Transcriptomic basis and evolution of the ant social interactome

Michael R. Warner[1], Alexander S. Mikheyev[2,3], Timothy A. Linksvayer[1]

[1]University of Pennsylvania, Philadelphia, Pennsylvania, USA

[2]Okinawa Institute of Science and Technology, Okinawa, Japan

[3]Research School of Biology, Australian National University, Canberra, Australia

Abstract

Social interactions are a fundamental feature of life for most organisms. Organismal development is often strongly regulated by interactions among close relatives, yet little is known about how genes expressed in social partners indirectly affect developmental trajectories and trait expression. In eusocial insects, social interactions between caregiving worker nurses and larvae strictly regulate larval development and resultant adult phenotypes. Here, we study the social interactome regulating larval development by collecting and sequencing interacting nurses and larvae across a time course of larval development. First, we find that the majority of nurse and larval transcriptomes exhibit parallel expression dynamics across larval development, providing a strong transcriptomic signature of the social interactome regulating larval development. Next, we leverage this widespread nurse-larva gene co-expression to infer social gene regulatory networks acting between nurses and larvae. We find that genes with the strongest social effects tend to be peripheral elements of within-tissue regulatory networks and are often known to encode secreted proteins. For example, our data suggest that the gene *giant-lens* expressed by nurses may inhibit larval epidermal growth factor signaling, which is known to affect various aspects of insect development, including caste in honey bees. Finally, we find that genes recruited for social regulatory processes tend to be relatively evolutionarily young and tend to experience relaxed selective constraint. Overall, our study provides a first glimpse into the molecular details and evolutionary features of the social mechanisms that regulate all aspects of life in eusocial insects.

Author Summary

From single-celled bacteria to complex plants and animals, most organisms experience and are strongly impacted by social interactions. Despite their obvious importance, little is known about the genetic basis of social traits and interactions. In eusocial insects, the development of larvae is strictly regulated by caregiving nurse workers through a myriad of social interactions occurring across larval development. In this paper, we study ant nurse-larva social interactions and the effect of interactions on gene expression

by simultaneously collecting and sequencing interacting nurses and larvae across larval development. We find that broad-scale gene expression patterns reflect ongoing social interactions. Based on nurse-larva gene co-expression, we identify genes expressed in nurses that exhibit strong putative effects on the expression of genes in larvae. Interestingly, such genes with strong social roles tend to be less important for pathways operating within individuals, and such highly social genes tend to experience relatively weaker natural selection in comparison to less social genes. This suggests that genes underlying social traits are characterized by distinct evolutionary and network features. Overall, this study represents a significant advance in our understanding of the intersection of genetics, behavior, and evolution.

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 generationally-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 strictly 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 manipulate 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

77 one individual but impact the fitness of other individuals, social behavior and socially-influenced traits
78 experience distinct forms of selection, including kin selection and social selection [23,24]. Altogether,
79 these distinct genetic features and patterns of selection are often thought to lead to distinct evolutionary
80 features, such as rapid evolutionary dynamics in comparison to other traits [25–27]. In eusocial insects,
81 previous studies show that variation in larval developmental trajectories and ultimate adult phenotypes
82 (including reproductive caste, body size, etc.) depends on the combination of larval and nurse genotypes
83 [28–34]. However, the identity of specific genes and molecular pathways that are functionally involved in
84 the expression of social interactions (e.g., genes underlying nurse and larval traits affecting nurse-larva
85 interactions), and patterns of molecular evolution for these genes, have remained less well studied
86 [15,16,35,36].

87 Transcriptomic studies are often used to identify sets of genes underlying the expression of
88 particular traits by performing RNA-sequencing on individuals that vary in the expression of such traits.
89 For example, in social insects, recent studies have compared the transcriptomes of workers that perform
90 nursing versus foraging tasks [37–39], or nurses feeding larvae of different stages or castes [35,40].
91 However, given the phenotypic co-regulation known to occur between interacting social partners (here,
92 nurses and larvae), it is likely that genes expressed in one social partner affect the expression of genes in
93 the other social partner, and vise-versa, such that interacting social partners are connected by "social"
94 gene regulatory networks [14,32,41,42]. Thus, identifying the genes important for *social interactions* such
95 as nurse-larva interactions is only possible by studying the transcriptomic dynamics of both interacting
96 social partners across a time series of interactions. Similarly, host-symbiont interactions result in gene
97 regulation between interacting partners, and recent studies have sought to reconstruct gene regulatory
98 networks acting between hosts and symbionts by collecting and profiling the transcriptomes of each social
99 partner across a time series of interactions [43–47].

100 Here, we use analogous methodology to study transcriptomic signatures of nurse-larva
101 interactions in the pharaoh ant, *Monomorium pharaonis*. We sample a developmental time series of larvae
102 as well as the nurses that feed each larval stage in this series, collecting individuals at the moment of

103 interaction in order to identify genes involved in the expression of nurse-larva interactions, as well as
104 genes affected by these interactions (i.e. the full "social interactome" [14]). Given that pharaoh ant nurses
105 tend to specialize on feeding young versus old larvae and nurses feeding young versus old larvae show
106 different transcriptomic profiles [40], and larval transcriptomic profiles also change over development
107 [48,49], we predicted that if nurse-larva interactions have functional implications as described above, we
108 would observe concerted changes in broad-scale gene expression in larvae and their nurses across larval
109 development (Fig 1). Based on our dual RNA-seq data, we infer social gene regulatory networks acting
110 between nurses and larvae to identify genes predicted to have important social regulatory effects. Finally,
111 we combine our measures of social regulatory effects with available population genomic data [48] to
112 characterize the patterns of molecular evolution of genes underlying nurse-larva interactions.
113

114 **Results**

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

116 To elucidate transcriptomic signatures of nurse-larva interactions, we performed RNA-sequencing on
117 worker-destined larvae across five developmental stages and nurses that fed larvae of each developmental
118 stage (termed "stage-specific" nurses; see Fig S1 for sampling scheme, Table S1 for list of samples),
119 building upon a previously published dataset focused on caste development in *M. pharaonis* [48]. We
120 hypothesized that if genes expressed in larvae regulate the expression of genes in nurse and vise versa, we
121 would observe correlated expression profiles across development in larvae and nurses (Fig 1). As a
122 biological control, we also collected "random nurses" that we observed feeding any stage of larvae in the
123 colony, and hence would not be expected to show correlated expression dynamics with larvae across the
124 five larval developmental stages. We also concurrently collected reproductive-destined larvae across the
125 last four developmental stages (reproductive-destined larvae can only be identified after the first larval
126 stage [50]), but unless clearly stated otherwise, all analyses were conducted only on worker-destined
127 larvae. To minimize experimental variance, we pooled ten individuals per sample. We sequenced whole
128 bodies of larvae but separated nurse heads and abdomens prior to sequencing.

129 We grouped genes into co-expression profiles or "modules" using an algorithm designed to
130 characterize gene co-expression dynamics across a short time series [51]. Each module represents a
131 standardized pre-defined expression profile, consisting of five values that each represent the $\log_2$ fold-
132 change between the given developmental stage and the initial stage (see Fig S2; this results in a total of 81
133 possible modules). We sorted genes into the module that most closely represented their expression profile
134 by Pearson correlation. We identified modules containing a greater than expected number of genes, where
135 we formed null expectations using permutation tests across developmental stage [51]. We identified such
136 significantly-enriched modules separately for larvae, stage-specific nurse heads, stage-specific nurse
137 abdomens, random nurse heads, and random nurse abdomens. We focused on both parallel (i.e. positive
138 regulation or activation) and anti-parallel (i.e. inhibitory) correlated expression patterns by identifying
139 significantly-enriched modules that were shared in both larvae and nurses (parallel), as well as
140 significantly-enriched modules for which the inverse of the module was identified as significantly-
141 enriched in the social partner (anti-parallel).
142 Larvae and stage-specific nurses shared many significantly-enriched modules (Table S2). These
143 modules contained the majority of genes expressed in nurses (stage-specific nurse head: 65%; abdomen:
144 76%) and a substantial proportion of the larval transcriptome (shared with stage-specific nurse head: 22%;
145 abdomen: 60%), such that there was a general widespread signature of correlated transcriptional patterns
146 between stage-specific nurses and larvae across larval development (Fig 2A-D). These coordinated
147 dynamics were dominated by parallel associations in nurse abdomens (possibly reflecting shared
148 metabolic pathways) but anti-parallel associations in nurse heads (possibly reflecting the social regulation
149 of larval growth). In contrast to stage-specific nurses, random nurses (our biological control) shared few
150 significantly-enriched modules with larvae (Table S2), and modules shared between random nurses and
151 larvae contained significantly fewer genes than modules shared between stage-specific nurses and larvae
152 (Fig 2E).
153
154 *Identification of genes putatively involved in social interactions*

