Comparative transcriptomics reveals unique patterns of convergence in the evolution of eusociality

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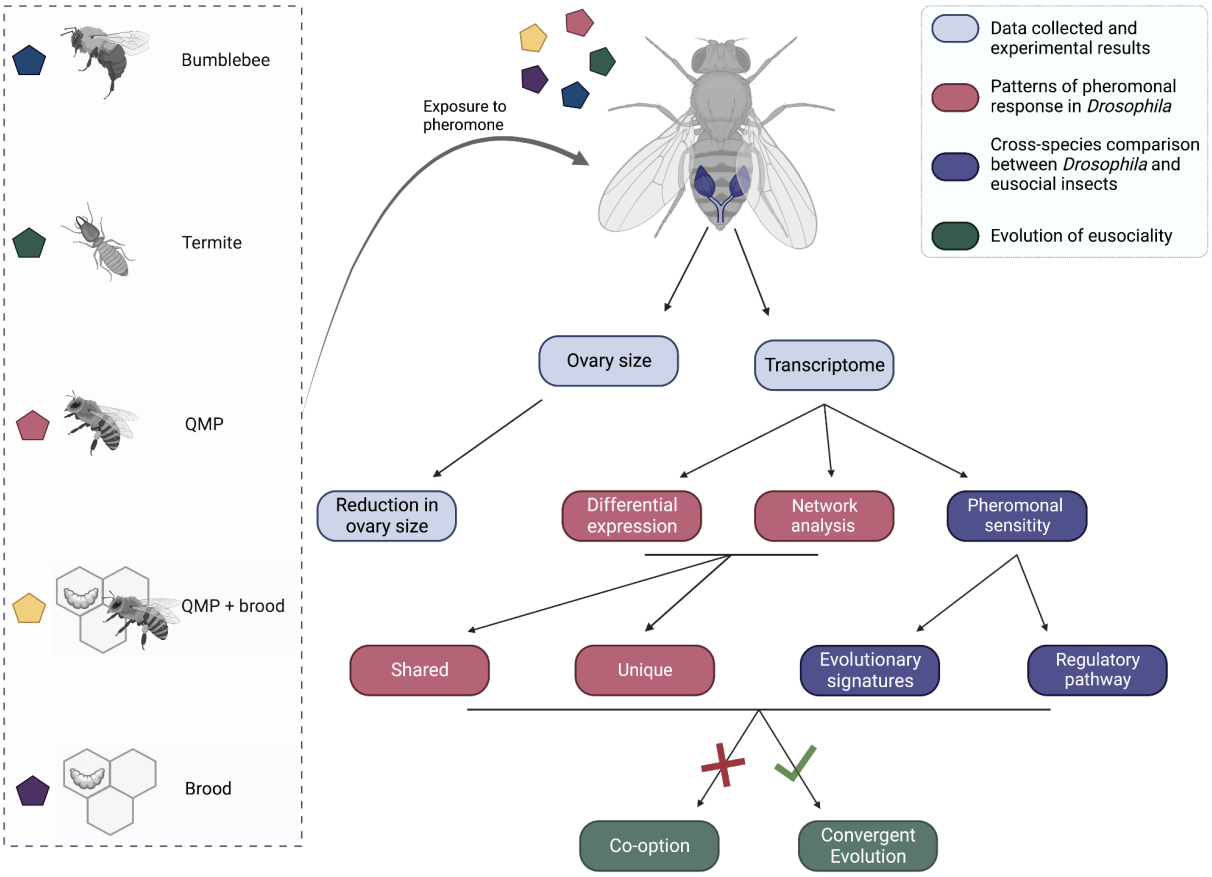
Abstract

Eusociality represents a major evolutionary transition that arose independently in at least 12 insect lineages. Despite this prevalence, there remains considerable uncertainty surrounding the catalysing event and underlying genomic changes that enable such modifications. Commonly associated with this evolutionary transition is establishing and maintaining the reproductive division of labour (e.g. a reproductive queen and no-reproductive workers). This division is, at least in part, induced and maintained by highly species-specific pheromones. However, genomic analysis remains conflicted on the role of pheromones in this evolutionary transition. Specifically, if there was co-option of a common pheromone-sensitive genetic pathway present in all progenitor species or strong lineage-specific selection converging on similar transcriptomic signatures.

Using a solitary insect model, we sought to determine if various species-specific pheromones induced similar transcriptomic responses, thus activating similar pathways. We measured the transcriptomic and physiological response of a solitary insect, *Drosophila melanogaster*, to pheromones from bumblebees, honey bees, and termites. Each treatment induced the same strong physiological response - a decreased ovary size. However, employing several methods of transcriptomic analysis, we did not observe conservation in pheromone-mediated gene/pathway regulation.

