens separately, we perform differential expression analysis in stage-specific and

750 random nurses to identify genes differentially expressed according to larval stage fed, using a nominal P-
751 value of 0.05. We remove genes differentially expressed in random nurses, as these correspond to colony-
752 specific environmental effects unrelated to social regulation of larval development. Next, we select the
753 top 1000 differentially expressed genes by P-value in stage-specific nurses (after removing those DE in
754 random nurses) as well as the top 1000 differentially expressed genes in larvae. From these genes, we
755 create "meta-samples" by combining gene expression of larvae and stage-specific nurses collected from
756 the same colony (separately for heads and abdomens), and labeling genes by the tissue they are expressed
757 in. Using these meta-samples, we perform gene regulatory reconstruction (right) to identify genes
758 expressed in nurses that regulate larval gene expression, and vise-versa. We repeat gene regulatory
759 reconstruction 1000 times and average connection strength across runs, as the algorithm is non-
760 deterministic. The output of gene regulatory reconstruction is a matrix of regulatory connections acting
761 between genes. From this matrix, we calculate the average connectivity for each gene, separating within-
762 tissue (larva-larva or nurse head-nurse head) from social (nurse-larva) connections. Genes with high
763 connectivity are predicted to interact with many genes, i.e. are central to the network. Finally, we
764 calculate each genes' sociality index as the difference between social connectivity and within-tissue
765 connectivity.
766
767 **S4 Fig. Genes highly connected in social regulatory networks are loosely connected in within-tissue**
768 **regulatory networks**
769 Connectivity is representative of the number and strength of regulatory connections each gene makes.
770 Points indicate the average connectivity for a given gene, as measured within-tissue (x-axis; i.e. larva-
771 larva or nurse-nurse) or socially (y-axis; i.e. larva-nurse). Points are colored by tissue the connectivity is
772 measured in (e.g., dark blue indicates genes expressed in larvae, with connectivity measured in networks
773 constructed with nurse abdomens). Spearman rho = -0.166, -0.374, -0.276, -0.342 for the four tissues as
774 ordered in legend; $P < 0.001$ in all cases.
775

776 **S5 Fig. Expression of *giant-lens* in nurse heads and worker-destined larvae.** Expression at stage *i* is
777 equal to $\log_2(\text{expression}_i/\text{expression}_1)$, i.e. the ratio of expression at the given stage to expression at the
778 initial (L1) stage. **: $P < 0.01$, ns: $P > 0.05$ (Wilcoxon test at each stage).
779
780 **S6 Fig. Expression of *eps8* (epidermal growth factor receptor substrate 8) in worker-destined and**
781 **reproductive-destined larvae.** Expression at stage *i* is equal to $\log_2(\text{expression}_i/\text{expression}_1)$, i.e. the ratio
782 of expression at the given stage to expression at the initial (L1) stage. Expression of *eps8* changed
783 differently over time in worker-destined versus reproductive-destined larvae (linear model with
784 developmental stage treated as an ordinal variable; LRT; $\chi^2 = 12.574$, $P = 0.014$ for the interaction term
785 stage*caste).
786
787 **S1 Table. Description of samples included in study.** Worker-destined larvae are indicated by larva (W),
788 and reproductive-destined larvae are indicated by larva (R). Larval caste cannot be distinguished at the L1
789 stage, so L1 larvae are labeled larva (W/R). For network reconstruction, "meta" samples were used as
790 input for network reconstruction, in which genes were labeled by sample type and grouped such that each
791 gene contained a measurement of expression in worker-destined larvae, nurse heads, and nurse abdomens.
792 After sample collection and RNA extraction, some samples exhibited clearly degraded RNA according to
793 an Agilent Bioanalyzer assay. Removing these samples caused sampling to be uneven, so we used the
794 minimum number of samples contained across tissues at a given stage for stage-specific nurse heads and
795 abdomens, and randomly dropped excess samples. Overall, 25 "aggregate" samples were used as input for
796 gene regulatory network reconstruction.
797
798 **S2 Table. Number of nurse significantly-enriched modules shared with larvae.**
799 Significantly-enriched modules are defined as modules with a statistically significant number of genes
800 assigned, as determined by a permutation test (FDR < 0.05). Left column is the total number of
801 significant modules for each tissue, while the second and third columns indicate number shared with

802 larvae (out of 24 larval significantly-enriched modules). The last column indicates the total number of
803 genes in these shared modules.
804
805 **S3 Table. Nurse head social connectivity GO terms based on GSEA of social connectivity.**
806 P-value (unadjusted) is from Kolmogorov Smirnov (K-S) test. Enriched terms have higher than expected
807 social connectivity in nurse heads.
808
809 **S4 Table. Nurse abdomen GO terms based on GSEA of social connectivity.**
810 P-value (unadjusted) is from Kolmogorov Smirnov (K-S) test. Enriched terms have higher than expected
811 social connectivity in nurse abdomens.
812
813 **S5 Table. Top 20 genes by social connectivity in nurses.**
814 SwissProt ID is listed from automated annotation where a term was found.
815
816 **S6 Table. SwissProt annotations for the top genes coding for secreted proteins, sorted by social**
817 **connectivity.**
818 Only genes with SwissProt annotations are included. All genes listed encode for secreted proteins in *D.*
819 *melanogaster*.
820
821 **S1 Dataset. Complete list of all differentially expressed genes.**
822 Each gene can be differentially expressed in three tissues: worker larva, nurse head, or nurse abdomen. P-
823 values are listed for each tissue. The top 1000 differentially expressed genes (by P-value) were used for
824 regulatory network reconstruction. Social connectivity is the sum of all regulatory interactions in the
825 direction specified by "estimated regulatory direction".
826
827 **S2 Dataset. List of species used for phylostratigraphy.**