155 Given that we observed transcriptome-wide patterns consistent with nurse-larva transcriptional co-
156 regulation across larval development, we next identified the genes that might be driving these patterns
157 (see Fig S3). We performed differential expression analysis to identify genes that varied in larval
158 expression according to larval developmental stage, as well as genes that varied in nurse expression
159 according to the developmental stage of larvae they fed. We identified 8125 differentially expressed
160 genes (DEGs) in larvae (78% of 10446 total genes). We identified 2057 and 1408 DEGs in stage-specific
161 nurse heads and abdomens, respectively, compared to 599 and 520 DEGs in random nurse heads and
162 abdomens, respectively. We removed genes differentially expressed in both stage-specific and random
163 nurses (N = 272 DEGs in heads, N = 140 DEGs in abdomens), which might differ among our colony
164 replicates due to random colony-specific effects that were not consistently associated with social
165 regulation of larval development. After this removal, we retained the top 1000 DEGs, sorted by P-value,
166 for each sample type other than random nurses (larvae, stage-specific nurse heads, stage-specific nurse
167 abdomens) for social gene regulatory network reconstruction.
168
169 *Reconstruction of social gene regulatory networks*
170 To infer gene-by-gene social regulatory relationships between nurses and larvae, we reconstructed gene
171 regulatory networks acting within and between nurses and larvae (Fig S3). To identify the most highly
172 connected (i.e. centrally located, upstream) genes of regulatory networks, we calculated within-tissue
173 connectivity and social connectivity by averaging the strength of connections across each connection a
174 gene made, differentiating between within-tissue (nurse-nurse or larva-larva) and social connections
175 (nurse-larva) (Fig 1B). On average, within-tissue connectivity was higher than social connectivity
176 (Wilcoxon rank-sum test; $P < 0.001$ in all tissues), and within-tissue connectivity was negatively
177 correlated with social connectivity in each tissue (Fig S4). The top enriched gene ontology terms based on
178 social connectivity in nurses were entirely dominated by metabolism (Tables S3,S4).
179
180

181 *Secreted proteins and social gene regulation*

182 While based on our data it is not possible to distinguish between genes that code for protein products that
183 are actually exchanged between nurses and larvae versus genes that affect behavior or physiology within
184 organisms (Fig 1A), proteins that are known to be cellularly secreted represent promising candidates for
185 the social regulation of larval development (40). Genes coding for proteins that are cellularly secreted in
186 *Drosophila melanogaster* had higher social connectivity than genes coding for non-secreted orthologs in
187 nurse heads (Fig 3A; Wilcoxon rank-sum test; P = 0.011), though not for nurse abdomens (P = 0.094).
188 While many of the genes with the highest social connectivity have unknown function, one of the genes
189 with the highest social connectivity within nurse heads was the protein *giant-lens* (Table S5). *Giant-lens*
190 (also known as *argos*) is a secreted protein that is known to inhibit EGFR signalling in *D. melanogaster*
191 [52]. *Giant-lens* expression in nurse heads was negatively correlated with the expression of the homolog
192 of *eps8*, human EGFR substrate 8 (Fig 3B; note however that this pattern was not seen for all genes in the
193 EGFR pathway). Furthermore, *giant-lens* expression in larvae steadily drops throughout development
194 (Fig S5), suggesting that *giant-lens* expressed in nurses and passed to larvae may inhibit EGFR signaling
195 in larvae, even as EGFR inhibition within larvae via *giant-lens* decreases over the course of development.
196 Interestingly, *eps8* does not exhibit a similar peak and drop in expression level in reproductive-destined
197 larvae in comparison to worker-destined larvae (Fig S6), suggesting that this pathway could be involved
198 in the regulation of caste development.

199

200 *Molecular evolution of social gene regulatory networks*

201 To investigate the selective pressures shaping social regulatory networks, we used population genomic
202 data from 22 resequenced *M. pharaonis* workers, using one *M. chinense* worker as an outgroup [48].
203 Using polymorphism and divergence data, we estimated gene-specific values of selective constraint,
204 which represents the intensity of purifying selection that genes experience [53]. To identify genes
205 disproportionately recruited to the core of social regulatory networks, we calculated "sociality index" as
206 the difference between social connectivity and within-tissue connectivity for each gene. Sociality index

207 was negatively correlated to selective constraint due to a positive correlation between within-tissue
208 connectivity and constraint and a negative correlation between social connectivity and constraint (Fig 4A-
209 C). Additionally, genes differed in sociality index according to their estimated evolutionary age, with
210 ancient genes exhibiting lower sociality indices than genes in younger age categories (Fig 4D). Finally,
211 while evolutionary age and evolutionary rate appear to be somewhat empirically confounded [54],
212 selective constraint and evolutionary age were each independently associated with sociality index, based
213 on a model including both variables as well as tissue type (GLM; LRT; evolutionary age: $\chi^2 = 21.536$, $P <$
214 0.001; selective constraint: $\chi^2 = 22.191$, $P < 0.001$).
215

216 **Discussion**

217 In organisms with extended offspring care, developmental programs are controlled in part by socially-
218 acting gene regulatory networks that operate between caregivers and developing offspring [14,41]. In this
219 study, we sequenced the transcriptomes of ant nurses and larvae as they interacted across larval
220 development to assess the effects of social interactions on gene expression dynamics. We found that large
221 sets of genes (i.e. modules) expressed in ant larvae and their caregiving adult nurses show correlated
222 changes in expression across development (Fig 2). The majority of nurse and larval transcriptomes was
223 represented in these correlated modules, indicating that the tight phenotypic co-regulation characterizing
224 nurse-larva interactions over the course of larval development is strongly reflected at the molecular level.
225 To characterize the overall network and evolutionary patterns of genes involved in nurse-larva
226 interactions, we reverse engineered nurse-larva gene regulatory networks and calculated the "social
227 connectivity" for each gene, defined as the sum of inferred social regulatory effects on all genes
228 expressed in social partners. We found that genes with high social connectivity tended to have low
229 within-individual connectivity (Fig S4; where within-individual connectivity is defined as the sum of
230 inferred regulatory effects acting within a given tissue). Nurse-expressed genes with higher sociality
231 indices (i.e disproportionately higher social connectivity than within-individual connectivity) tended to be

232 evolutionarily young and rapidly evolving due to relaxed selective constraint (Fig 4). Genes with high
233 social connectivity were enriched for a number of Gene Ontology (GO) categories associated with
234 metabolism (Tables S3,S4), consistent with the idea that molecular pathways associated with metabolism
235 are involved in the expression of social behavior [55,56]. Finally, we found that genes encoding for
236 orthologs of cellularly-secreted proteins important for intercellular signaling in *Drosophila melanogaster*
237 tended to exhibit higher levels of social connectivity than their non-secreted counterparts (Fig 3A).

238 One gene that stands out in terms of being cellularly secreted and exhibiting a relatively high
239 social connectivity is the gene *giant-lens*, which inhibits epidermal growth factor receptor (EGFR)
240 signaling [52]. EGFR signaling affects eye and wing development [57] and body size in *D. melanogaster*
241 [58], caste development in the honey bee *Apis mellifera* [58,59] via the transfer of royalactin from nurses
242 to larvae [58], and worker body size variation in the ant *Camponotus floridanus* [60]. While further
243 experimental work is necessary to ascertain whether *giant-lens* is actually orally secreted by nurses and
244 transferred to larvae, gene expression dynamics suggest that *giant-lens* transferred from nurses to larvae
245 may inhibit EGFR signaling at the end of larval development in worker-destined larvae (Fig 3B), but not
246 in reproductive-destined larvae (Fig S6). While caste in *M. pharaonis* is socially regulated in the first
247 larval stage [49], social inhibition of EGFR signaling could play a role in the regulation of worker body
248 size [60] or secondary caste phenotypes such as wings [61,62].