Thus, despite a conserved phenotypic response, the underpinning transcriptome was vastly different. This suggests that pheromone-mediated eusociality is the result of convergent evolution. We propose that mechanisms maintaining eusociality (i.e. proto-pheromone) in early stages of eusocial evolution in each group, thus, acting as a primer for eusociality. This early state is then refined through strong selective pressure, resulting in a converging eusocial phenotype.

Visual Abstract



Introduction

The transition from solitary to group living is a major evolutionary jump (1). However, it requires complex communication mechanisms to convey essential information to the group, such as the presence of predators and resource location (2, 3). Although there are multiple methods to transmit and receive information (e.g. visual, auditory, electric, olfactory), chemical cues are one of the most important and earliest to evolve (2, 4).

Insects use chemical components for recruitment, defence, predator recognition, and mate attraction, as well as to establish and shape social interactions (5–7). However, some eusocial organisms have evolved beyond context-dependent messaging. Acting as primer pheromones, they induce long-term behavioural and physiological effects by regulating colony structure and dynamics (8–11). This ranges from modulating aggression and attraction to monopolising colony reproduction through regulating female fecundity (12, 13). This monopolisation of reproduction by a few or one groups (queens/kings) creates a reproductive division of labour (8, 9). This is the cornerstone of eusociality (14–17).

Pheromone-mediated eusociality is not unique to honey bees as it has independently evolved at least 12 times in arthropods (18). The diverse evolutionary history of eusociality is also reflected in the production and structure of pheromones controlling the process (19–21). For instance, in several species, cuticular hydrocarbons (CHCs), a component ubiquitously produced in insects to prevent desiccation and infections, can act as queen pheromones (22–24). Such as in stingless and bumble bees, *Bombus terrestris,* where CHCs act as a queen pheromone (22, 25). In other species, such as the subterranean termite *Reticulitermes speratus,* queen pheromone is a mixture of CHCs, n-butyl-n-butyrate and 2-methyl-1butanol. However, the use of CHCs is not conserved in other eusocial animals. In *Apis mellifera,* queen pheromone comprises a complex blend of fatty acids secreted by the (26–28) mandibular gland (26–28). Moreover, pheromones produced by the brood (brood pheromone – Brood) were also found to regulate behaviour and worker reproduction in several species, such as *A. mellifera* (29–31).

In eusocial animals, the reproductive division of labour usually initiates during early larval development and is maintained throughout adulthood via exposure to queen pheromone (32, 33). Hence, in the absence of such pheromones, the division of labour breaks down as young workers activate their ovaries and engage in more selfish behaviour (34–38).

In eusocial animals, the division of labour and reproduction are suggested to be intertwined, arising from modifications of the reproductive life cycle of solitary insects (39, 40). Comparative genomics of multiple eusocial insect species corroborates this hypothesis and suggests that eusocial traits evolved from the co-option of pathways present in solitary ancestors (41–45). Moreover, there is strong evidence that specific queen pheromones evolved from conserved signals of solitary ancestors (22, 45, 46). It has been proposed that eusociality requires a highly conserved group of core genes, present in the solitary ancestor, that serves as a ground plan for eusociality (43). However, clear unambiguous evidence of this co-option hypothesis remains elusive (42, 47).

Further expansion of comparative genomics has added to this ambiguity. Genome analysis revealed branch-specific positive selection in five bee species from the *Apis* genus (48). Specifically, a greater proportion of species-specific genes were under positive selection than *Apis*-specific (45, 48). Furthermore, despite similar eusocial behaviour, three eusocial termite species demonstrated vastly different underpinning genetic mechanisms when contrasted against a wide diversity of non-eusocial and eusocial insect species (49). Thus, eusociality may be induced and maintained through diverse genetic means.

Pheromone-induced transcriptomic changes reflect extensive evolutionary optimisation of affected networks. Thus, cross-species transcriptomic comparisons are complicated due to the evolutionary distances between eusocial insect species, as inter-species gene orthologs may have vastly different functions (44, 50–52). The birth of novel genes and the expansion or contraction of gene families can make clear orthologue identification impossible and can be conflated with evolutionary forces (52–54). Therefore, differences or similarities in expression patterns between two highly divergent species can be highly misleading (55). One approach to overcome this

problem is by using a single species to investigate transcriptomic changes induced by various pheromones (56–58). This permits the study of conserved and co-opted transcriptomic changes and identifies molecular signatures of these rare evolutionary events.

Here, we investigate pheromone-mediated gene expression in a solitary species. Using *Drosophila melanogaster*, we examine if primer pheromones, regulating eusociality, evolved from the co-option of similar pathways present in a solitary ancestor. Specifically, whether distinct pheromones have a similar transcriptomic and physiological profile across multiple instances on which they evolved, as previously suggested. We present a comprehensive transcriptome dataset set investigating gene expression associated with the reproductive division of labour. We show the variation in differential gene expression induced termite (*Reticulitermes speratus*), bumblebee (*Bombus terrestris*) and honey bee (*Apis mellifera*) queen pheromone and honey bee brood pheromone. We leverage this data set to perform an up to date and comprehensive comparison of differentially vs distinct (i.e. non-homologous) expressed genes associated with the maintenance of eusociality.