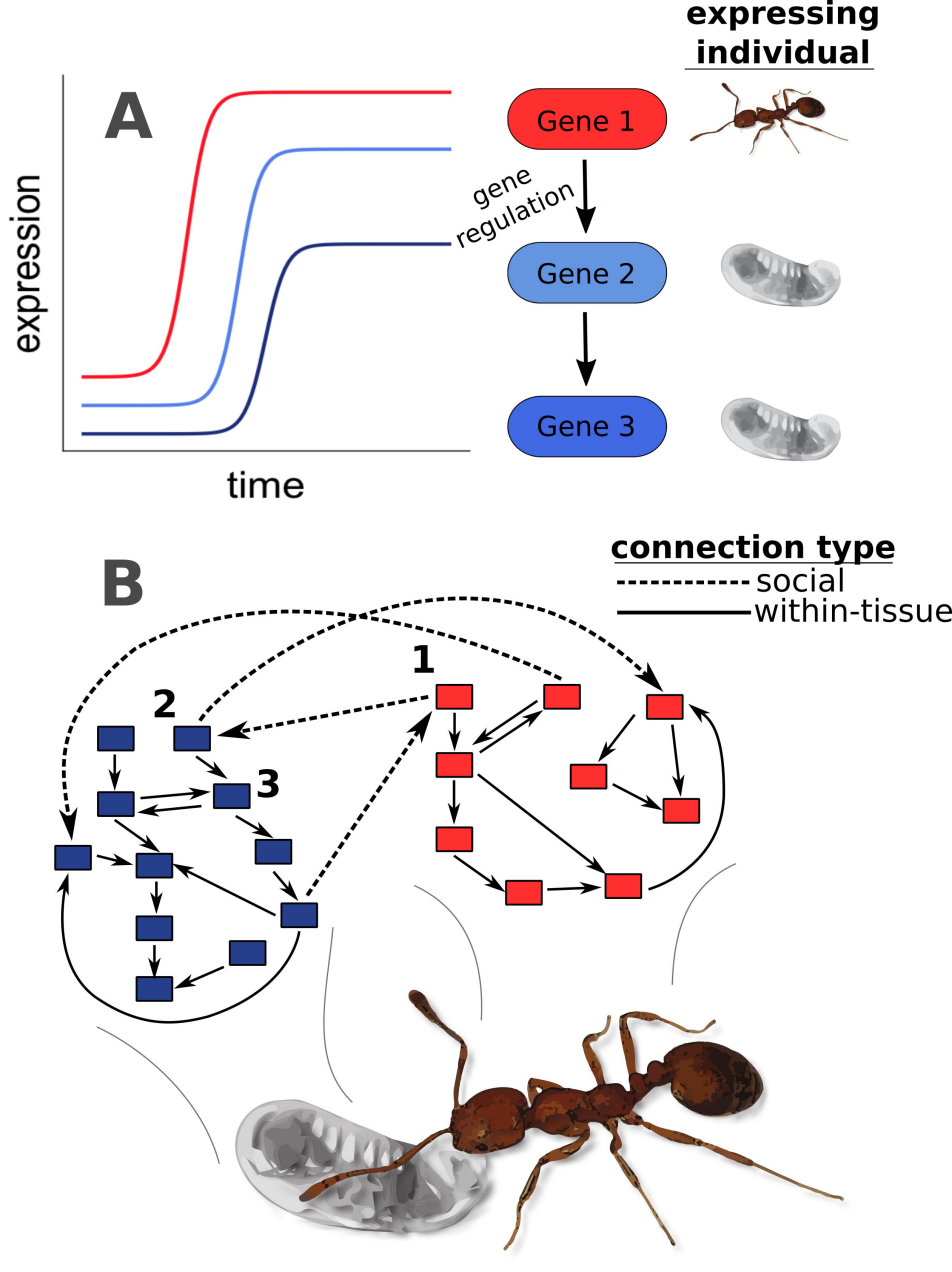
828 Each species listed, with their NCBI taxonomic ID, was used in the construction of the phylostratigraphic

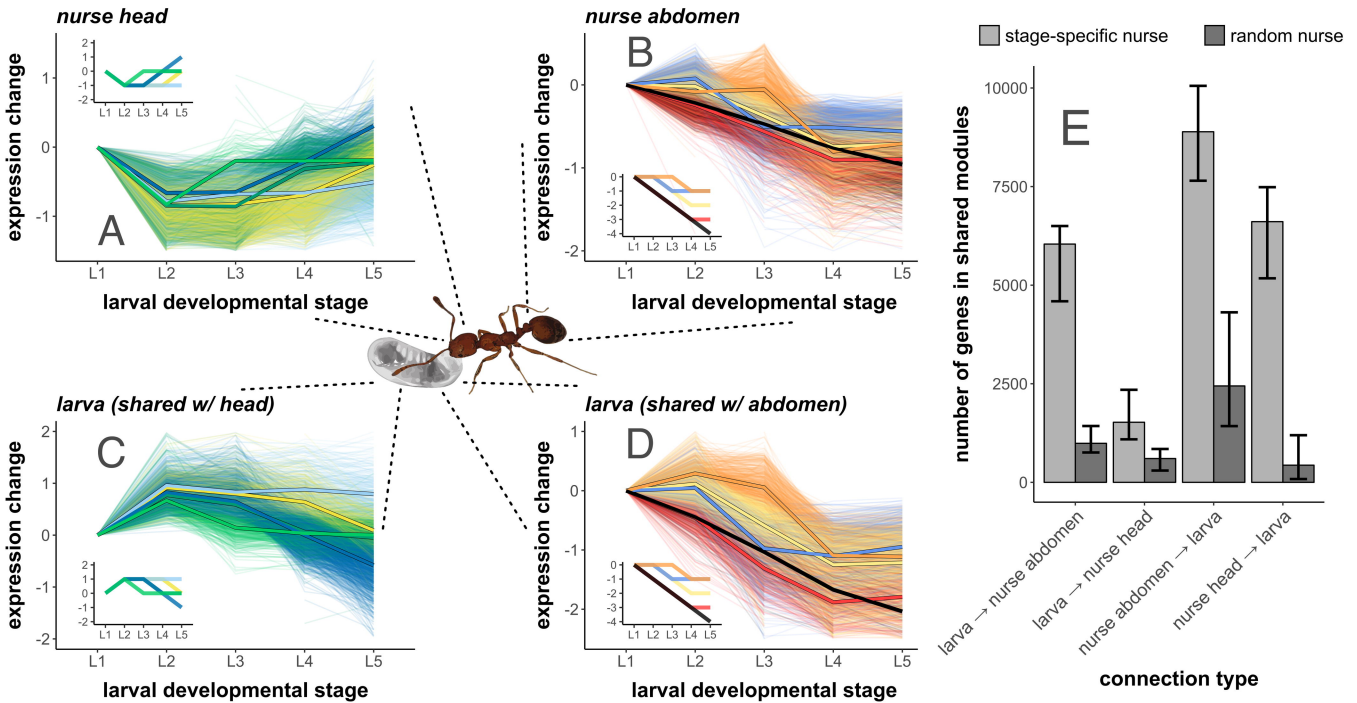
829 database to estimate evolutionary ages of genes.