249 In terms of broad evolutionary patterns, our study complements previous results suggesting genes
250 with worker-biased expression (in comparison to genes with queen-biased expression) tend to be rapidly
251 evolving, evolutionarily young, and loosely connected in regulatory networks [37,48,49,63–65]. Because
252 workers are obligately sterile, their traits are shaped indirectly by kin selection, based on how they affect
253 the reproductive success of fertile relatives (i.e. queens and males) [23,66]. As a result, all-else-equal,
254 genes associated with worker traits are expected to evolve under relaxed selection relative to genes
255 associated with queen traits [67,68]. In general, the suite of genic characteristics commonly associated
256 with worker-biased genes (rapidly evolving, evolutionarily young, loosely connected) are all consistent
257 with relaxed selection acting on genes associated with workers [49]. Here, we show that within the

258 worker caste, genes that appear to be functionally involved in the expression of social behavior (i.e.
259 nursing) experience relaxed selective constraint relative to genes important for within-worker processes.
260 Therefore, the combination of kin selection as well rapid evolution thought to be characteristic of social
261 traits [25] likely act in concert to shape the labile evolutionary patterns commonly associated with
262 worker-biased genes.

263 In this study, we reconstructed regulatory networks acting between nurses and larvae, which of
264 course assumes that nurse gene expression changes as a function of larval stage fed. This is much more
265 likely to be the case when nurses are specialized on feeding particular larval stages. About half of all
266 feeding events were performed by specialist nurses in a previous study in *M. pharaonis* [40], so our
267 pooled nurse samples likely contained a mix of generalist and specialist nurses. This would serve to
268 effectively dilute the signal of nurse-larva interactions. Clearly, future studies would ideally focus entirely
269 on specialists, as well as on tissues such as brains and the specific exocrine glands [69] known to be
270 important for social behavior and communication. Despite these limitations, we were still able to observe
271 strong transcriptomic signatures of social interactions.
272

273 **Conclusions**

274 In this study, we uncovered strong transcriptomic signatures of social interactions (i.e. co-regulation of
275 nurse and larval genes and modules) and identified distinct evolutionary features of genes that were
276 inferred to have strong social regulatory roles. Because we simultaneously collected nurses and larvae
277 over a time series of interactions, we were able to elucidate the molecular basis of social interactions. This
278 is a promising approach that could be readily extended to study the molecular underpinnings of all forms
279 of social regulation in social insect colonies, including regulation of foraging, regulation of reproduction,
280 etc.. Furthermore, by adapting the methodology presented here (i.e. simultaneous collection over the
281 course of interactions followed by sequencing), the molecular mechanisms and evolutionary features of
282 genes underlying a diverse array of social interactions, including courtship behavior, dominance hierarchy
283 formation, and regulation of biofilm production could all be investigated. Overall, this study provides

empirical, theoretical, and methodological foundations on which future research can build upon to realize the full power of the genomics revolution for understanding the genetic underpinnings and evolution of interacting phenotypes.

Methods

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

Study Design

We created thirty total replicate experimental colonies of approximately equal sizes (~300-400 workers, ~300-400 larvae) from a mixture of many genetically heterogeneous colonies of the ant *Monomorium pharaonis*. We removed queens from ½ the study colonies to promote the production of reproductive-destined larvae. In this study, we pooled samples across colonies with and without queens, as queen presence appeared to have a negligible effect on gene expression (0 DEGs at FDR < 0.1 for each sample type). We pre-assigned colonies to one of five larval developmental stages (labeled L1-L5, where L1 and L2 refer to 1st-instar and 2nd-instar larvae and L3, L4, and L5 refer to small, medium, and large 3rd-instar larvae [50]). We sampled individuals (larvae as well as nurses) across larval development time (3-4 days between each time point based on the developmental stage of the youngest larvae in colonies without queens). From each colony, we sampled stage-specific nurses and worker-destined larvae, as well as random nurses from colonies with queens and reproductive-destined larvae from colonies without queens (starting at the L2 stage, because at L1 caste cannot be distinguished [50,70]. See Table S1 for full sample list.

310 For each time point in each assigned colony, we collected stage-specific nurses, nurses feeding
311 larvae of the specified developmental stage (L1, L2, etc). Concurrently, we haphazardly collected random
312 nurses, nurses we observed feeding a larva of any developmental stage. After collecting nurses, we
313 anaesthetized the colony using carbon dioxide and collected larvae of the specified developmental stage.
314 We separated heads and abdomens of nurses to sequence separately and sequenced whole bodies of
315 larvae. We collected ten samples of each type to pool into single samples (separated by sample type and
316 tissue) for each colony.
317 We performed RNA-sequencing on all samples concurrently using Illumina HiSeq 2000 at
318 Okinawa Institute of Science and Technology Sequencing Center. Reads were mapped to the NCBI
319 version 2.0 *M. pharaonis* assembly [37], and we used RSEM [71] to estimate counts per locus and
320 fragments per kilobase mapped (FPKM) for each locus. For further details on RNA extraction and library
321 preparation, see [48].
322
323 *Transcriptome-wide signatures of nurse-larva co-expression across larval development*
324 We used an algorithm that categorizes genes based on their expression dynamics over time into a number
325 of modules represented by pre-defined expression profiles [51]; see Fig S2 for workflow). To create
326 modules, we started at 0 and either doubled, halved, or kept the expression level the same at each
327 subsequent stage, resulting in 81 possible modules (3*3*3*3 = 81; four stages after L1). To generate
328 gene-specific expression profiles based on real results, we calculated the average $\log_2$ fold change in
329 expression (FPKM) of the gene at each developmental stage compared to the initial expression level at
330 stage L1. We then assigned each gene to the closest module by Pearson correlation between gene
331 expression profile and module expression profile [51]. To identify significantly-enriched modules, we
332 generated null distributions of the number of genes present in each module (based on permutation of
333 expression over time), and retained modules with a significantly greater than expected number of genes
334 based on these null distributions (FDR < 0.05 after Bonferroni multiple correction [51]).
335

336 *Identification of genes putatively involved in social interactions*

337 We used the package EdgeR [72] to construct models including larval developmental stage and replicate
338 and performed differential expression analysis for each sample type separately. We retained genes
339 differentially expressed according to a nominal P-value of less than 0.05 (i.e. no false discovery
340 correction), as the purpose of this step was simply to identify genes that could be involved in interactions
341 that shape larval development (rather than spurious interactions arising from replicate-specific effects).

342

343 *Social regulatory network reconstruction*

344 We normalized expression for each gene using the inverse hyperbolic sine transformation of FPKM. As
345 input to the algorithm, we constructed "meta-samples" by combining expression data within the same
346 replicate and time point from nurses and larvae and labeling genes according to the tissue they were
347 expressed in, along the lines of previous host-symbiont studies [46,47]. We utilized the program GENIE3
348 [73], the top-performing individual program in a wide survey of GRN reconstruction programs [74], to
349 construct two types of networks: those acting between larvae and nurse heads, and those acting between
350 larvae and nurse abdomens. We repeated the reconstruction process 1000 times and averaged pairwise
351 connection strengths across runs. To capture the total effect of each gene on the transcriptome dynamics
352 within tissues, we averaged the regulatory effects each gene had on all other 999 genes expressed in the
353 same tissue ("within-individual connectivity"). Similarly, to capture the effect each gene had on the
354 transcriptome of social partners, we averaged regulatory effects each gene had on the 1000 genes
355 expressed in social partners ("social connectivity").