Results

Pheromone-induced Ovary Changes

We identified a significant change in ovary size across all pheromone-treated *D. melanogaster* (Repeated measures ANOVA $F_{7,180}$, $p < 0.0001$; Figure 1; Tables S1 & S2). Although all pheromone treatments strongly inhibited ovary size, their effects varied in strength. Bumblebee and Termite pheromone had similar ($p = 1$) severe pheromone-mediated ovary reductions of 0.32 mm^2 and 0.35 mm^2 (mean; n=10), respectively (Figure 1; Table S1). Pheromone treatments from *A. mellifera* queen mandibular pheromone (QMP), Brood and the QMP and Brood mix pheromone induced similar reductions in ovary size (QMP and Brood vs QMP $p =1$; QMP and Brood vs Brood $p =1$; QMP vs Brood $p = 0.064$) of 0.51 mm^2, 0.65 mm^2 and 0.59 mm^2 (mean; n=10) respectively (Figure 1; Table S1).

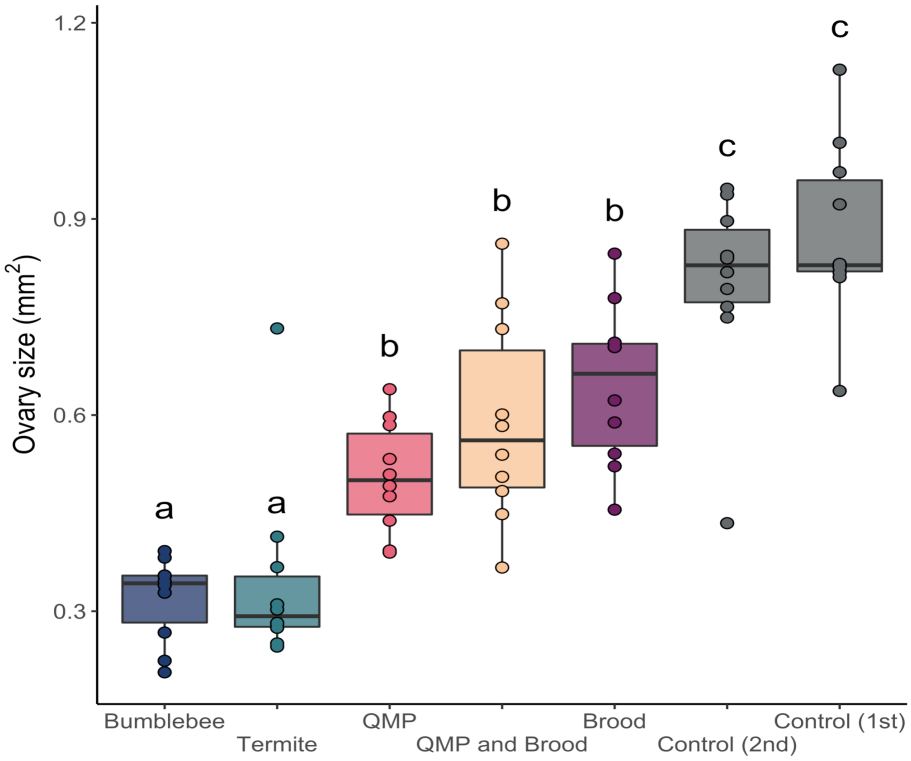


Figure 1. Pheromone treatment resulted in reduced *Drosophila melanogaster* ovary size. Treatments were queen pheromone produced by termites (*Reticulitermes speratus*), bumblebees (*Bombus terrestris*) and honey bees (*Apis mellifera*) (QMP), brood pheromone produced by honey bee larvae and a combination of both brood and QMP from honey bees. The pheromones were introduced to *D. melanogaster* from egg to adult via supplementation in food. Letters, either a, b or c, denote statistical significance with varying letters being significantly different (p-value < 0.05; Multiple Comparisons of Means: Tukey Contrasts; Table S2). Ten biological replicates were performed for each treatment or control with each biological replicate. Ovaries were measured from three flies per each replicate.