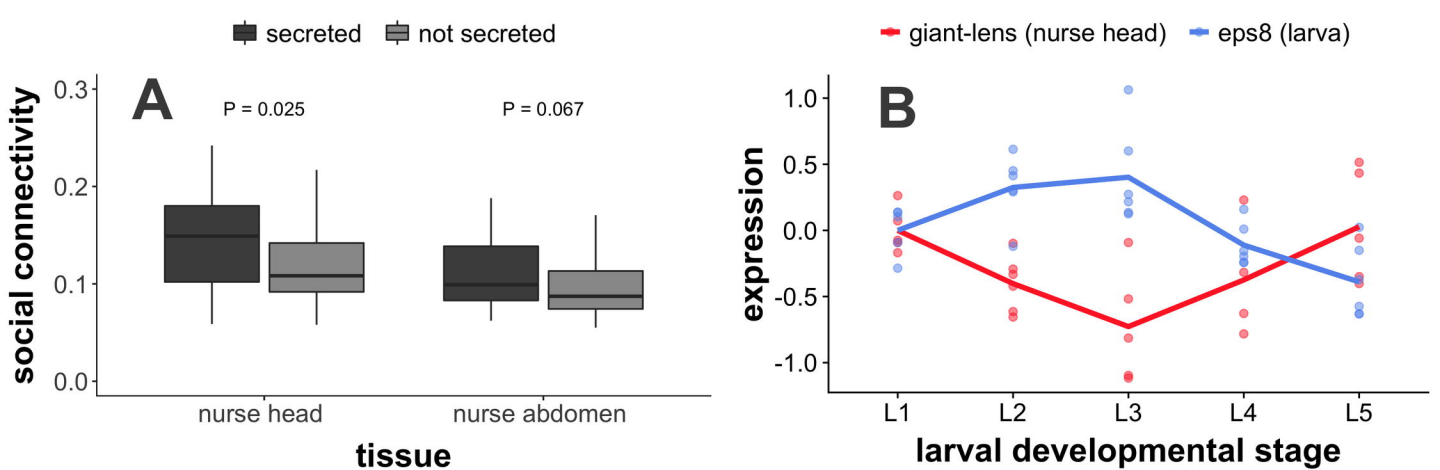
830

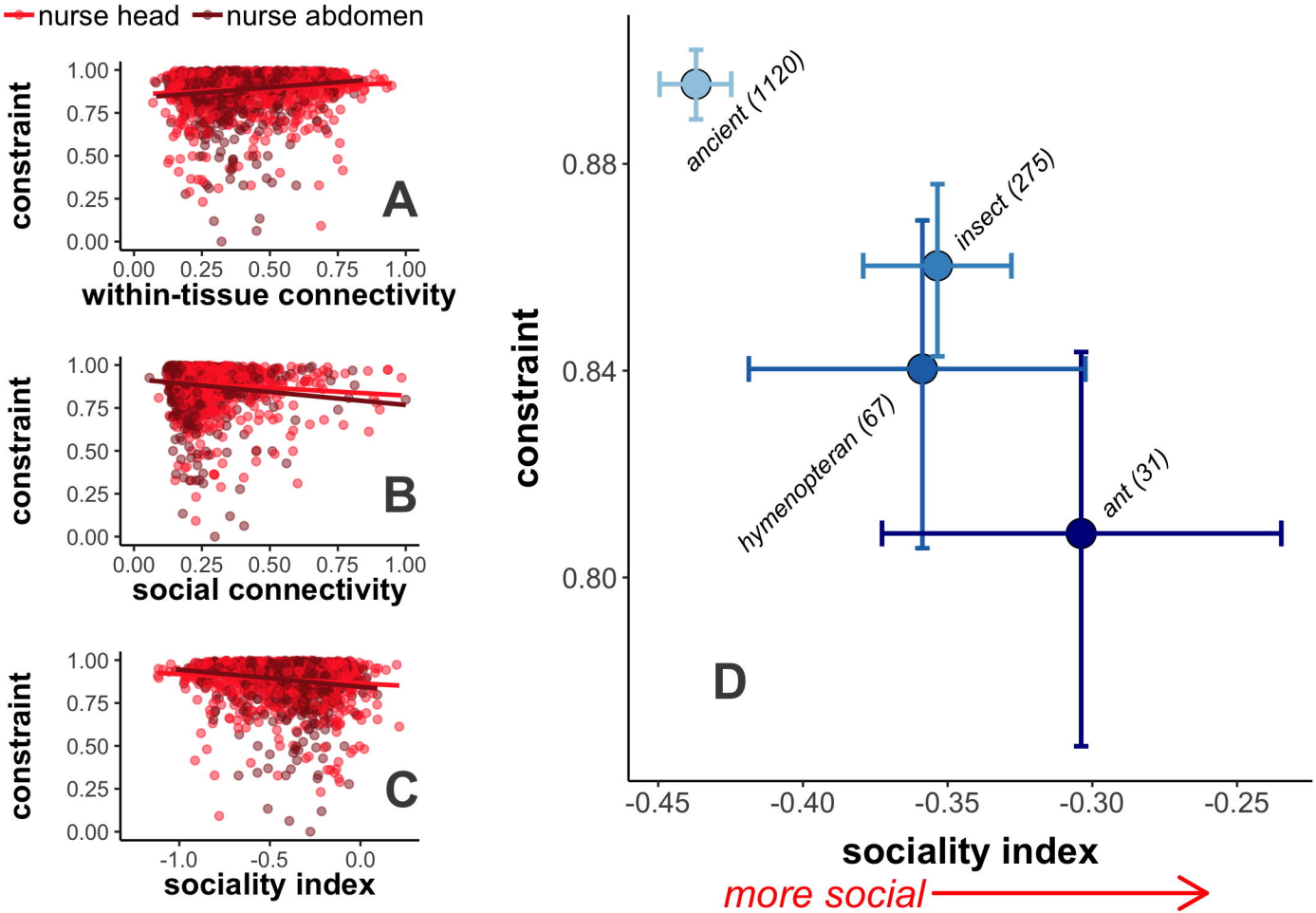
831 **S3 Dataset. Data files used to construct all figures.**