356

357 *Estimation of selective constraint, and evolutionary rate*

358 Previously, we performed whole-genome resequencing on 22 diploid *M. pharaonis* workers as well as
359 one diploid *M. chinense* worker to serve as an outgroup [48]. We estimated selective constraint using
360 MKtest2.0 [75], assuming an equal value of alpha (an estimate of the proportion of nonsynonymous
361 substitutions fixed by positive selection) across all genes. Selective constraint is the estimate of the

362 proportion of nonsynonymous mutations that are strongly deleterious and thereby do not contribute to
363 polymorphism or divergence [75]. Selective constraint is estimated using polymorphism data, so it
364 represents the strength of purifying selection genes experience within the study population [53].
365
366 *Phylostratigraphic Analysis*
367 Phylostrata are hierarchical taxonomic categories, reflecting the most inclusive taxonomic grouping for
368 which an ortholog of the given gene can be found [76–79]. We focused on distinguishing between genes
369 that were evolutionarily "ancient", present in non-insect animals, versus genes present in only insects,
370 hymenopterans, or ants [49]. We constructed a database containing 48 hymenopteran genomes, 10 insect
371 non-hymenopteran genomes, and 10 non-insect animal genomes, and estimated evolutionary age based on
372 the most evolutionarily distant identified BLASTp hit (E-value 10^{-10}).
373
374 *Gene Set Enrichment Analysis*
375 We performed gene set enrichment analysis based on social connectivity for each gene in each tissue
376 separately using the R package topGO [80]. We identified enriched gene ontology terms using
377 Kolmogorov-Smirnov tests ($P < 0.05$).
378
379 *General Analyses*
380 We performed all statistical analyses and generated all plots using R version in R version 3.4.0 [81], aided
381 by the packages "reshape2" [82], "plyr" [83], and "ggplot2" [84].
382
383
384 **Acknowledgments**
385 We would like to thank the following: Mandy Tin for constructing RNA-sequencing libraries and
386 performing RNA-sequencing, Luigi Pontieri for images of pharaoh ants, Chao Tong for compiling
387 hymenopteran genomes for use in phylostratigraphy, and Rohini Singh for comments on the manuscript.

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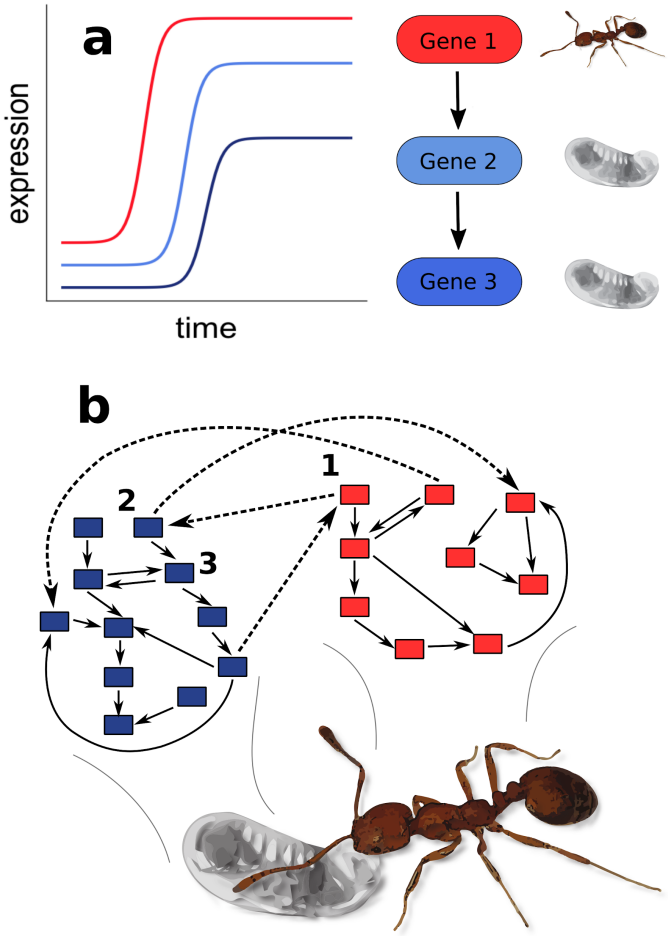

Fig 1. Social regulation of gene expression between ant nurses and larvae.

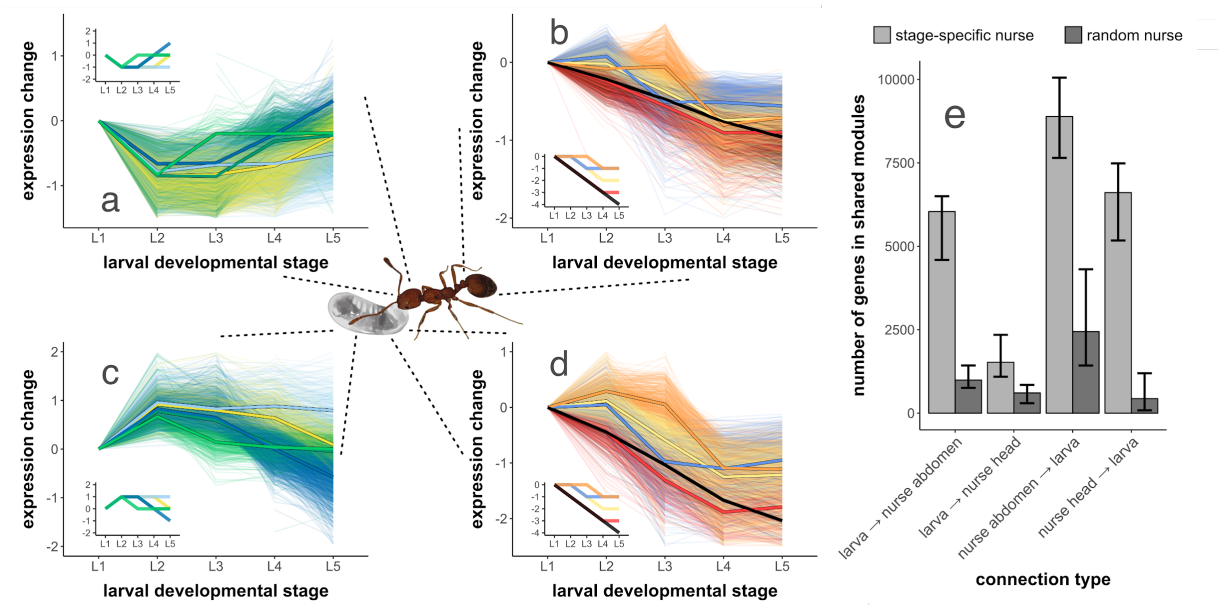

Fig 2. Nurse and larval transcriptomes show strong signatures of gene co-expression across larval development.

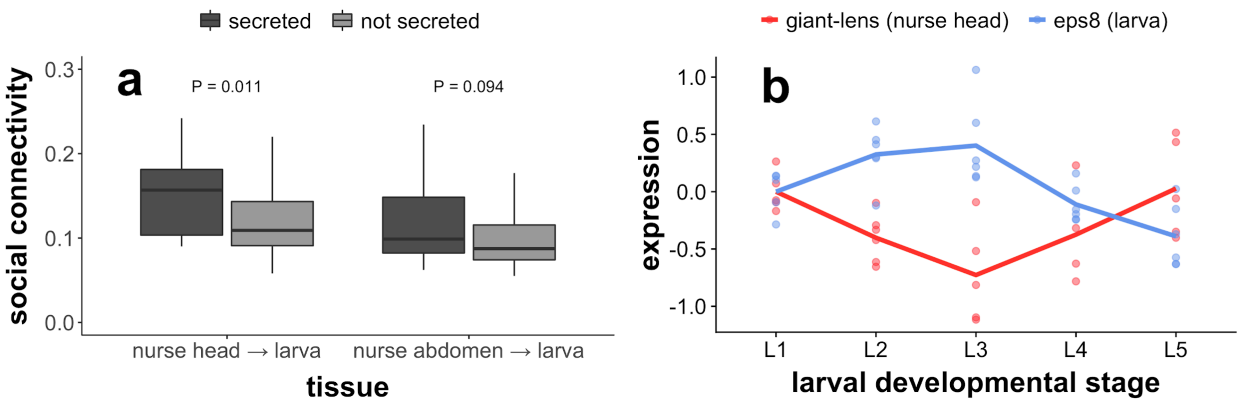

Fig 3. Genes encoding secreted proteins such as *giant-lens* are important for social gene regulation.