Pheromone-induced Transcriptomic Changes

Differentially expressed genes and networks

Despite phenotypic similarities, we identified no transcriptomic overlap between pheromone treatment groups. Within each treatment, we identified similar numbers of differentially expressed genes with brood pheromone treatment being elevated: QMP (180 genes), Brood (640 genes), QMP and Brood (293 genes), Termites (207 genes) and Bumblebees (102 genes). There was no shared core of pheromone response genes and negligible inter-treatment overlap (Figure 2A; Figure S1). Due to their

157 potential in eusociality regulation, differential expression of the Notch signalling pathway
158 and the yellow gene family was specifically examined (59, 60). No differential
159 expression of any genes encoding the 12 Notch signalling pathway core components or
160 any of the 14 genes in the yellow family was detected in any treatment.
161 As we were unable to identify a core of differentially expressed genes, we
162 investigated if pheromone-mediated changes in gene expression affected reproductive
163 physiology through similar cellular processes. We compared gene enrichment of
164 pheromone-induced changes in the transcriptome using GO (gene ontology) and KEGG
165 (Kyoto Encyclopedia of Genes and Genomes) annotations. Although we identified some
166 inter-treatment pathway enrichment, again, no core pathways were identified (Figure
167 2B).
168 In the absence of a conserved transcriptomic core, a broader general correlation
169 between treatments was undertaken. There was a high degree correlation between the
170 log_2-fold-change (Log_2FC) of all significantly expressed genes in each treatment (Figure
171 2C). To further investigate pheromone-mediated changes in genetic pathways and
172 transcriptomic networks, we employed two different network analysis approaches.
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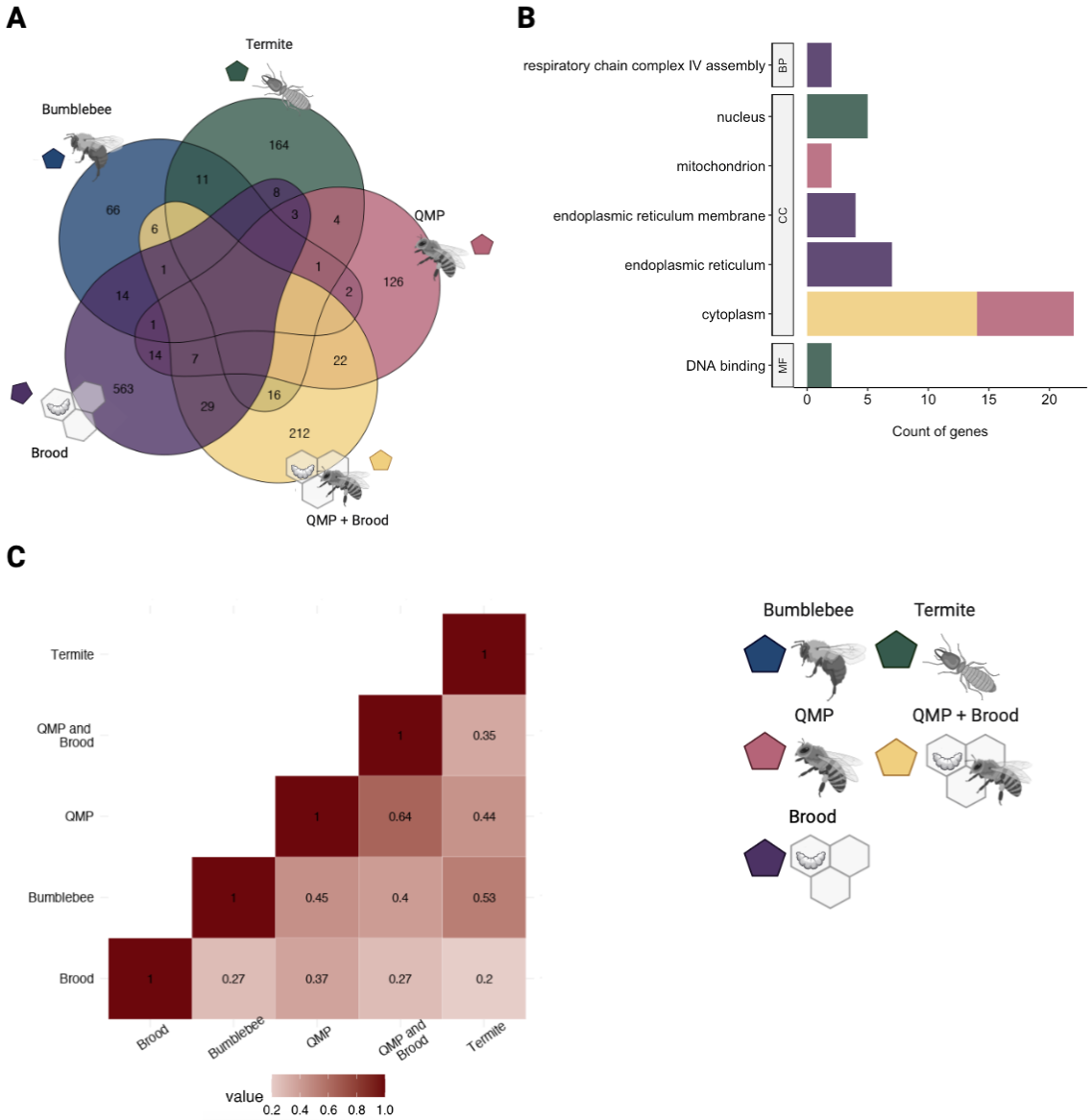