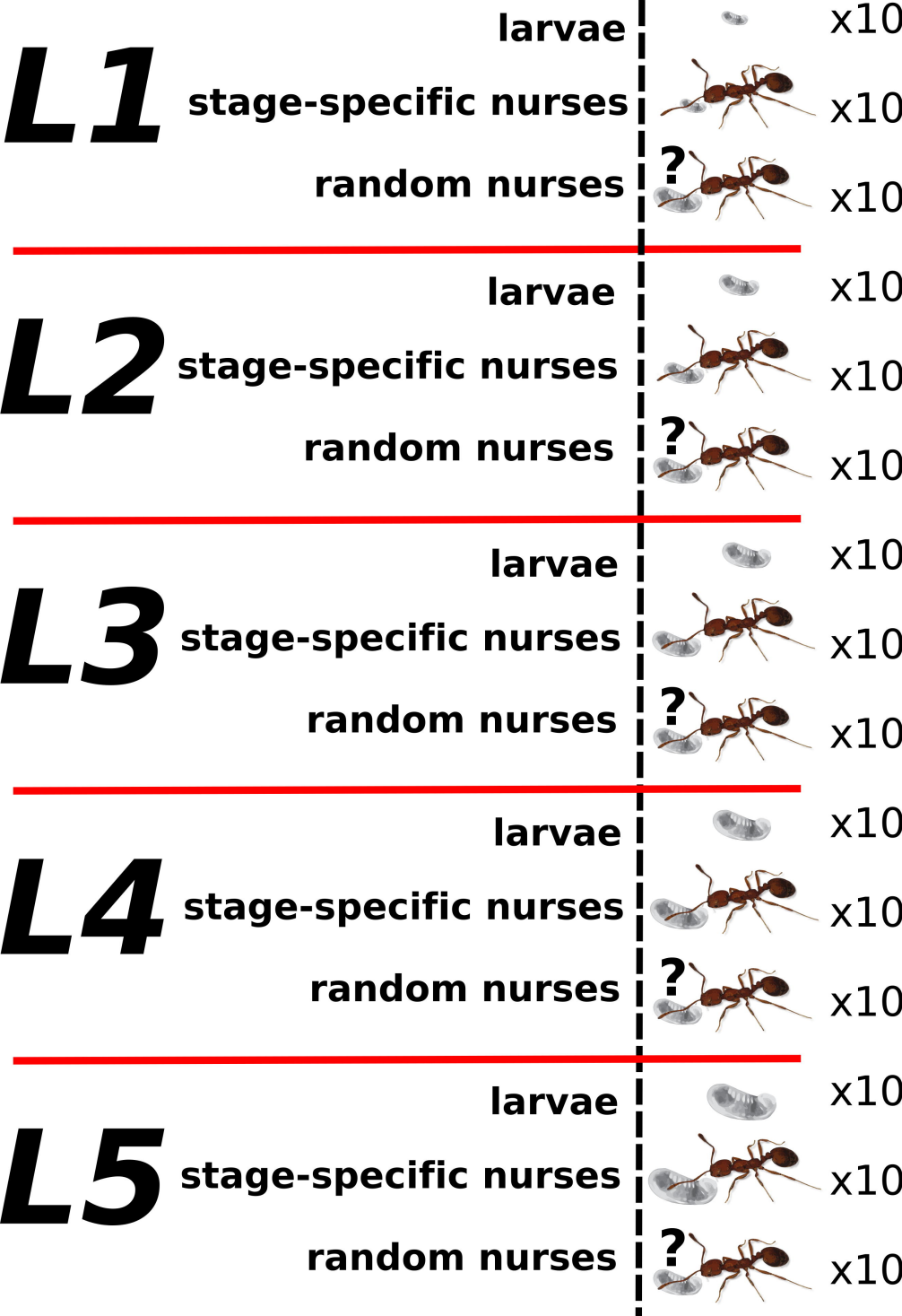
832 All data are organized in text files, with the relevant figure listed in the title.