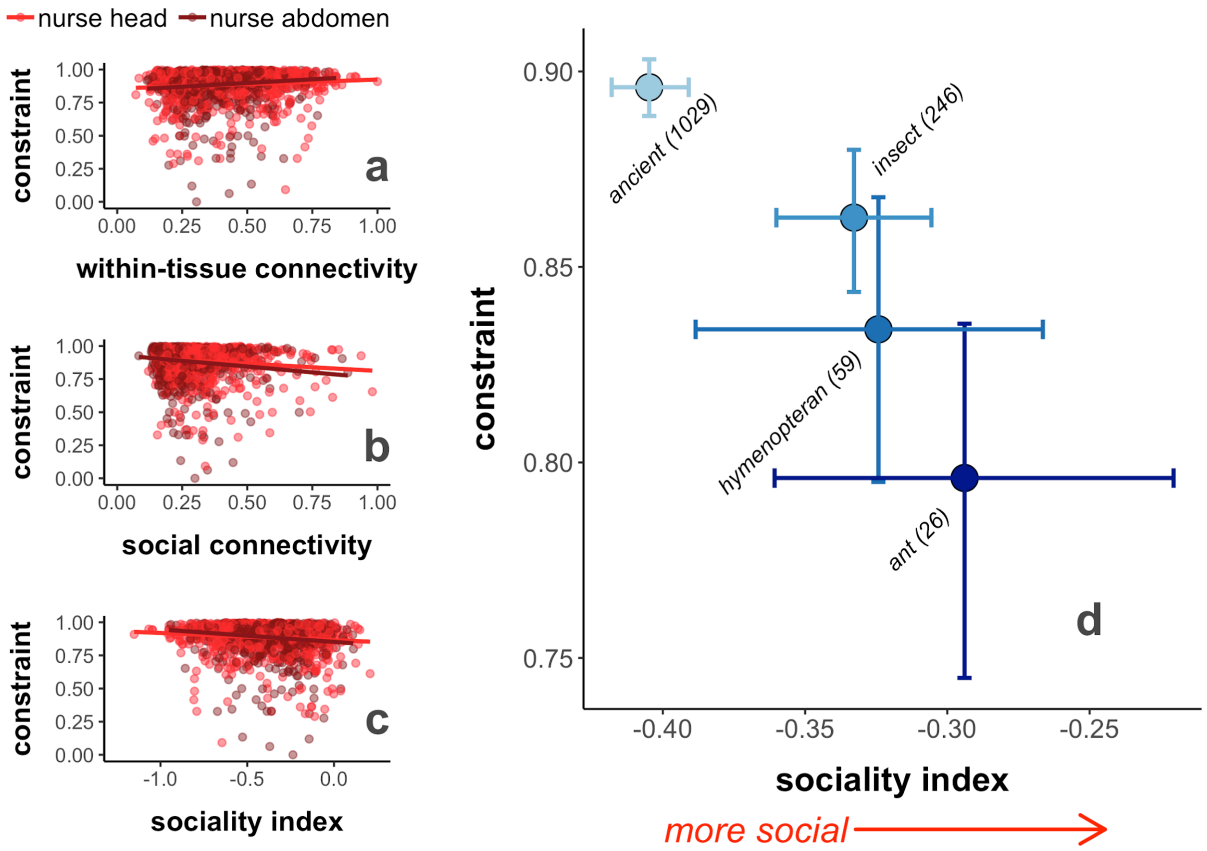

Fig 4. Highly social genes tend to be less evolutionarily constrained.

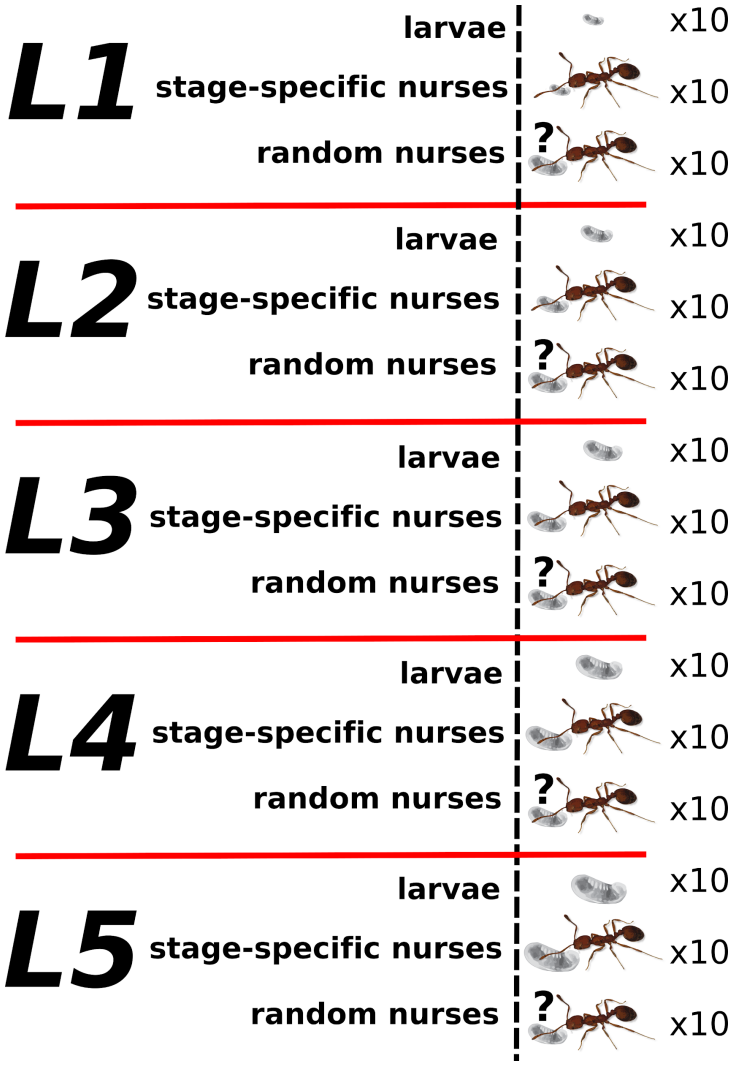

Fig S1. Diagram of sampling scheme.

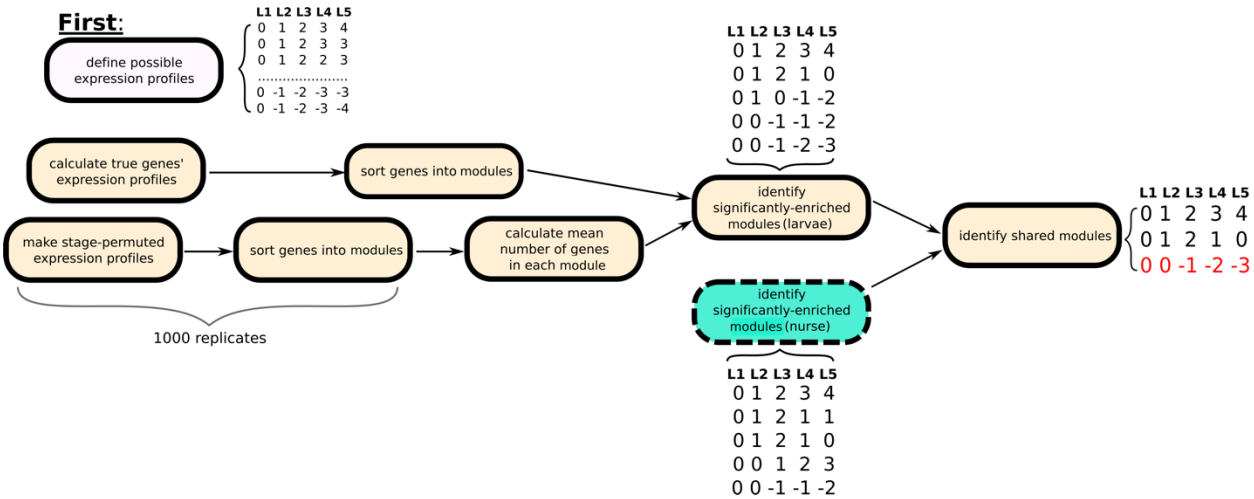

Fig S2. Identification of significantly-enriched modules shared between larvae and nurses.