Figure 2. Differential gene expression of pheromone-sensitive pathways and genes in *Drosophila melanogaster* does not show a shared response. The DeSeq package was employed to determine differentially expressed genes in each of the pheromone treatments. A) Venn diagram showing the number of significantly differentially expressed genes per pheromone treatment. B) Results of signalling pathway analysis of differentially expressed genes across different pheromone treatments. C) Pearson's correlation coefficient between the Log_2-FC of significantly expressed transcripts in each treatment. Treatments were queen pheromone produced by termites (*Reticulitermes speratus*), bumblebees (*Bombus terrestris*) and honey bees (*Apis mellifera*), brood pheromone produced by honey bee larvae and a combination of both brood and queen pheromone from honey bees.

186 Network Analysis

187 Employing network-based transcriptomic analysis, and weighted gene co-
188 expression network analysis (WGCNA), no shared regulatory network common among
189 all pheromone treatments was identified (Figure 3). We used WGCNA to screen and
190 build a gene co-expression network, screen important modules, and identify candidate
191 genes associated with each pheromone treatment (hub genes). We identified candidate
192 genes by estimating the correlation between each gene and the pheromone treatment
193 using Gene Significance analysis (61–63). WGCNA identified statistically significant
194 differentially co-expressed genes between control groups and *D. melanogaster* treated
195 with QMP (219 genes), Brood (219 genes), the mix of QMP and Brood (396 genes),
196 Termites (307 genes) and Bumblebees (276 genes) (Figure 3). Once again, each
197 treatment had limited overlap (Figure 3A; Figure S2). Employing GO enrichment and
198 gene set enrichment analysis (GSEA) on the WGCNA differentially co-expressed genes
199 also failed to identify high levels of overlap between functions (Figure 2B).

200 Once again, there was a high degree correlation between the log_2-fold-change
201 (Log_2FC) of all significantly expressed genes in each treatment (Figure 2C). However,
202 Brood pheromone treatment resulted in a negative correlation for the WGCNA-identified
203 transcripts. This was not observed in the mix of QMP and Brood treatment.