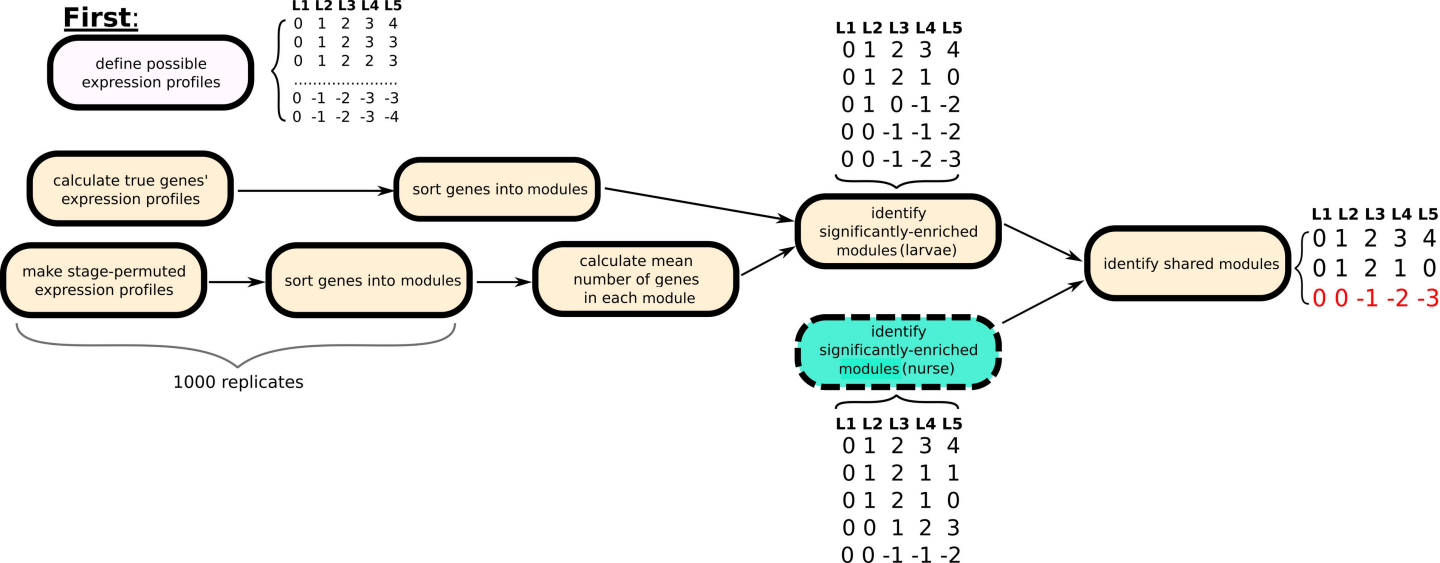

expressing individual
A
expression
time
Gene 1
gene regulation
Gene 2
Gene 3
B
connection type
social
within-tissue
1
2
3

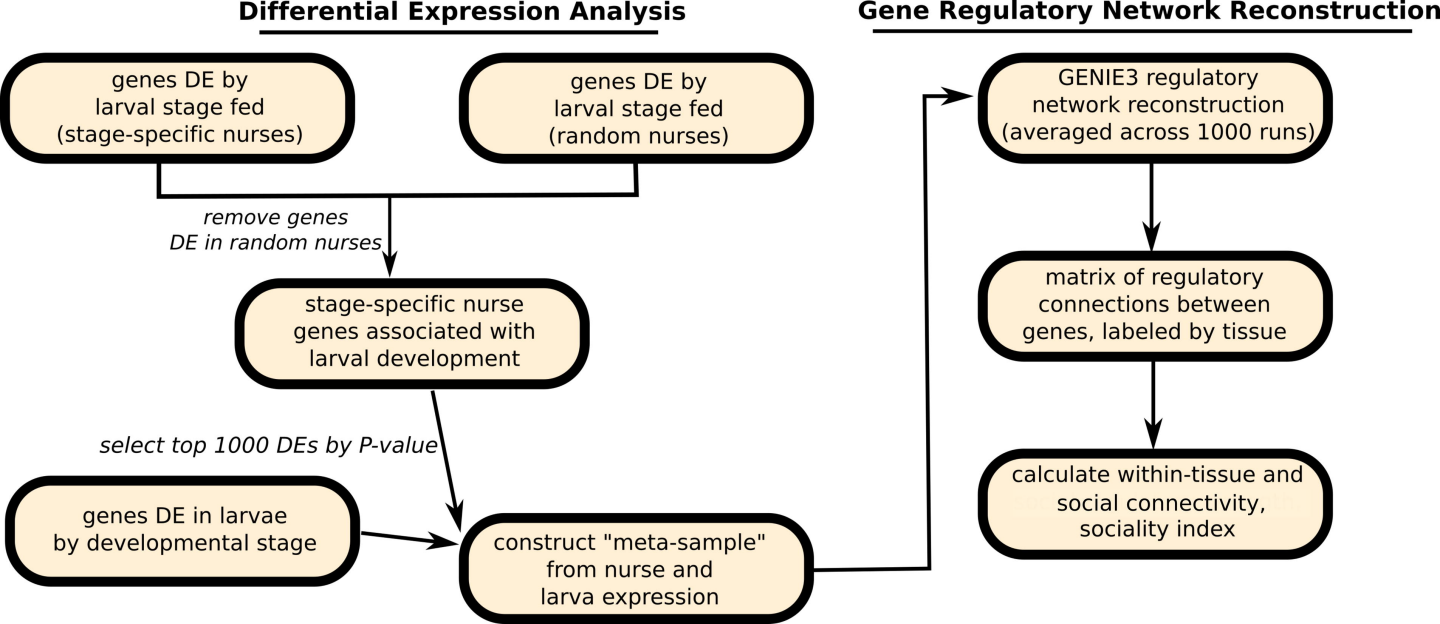

nurse head
A
expression change
larval developmental stage
L1 L2 L3 L4 L5
nurse abdomen
B
larva (shared w/ head)
C
larva (shared w/ abdomen)
D
stage-specific nurse
random nurse
E
number of genes in shared modules
larva → nurse abdomen
larva → nurse head
nurse abdomen → larva
nurse head → larva
connection type