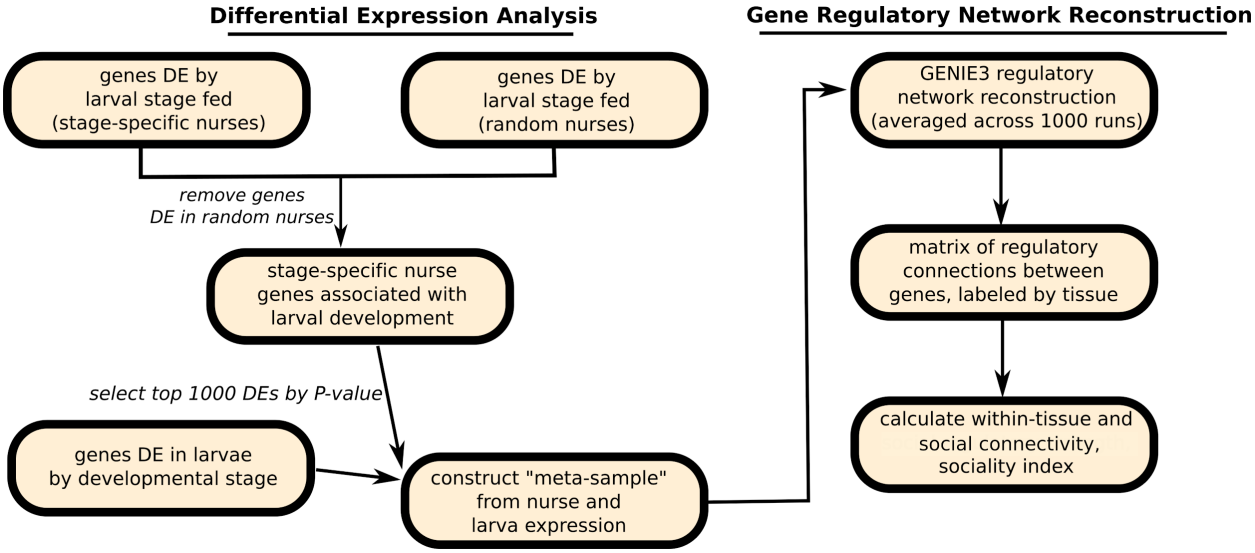

Figure S3. Workflow of preliminary differential expression analysis and gene regulatory network reconstruction.

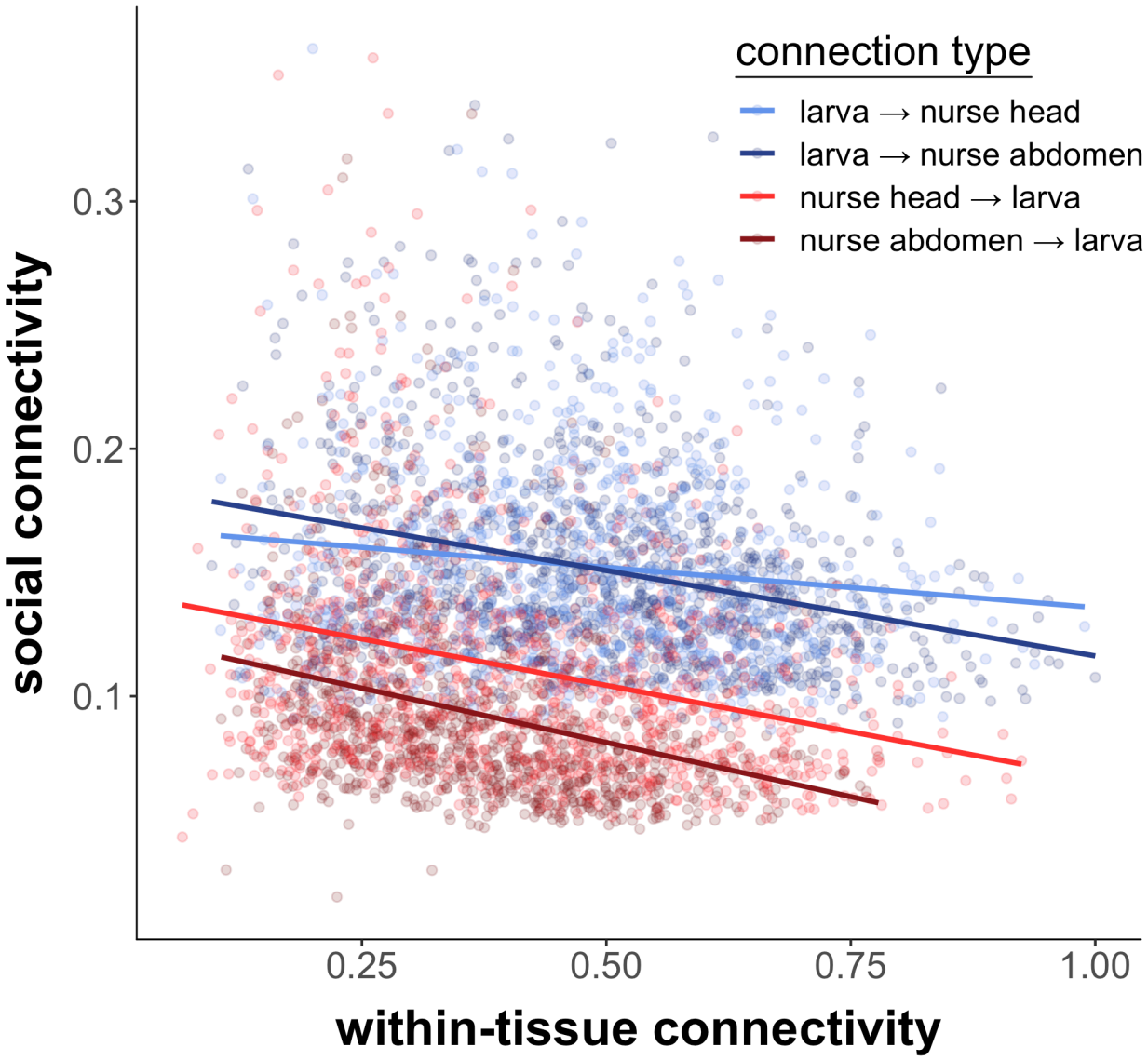

Figure S4. Genes highly connected in within-tissue networks tended to have low values of social connectivity.