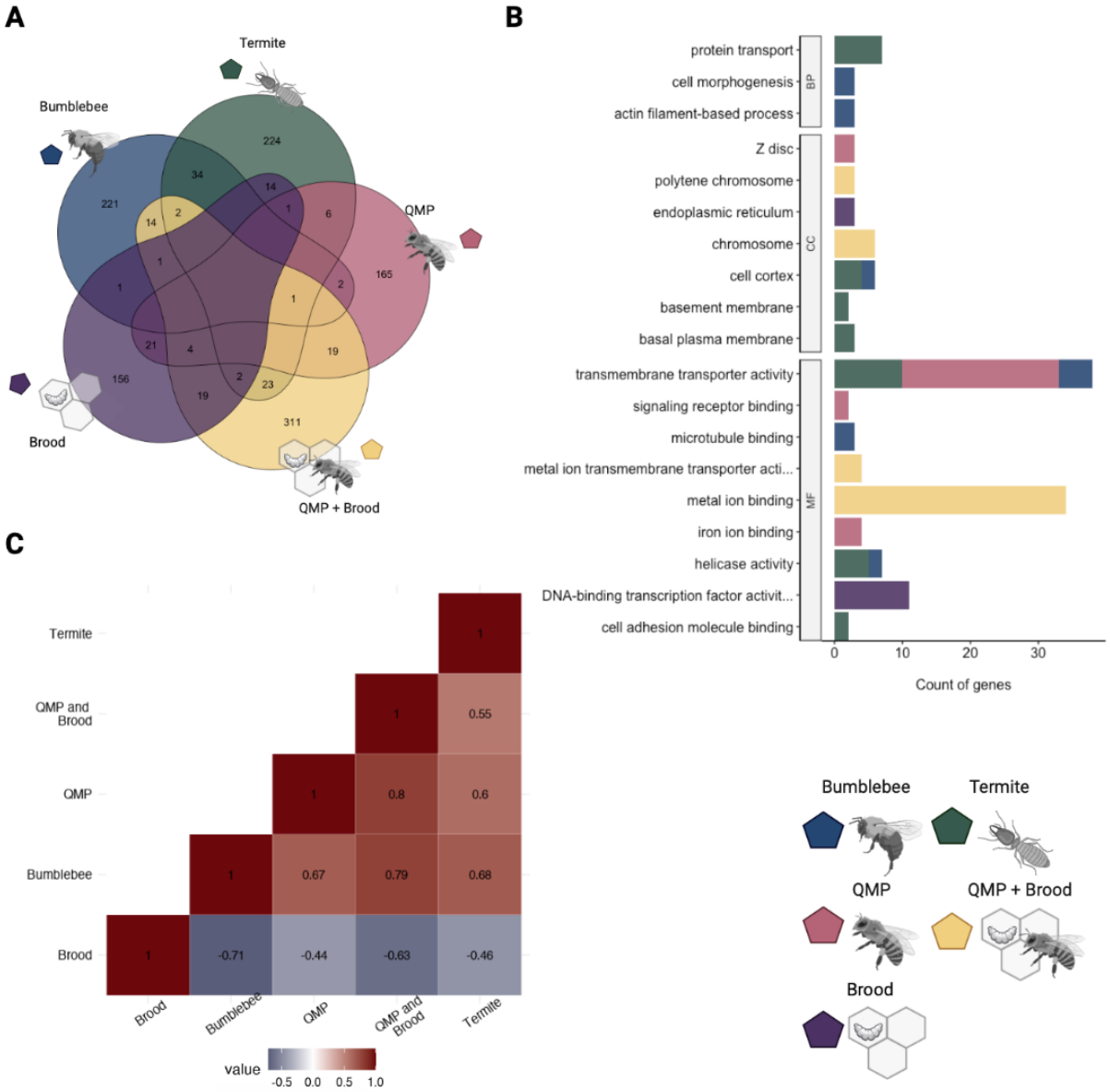

Figure 3. Network analysis showing pheromone-sensitive pathways and genes in *Drosophila melanogaster* does not show a shared response. Weighted gene co-expression analysis was used to screen and build a gene co-expression network, screen important modules, and identify candidate genes associated with each pheromone treatment. A) Venn diagram showing the number of significantly differentially expressed genes per pheromone treatment. B) Results of signalling pathway analysis of differentially expressed genes across different pheromone treatments. C) Pearson's correlation coefficient between the Log_2-FC of significantly expressed transcripts in each treatment. Treatments were queen pheromone produced by termites (*Reticulitermes speratus*), bumblebees (*Bombus terrestris*) and honey bees (*Apis mellifera*), brood pheromone produced by honey bee larvae and a combination of both brood and queen pheromone from honey bees.

215 Cross-species comparison

216 We found that pheromone-mediated transcriptomic changes between eusocial
217 insects and *D. melanogaster* were preserved. Although *D. melanogaster* has been
218 previously used as a model organism in the study of eusociality (64), there are no direct
219 comparisons between pheromone-mediated changes in gene expression for flies
220 compared to eusocial insects. Holman et al. studied the transcriptomic response of
221 several species, including *A. mellifera* and *B. terrestris,* treated with their own species'
222 pheromone (44). Using WGCNA module preservation, we determined the conservation
223 of pheromone-mediated network signatures in our *D. melanogaster* experiments to their
224 corresponding equivalents from Holman et al. Specifically, between our *D.*
225 *melanogaster* exposed to QMP (*A. mellifera*) and Holman et al. *A. mellifera* exposed to
226 QMP (*A. mellifera*), and our *D. melanogaster* exposed to bumblebee QMP (*B. terrestris*)
227 and Holman et al. *B. terrestris* exposed to bumblebee QMP.

228 Comparing these datasets, we identified a high degree of WGCNA module
229 preservation (Table 1; Figure 4; Figure S3). Overall, the *A. mellifera* comparison had a
230 higher number of preserved modules and, thus, a greater number of shared genes in
231 these modules. However, *A. mellifera* preservation also had a greater proportion of
232 preserved modules with strong and moderate preservation (Figure 4A; Figure S3). The
233 contrast between these two comparisons is likely down to the greater number of
234 identified orthologues in *A. mellifera*. Regardless of the number, the preservation of
235 these modules does demonstrate, in a broad sense, a similar transcriptomic response,
236 and those serve as an external control for our analysis.
237

238 **Table 1. Cross-species model preservation of pheromone-response.** The WGCNA module-
239 preservation function facilitated comparisons between *Drosophila melanogaster* and *Apis mellifera*
240 pheromone response to *A. mellifera* queen pheromone and *D. melanogaster* and *Bombus terrestris*
241 pheromone-response to *B. terrestris* queen pheromone. Number of shared modules between the two.
242 Total number of genes identified.

Species	Number of Modules	Number of Genes
Strong		
A. mellifera	30	2102
B. terrestris	9	721
Moderate		
A. mellifera	41	2636
B. terrestris	15	1310
Low		
A. mellifera	16	769
B. terrestris	23	713
Dissimilar		
A. mellifera	1	43
B. terrestris	4	115