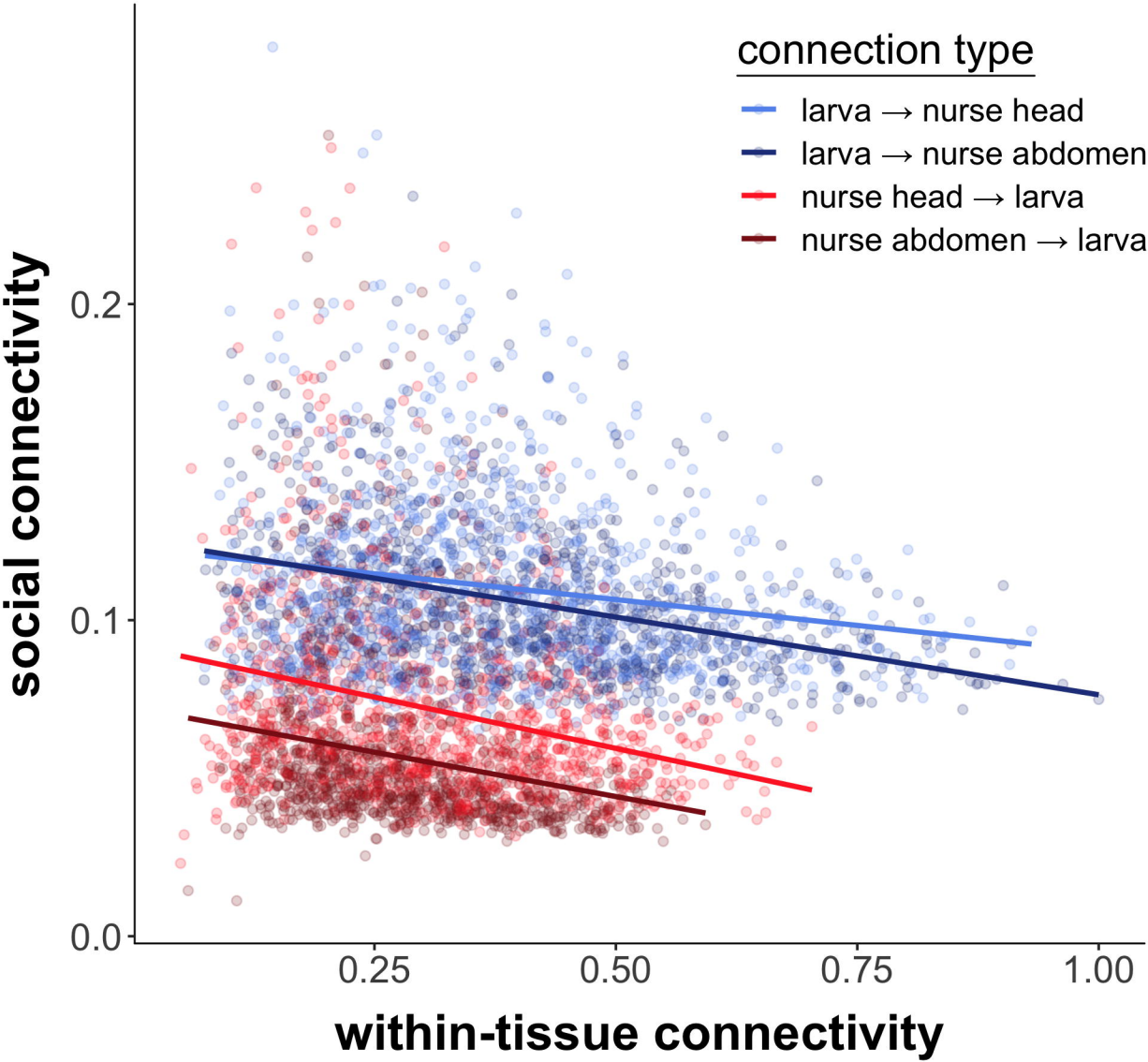

secreted
not secreted
A
P = 0.025
P = 0.067
social connectivity
0.3
0.2
0.1
0.0
nurse head
nurse abdomen
tissue
giant-lens (nurse head)
eps8 (larva)
B
expression
1.0
0.5
0.0
-0.5
-1.0
L1
L2
L3
L4
L5
larval developmental stage