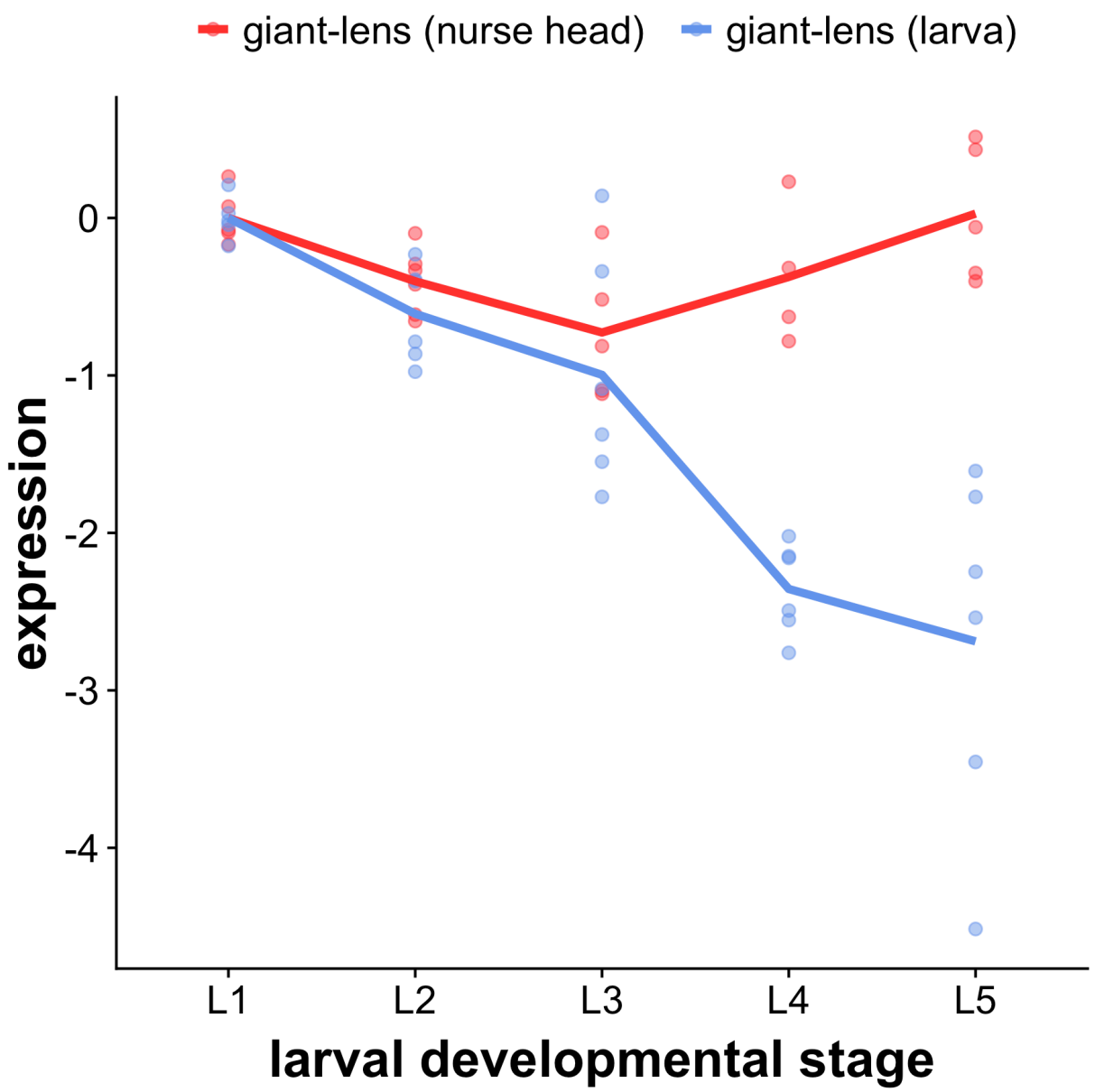

Fig S5. Expression of *giant-lens* in nurse heads and worker-destined larvae.

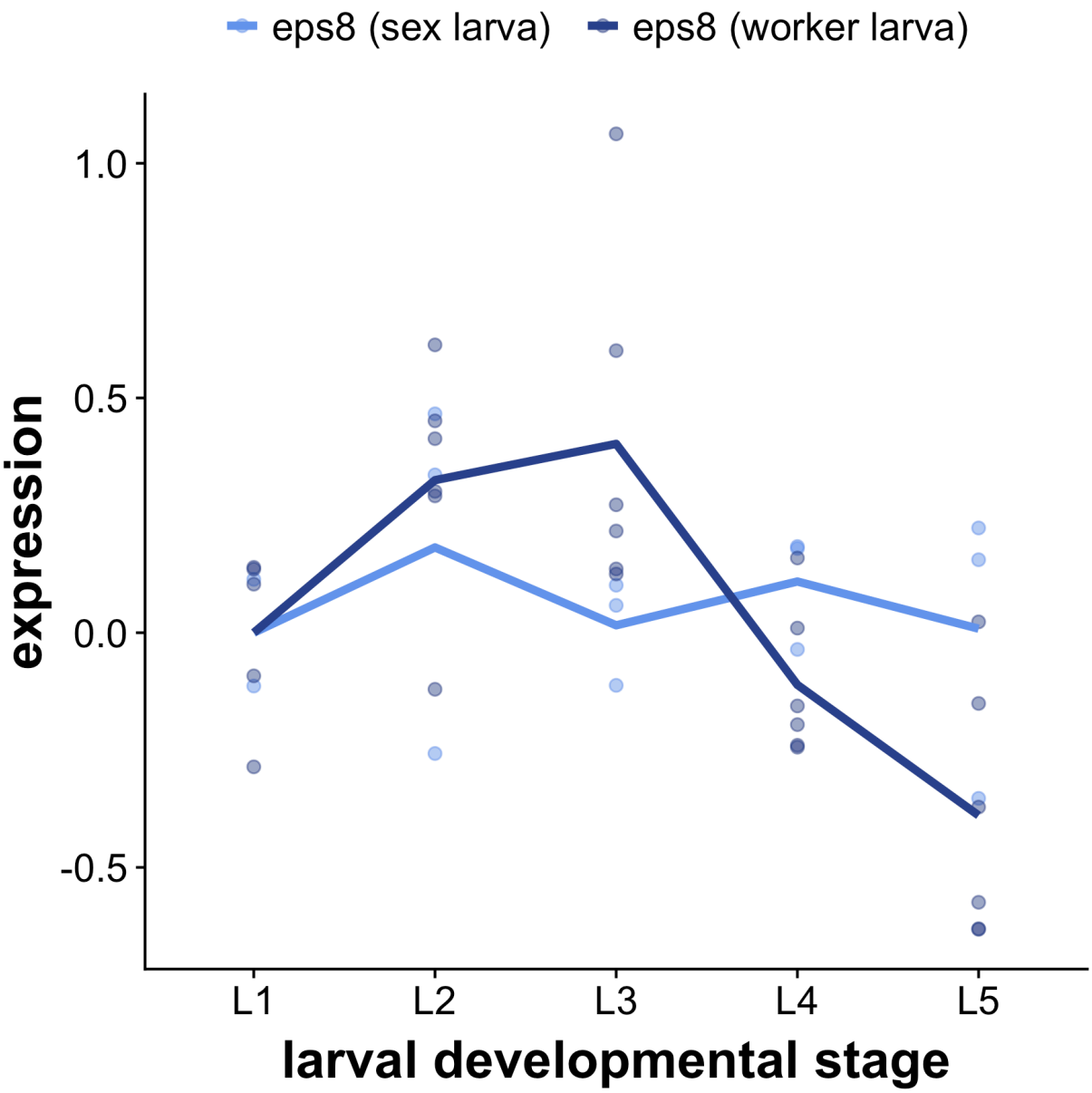

Fig S6. Expression of *eps8* (epidermal growth factor receptor substrate 8) in worker-destined and reproductive-destined larvae.

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

Table S1. Description of samples included in study.

	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

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677 **Table S2. Number of nurse significantly-enriched modules shared with larvae.**
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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

Table S3. Nurse head social connectivity GO terms based on GSEA of social connectivity.

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

723
724 **Table S4. Nurse abdomen GO terms based on GSEA of social connectivity.**
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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

Table S5. Swiss-Prot annotations for the top genes top genes coding for secreted proteins, sorted by social connectivity.

Figure Captions

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

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

Fig 2. Nurse and larval transcriptomes show strong signatures of gene co-expression across larval development.