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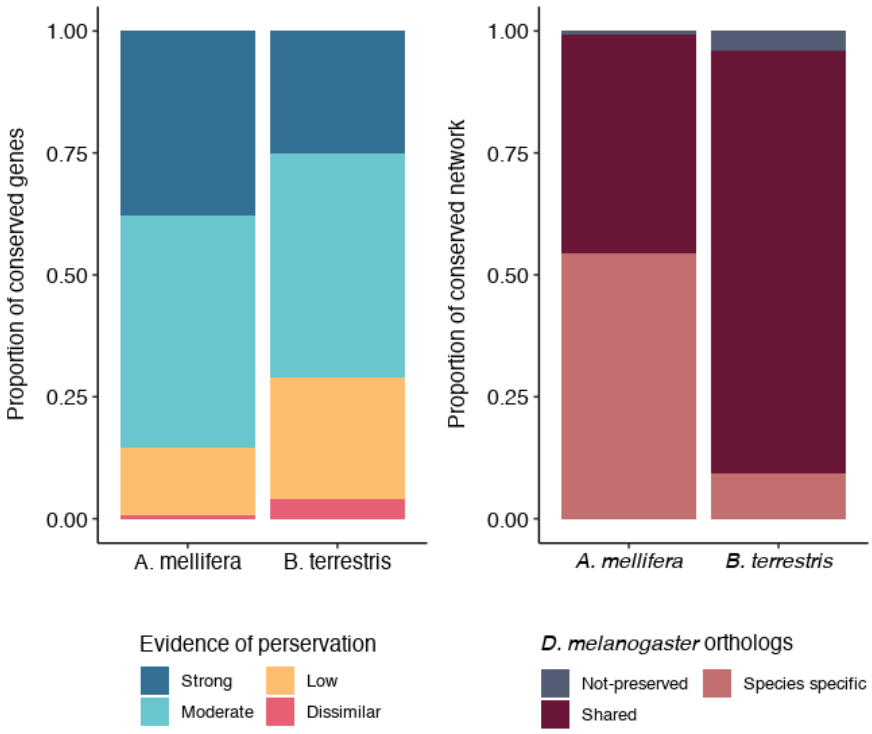
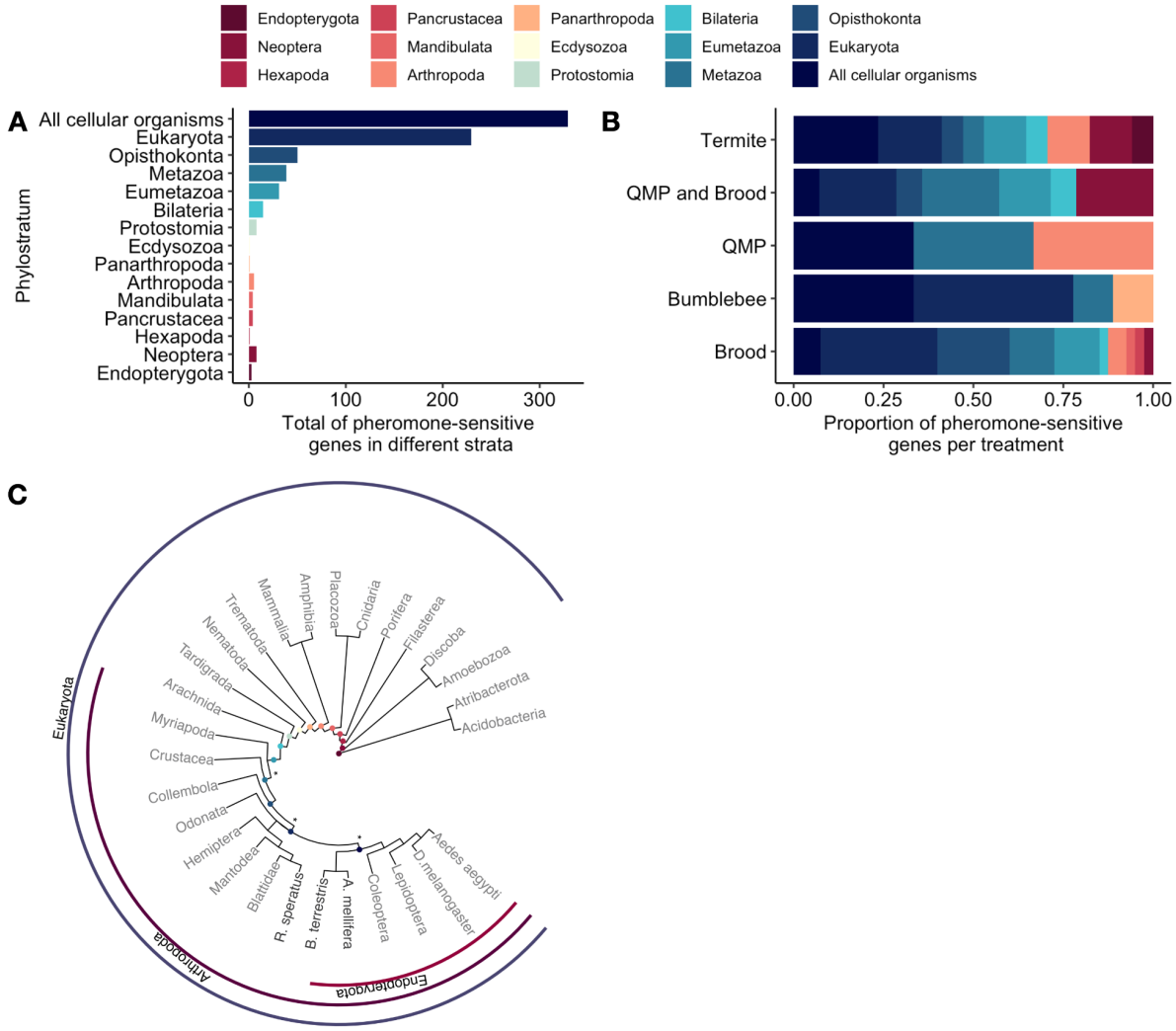