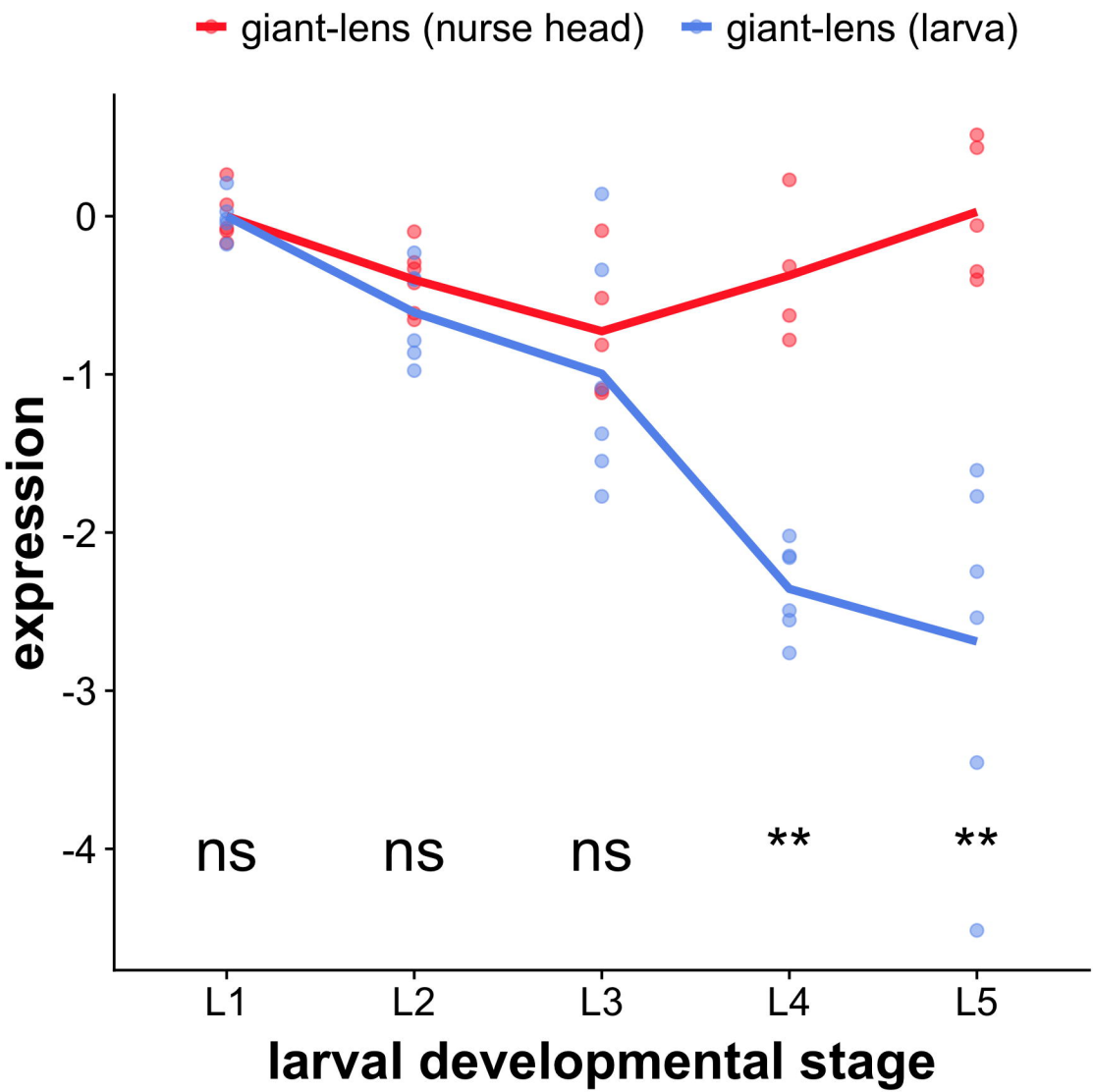

nurse head
nurse abdomen
constraint
1.00
0.75
0.50
0.25
0.00
A
0.00
0.25
0.50
0.75
1.00
within-tissue connectivity
B
social connectivity
C
-1.0
-0.5
0.0
sociality index
ancient (1120)
insect (275)
hymenopteran (67)
ant (31)
0.88
0.84
0.80
D
-0.45
-0.40
-0.35
-0.30
-0.25
more social

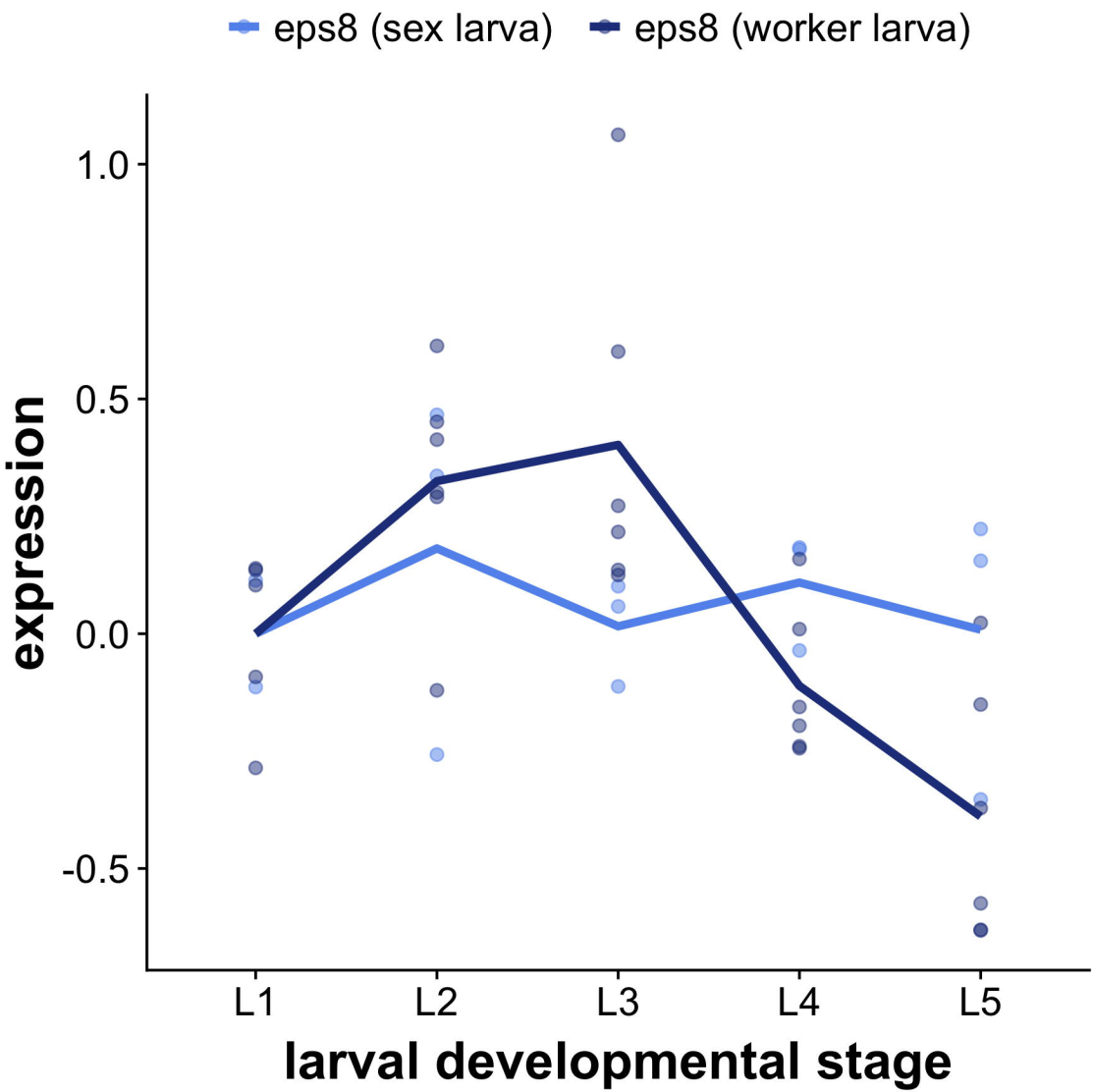

L1
larvae
x10
stage-specific nurses
x10
random nurses
?
x10
L2
larvae
x10
stage-specific nurses
x10
random nurses
?
x10
L3
larvae
x10
stage-specific nurses
x10
random nurses
?
x10
L4
larvae
x10
stage-specific nurses
x10
random nurses
?
x10
L5
larvae
x10
stage-specific nurses
x10
random nurses
?
x10


First:
define possible expression profiles
L1 L2 L3 L4 L5
0 1 2 3 4
0 1 2 3 3
0 1 2 2 3
0 -1 -2 -3 -3
0 -1 -2 -3 -4
calculate true genes' expression profiles
sort genes into modules
make stage-permuted expression profiles
sort genes into modules
calculate mean number of genes in each module
1000 replicates
L1 L2 L3 L4 L5
0 1 2 3 4
0 1 2 1 0
0 1 0 -1 -2
0 0 -1 -1 -2
0 0 -1 -2 -3
identify significantly-enriched modules (larvae)
identify significantly-enriched modules (nurse)
L1 L2 L3 L4 L5
0 1 2 3 4
0 1 2 1 1
0 1 2 1 0
0 0 1 2 3
0 0 -1 -1 -2
identify shared modules
L1 L2 L3 L4 L5
0 1 2 3 4
0 1 2 1 0
0 0 -1 -2 -3