Plots (A-D) depict the expression profiles of individual genes (light lines) as expressed in (A) nurse head, and (B) nurse abdomens, as well as larvae (C) larvae, shared with nurse heads, and (D) larvae, shared with nurse abdomens. Dark lines indicate the median expression values of all genes sorted into modules, with pre-defined expression profiles of modules depicted in plot insets. Only the five shared modules containing the most nurse-expressed genes are shown for clarity. Larval expression profiles are divided by the nurse tissue they are shared with, such that (C) depicts larval gene expression shared with nurse

803 heads (A), while (D) depicts larval gene expression shared with nurse abdomens (B). Note that nurse
804 heads and larvae shared inversely-related expression profiles. (E) Stage-specific nurses have more genes
805 than random nurses in modules shared with larvae than do random nurses, reflecting more broad-scale co-
806 expression across development. Error bars indicate 95% confidence intervals derived from systematic
807 drop-1 jackknifing of nurse samples. N = 10944 genes total.
808
809 **Fig 3. Genes encoding secreted proteins such as *giant-lens* are important for social gene regulation.**
810 (A) Genes encoding for proteins that are secreted in *Drosophila melanogaster* exhibit higher social
811 connectivity (i.e. more strongly socially regulate larval expression) in nurse heads than genes encoding
812 for non-secreted proteins (Wilcoxon rank-sum test). (B) The protein *giant-lens* is one of the genes coding
813 for secreted proteins with the highest social connectivity in nurse heads. Based on our data, *giant-lens*
814 expressed in stage-specific nurse heads (red) appears to inhibit the expression of the homolog of human
815 EGFR substrate 8 (*eps8*) expressed in worker-destined larvae (blue). Expression at stage *i* is equal to
816 $\log_2(\text{expression}_i/\text{expression}_1)$, i.e. the ratio of expression at the given stage to expression at the initial
817 stage.
818
819 **Fig 4. Highly social genes tend to be less evolutionarily constrained.**
820 Selective constraint, estimated from whole-genome polymorphism data, is (A) positively correlated with
821 within-tissue connectivity (Spearman correlation; head: $r = 0.122$, $P < 0.001$; abdomen: $r = 0.217$, $P <$
822 0.001), but negatively correlated with (B) social connectivity (head: $r = -0.090$, $P = 0.009$; abdomen: $r = -$
823 0.150, $P < 0.001$) and (C) sociality index (head: $r = -0.132$, $P < 0.001$; abdomen: $r = -0.223$, $P < 0.001$),
824 where sociality index is the difference between social and within-tissue connectivity per gene. Each point
825 in (a-c) indicates a single gene, as expressed in nurse heads or abdomens. Lines indicate trendline from
826 linear model. (D) Highly social genes also tend to be taxonomically restricted. Individual points depict
827 average values across nurse heads and abdomens for all genes within each estimated age class, indicated

828 by labels on points. Error bars depict 95% confidence intervals from bootstrapping. Numbers in
829 parentheses indicate number of genes in each age class.
830
831 **Fig S1. Diagram of sampling scheme.**
832 We collected ten worker-destined larvae, ten stage-specific nurses, and ten random nurses from each
833 colony (six colonies per time point, where time points represent larval developmental stages L1, L2, etc).
834 We collected stage-specific nurses when we observed them feeding larvae of the given developmental
835 stage. We collected random nurses when we observed them feeding any stage of larvae. We identified the
836 five separate stages of larvae as in [50].
837
838 **Fig S2. Identification of significantly-enriched modules shared between larvae and nurses.**
839 Inset tables depict expression profiles of sample modules genes can be assigned to. First, we construct
840 modules using all possible expression profiles (top left bubble). Expression profiles consist of five values,
841 starting at zero, that indicate the $\log_2$ fold-change in expression from the initial value (at stage L1). At
842 each subsequent stage, we either double, halve, or keep the expression level the same. This process is
843 repeated to produce 81 (four stages after L1; 3*3*3*3 = 81) total modules. Next, for each tissue
844 separately (here we depict workflow in larvae with yellow bubbles), we calculate individual gene
845 expression profiles as the $\log_2$ fold-change in expression from the initial value and assign genes to the
846 closest related module by Pearson correlation. Concurrently, we permute the developmental stage labels
847 for each gene and assign the stage-permuted genes to modules (repeated 1000 times). From these stage-
848 permuted results, we calculate the mean number of genes assigned to each module and treat this number
849 as a null expectation (as each expression profile is not equally likely to occur by chance). We then
850 identify significantly-enriched modules using a one-way binomial test (with the calculated mean as the
851 null), with a Bonferroni-corrected false discovery rate of 0.05. This entire process is repeated in a nurse
852 tissue and significantly-enriched modules are found (blue bubble). Finally, we compare significantly-
853 enriched modules between larvae and nurses and retain identical and inverse modules as shared profiles.

854 An example of an inversely related profile is shown in red, where larvae exhibit the enriched module [0,
855 0, -1, -2, -3] and nurses exhibit the inverse module, [0, 0, 1, 2, 3].
856
857 **Figure S3. Workflow of preliminary differential expression analysis and gene regulatory network**
858 **reconstruction.**
859 On the left, we identify putatively socially-acting genes through differential expression analysis. First, for
860 nurse heads and abdomens separately, we perform differential expression analysis in stage-specific and
861 random nurses to identify genes differentially expressed according to larval stage fed, using a nominal P-
862 value of 0.05. We remove genes differentially expressed in random nurses, as these correspond to colony-
863 specific environmental effects unrelated to social regulation of larval development. Next, we select the
864 top 1000 differentially expressed genes by P-value in stage-specific nurses (after removing those DE in
865 random nurses) as well as the top 1000 differentially expressed genes in larvae. From these genes, we
866 create "meta-samples" by combining gene expression of larvae and stage-specific nurses collected from
867 the same colony (separately for heads and abdomens), and labeling genes by the tissue they are expressed
868 in. Using these meta-samples, we perform gene regulatory reconstruction (right) to identify genes
869 expressed in nurses that regulate larval gene expression, and vise-versa. We repeat gene regulatory
870 reconstruction 1000 times and average connection strength across runs, as the algorithm is non-
871 deterministic. The output of gene regulatory reconstruction is a matrix of regulatory connections acting
872 between genes. From this matrix, we calculate the average connectivity for each gene, separating within-
873 tissue (larva-larva or nurse head-nurse head) from social (nurse-larva) connections. Genes with high
874 connectivity are predicted to interact with many genes, i.e. are central to the network. Finally, we
875 calculate each genes' sociality index as the difference between social connectivity and within-tissue
876 connectivity.
877
878 **Figure S4. Genes highly connected in within-tissue networks tended to have low values of social**
879 **connectivity.**

880 Connectivity is representative of the number and strength of regulatory connections each gene makes.
881 Points indicate the average connectivity for a given gene, as measured within-tissue (x-axis; i.e. larva-
882 larva or nurse-nurse) or socially (y-axis; i.e. larva-nurse). Points are colored by tissue the connectivity is
883 measured in (e.g., dark blue indicates genes expressed in larvae, with connectivity measured in networks
884 constructed with nurse abdomens). Spearman r = -0.166, -0.374, -0.276, -0.342 for the four tissues as
885 ordered in legend; $P < 0.001$ in all cases.
886 **Fig S5. Expression of *giant-lens* in nurse heads and worker-destined larvae.** Expression at stage *i* is
887 equal to $\log_2(\text{expression}_i/\text{expression}_1)$, i.e. the ratio of expression at the given stage to expression at the
888 initial stage.
889
890 **Fig S6. Expression of *eps8* (epidermal growth factor receptor substrate 8) in worker-destined and**
891 **reproductive-destined larvae.** Expression at stage *i* is equal to $\log_2(\text{expression}_i/\text{expression}_1)$, i.e. the ratio
892 of expression at the given stage to expression at the initial stage.
893
894 **Table S1. Description of samples included in study.** Worker-destined larvae are indicated by larva (W),
895 and reproductive-destined larvae are indicated by larva (R). Larval caste cannot be distinguished at the L1
896 stage, so L1 larvae are labeled larva (W/R). For network reconstruction, "meta" samples were used as
897 input for network reconstruction, in which genes were labeled by sample type and grouped such that each
898 gene contained a measurement of expression in worker-destined larvae, nurse heads, and nurse abdomens.
899 Because sampling was uneven after removing low-quality samples, we used the minimum number of
900 samples contained across tissues at a given stage for stage-specific nurse heads and abdomens, and
901 randomly dropped excess samples. Overall, 24 "aggregate" samples were used as input for gene
902 regulatory network reconstruction.
903
904 **Table S2. Number of nurse significantly-enriched modules shared with larvae.**

905 Significantly-enriched modules are defined as modules with a statistically significant number of genes
906 assigned, as determined by a permutation test (FDR < 0.05). Left column is the total number of
907 significant modules for each tissue, while the second and third columns indicate number shared with
908 larvae (out of 24 larval significantly-enriched modules). The last column indicates the total number of
909 genes in these shared modules.