Differential Expression Analysis
Gene Regulatory Network Reconstruction
genes DE by larval stage fed (stage-specific nurses)
genes DE by larval stage fed (random nurses)
remove genes DE in random nurses
stage-specific nurse genes associated with larval development
select top 1000 DEs by P-value
genes DE in larvae by developmental stage
construct "meta-sample" from nurse and larva expression
GENIE3 regulatory network reconstruction (averaged across 1000 runs)
matrix of regulatory connections between genes, labeled by tissue
calculate within-tissue and social connectivity, sociality index


connection type
larva → nurse head
larva → nurse abdomen
nurse head → larva
nurse abdomen → larva
social connectivity
0.0
0.1
0.2
0.25
0.50
0.75
1.00
within-tissue connectivity


giant-lens (nurse head)
giant-lens (larva)
expression
0
-1
-2
-3
-4
ns
ns
ns
**
**
L1
L2
L3
L4
L5
larval developmental stage


eps8 (sex larva)
eps8 (worker larva)
1.0
0.5
0.0
-0.5
expression
L1
L2
L3
L4
L5
larval developmental stage

development stage	sample type	number of samples
L1	larva (W/R)	5
	stage-specific nurse head	5
	stage-specific nurse abdomen	6
	random nurse head	3
	random nurse abdomen	2
L2	larva (W)	6
	larva (R)	3
	stage-specific nurse head	6
	stage-specific nurse abdomen	5
	random nurse head	3
	random nurse abdomen	3
L3	larva (W)	6
	larva (R)	3
	stage-specific nurse head	5
	stage-specific nurse abdomen	6
	random nurse head	3
	random nurse abdomen	3
L4	larva (W)	6
	larva (R)	3
	stage-specific nurse head	4
	stage-specific nurse abdomen	5
	random nurse head	2
	random nurse abdomen	2
L5	larva (W)	6
	larva (R)	3
	stage-specific nurse head	5
	stage-specific nurse abdomen	5
	random nurse head	3
	random nurse abdomen	3

	number of significantly-enriched modules	modules positively shared with larvae	modules negatively shared with larvae	number of genes in shared modules
stage-specific nurse head	9	0	5	6838
random nurse head	9	0	2	209
stage-specific nurse abdomen	21	13	4	7943
random nurse abdomen	10	0	1	1400

GO.ID	Term	Annotated	Significant	Expected	P-value
GO:0044710	single-organism metabolic process	81	14	7.82	0.00011
GO:0055114	oxidation-reduction process	45	11	4.35	0.00230
GO:0019637	organophosphate metabolic process	18	1	1.74	0.00506
GO:0044711	single-organism biosynthetic process	18	0	1.74	0.00506
GO:0006629	lipid metabolic process	12	2	1.16	0.00565
GO:0009117	nucleotide metabolic process	14	0	1.35	0.00755
GO:0006812	cation transport	18	2	1.74	0.00781
GO:0015672	monovalent inorganic cation transport	11	1	1.06	0.00898
GO:0090407	organophosphate biosynthetic process	11	0	1.06	0.01033
GO:0055086	nucleobase-containing small molecule metabolic process	16	0	1.55	0.01156

GO.ID	Term	Annotated	Significant	Expected	P-value
GO:0055114	oxidation-reduction process	52	7	4.96	0.022
GO:0008152	metabolic process	246	25	23.46	0.033

Gene	estimated regulatory direction	social connectivity	SwissProt ID
LOC105833299	nurse abdomen → larva	0.464	5'-nucleotidase domain-containing protein 1
LOC105838526	nurse head → larva	0.456	1 5-anhydro-D-fructose reductase
LOC105828583	nurse head → larva	0.433	Glycerol-3-phosphate dehydrogenase NAD( ) cytoplasmic
LOC105835488	nurse head → larva	0.433	Myosin regulatory light chain 2
LOC105837148	nurse head → larva	0.419	Short-chain dehydrogenase/reductase family 16C member 6
LOC105828656	nurse head → larva	0.413	N-acetyltransferase 6
LOC105837075	nurse head → larva	0.409	Hemolymph lipopolysaccharide-binding protein
LOC105834708	nurse head → larva	0.401	Glucose dehydrogenase FAD quinone
LOC105836140	nurse head → larva	0.399	Ankyrin-2
LOC105832040	nurse abdomen → larva	0.393	-
LOC105834910	nurse abdomen → larva	0.376	Pyruvate dehydrogenase E1 component subunit beta mitochondrial
LOC105835497	nurse abdomen → larva	0.373	Phenoloxidase 2
LOC105836690	nurse abdomen → larva	0.366	-
LOC105836193	nurse head → larva	0.365	-
LOC105833826	nurse head → larva	0.360	Probable tubulin polyglutamylase TTLL2
LOC105833938	nurse head → larva	0.357	-
LOC105836189	nurse abdomen → larva	0.348	Venom metalloproteinase 3
LOC105829511	nurse head → larva	0.341	La-related protein 1
LOC105840715	nurse abdomen → larva	0.336	-
LOC105833984	nurse abdomen → larva	0.331	Procollagen-lysine 2-oxoglutarate 5-dioxygenase 3

nurse head	nurse abdomen
Basement membrane-specific heparan sulfate proteoglycan core protein	Procollagen-lysine 2-oxoglutarate 5-dioxygenase 3
Collagen alpha-1(IV) chain	Collagen alpha-1(IV) chain
Spondin-1	Tubulointerstitial nephritis antigen-like
Serine proteinase stubble	Papilin
Angiotensin-converting enzyme (Fragment)	Semaphorin-2A
Thrombospondin-4	Transferrin
Protein giant-lens	Basement membrane-specific heparan sulfate proteoglycan core protein
Protein lev-9	Protein NPC2 homolog
Papilin	Testican-2
Glypican-6	Lysozyme