Figure 4. Pheromone-responsive module preservation between *Drosophila melanogaster* and *Apis mellifera* or *Bombus terrestris.* Comparing *D. melanogaster* pheromone-response data from this work and *A. mellifera* and *B. terrestris* pheromone-response data from Holman et al. Gene modules were determined using WGCNA module preservation. *A)* Proportion conserved pheromone sensitive genes in *D. melanogaster*, *A. mellifera* and *B. terrestris*. B) Summary of preservation evidence for pheromone response. Pheromonal sensitivity in *A. mellifera* and *B. terrestris* were compared with *D. melanogaster*. Shared genes indicate those highly conserved between the two comparison groups.

Origins of Pheromone Response Genes

The responsive genes of each pheromone treatment do not have a conserved evolutionary origin, and thus eusociality is not co-oping recently evolved genes. The absence of overlapping differentially expressed pheromone-sensitive genes between treatments led to querying the evolutionary origins of these responsive pathways. We employed phylostratigraphy and calculated the transcriptome age index (TAI) to infer the evolutionary origins of pheromone-activated networks and genes. When separated by treatment, each group of differentially expressed pheromone-responsive genes lack a statistically significant conversed age (Figure 4A). If each treatment was further separated into up- and down-regulated differentially expressed transcripts, up-regulated transcripts from both termite and QMP treatments were statistically significant (Figure 4B).

264 A phylogenetic tree was created to determine the evolutionary relationship
265 between target species homologues of the pheromone-responsive genes (Figure 4C).
266 The tree was built using pylostratigraphic gene ages and phylogenetic relationships
267 amongst a diverse collection of 182 species, with most species collapsed into
268 systematic order or higher classification.



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270 **Figure 4. The evolutionary origins of pheromone responsive genes.** The transcriptome age index
271 (TAI) was used to infer the evolutionary origins of pheromone-responsive genes. A) Summary of all
272 pheromone-sensitive genes per strata. B) Proportion of pheromone-sensitive genes in each treatment per
273 strata. C) A phylogenetic tree with pylostratigraphic gene ages and phylogenetic relationships amongst
274 182 species. Dots indicate the evolutionary age of different pheromone-sensitive genes. Asterisks
275 indicate phylogenetic levels (phylostrata) with significant changes in the expression of pheromone-
276 sensitive genes.

277 # Discussion

278 Although all pheromone treatments suppressed ovary development in *D.*
279 *melanogaster,* we found no evidence of a shared evolutionary pathway or conserved
280 mechanism underpinning this phenotype. In social insects, chemical communication has
281 been independently co-opted in numerous species to facilitate the inhibition of worker
282 reproduction by dominant females (4, 5). The prevailing hypothesis is that this induced
283 sterility evolved from modifications in conserved molecular pathways regulating the
284 physiology and behaviour of solitary species (44, 45, 59, 65, 66). Therefore, it was
285 reasonable to expect similar pheromone-induced responses between different insect
286 lineages and, thus, similar physiological and regulatory pathway responses. We
287 removed the complexities of cross-species analysis and millions of years of convergent
288 evolution by applying pheromones that inhibit worker reproduction from honeybees,
289 bumblebees, and termites to a single species.

290 In contextualising our work, we are mindful of conflating factors introduced
291 through cross-species analysis, especially when the species are separated by large
292 evolutionary distances (18, 20, 48, 67). Furthermore, transcriptomic enrichment
293 analyses, employed widely for cross-species studies, can potentially introduce biases
294 (68, 69). However, we employed relaxed differential gene filtering ($p<0.05$, irrespective
295 of expression fold-change) and diverse enrichment methods. Our view was that
296 subsequent manual curation would allow us to identify candidate genes acting as
297 universal eusocial catalysts. This proved unnecessary as we could not identify a core of
298 differentially expressed genes and minimal inter-treatment overlap (Figure 2). Even GO
299 enrichment and pathway analysis, highly permissive methods, only revealed minimal
300 inter-group overlap.

301 Unable to identify a core of pheromone-responsive transcripts, we employed a
302 candidate-based approach. Pheromone exposure has previously been shown to
303 activate pathways related to lipid biosynthesis, oogenesis and olfaction (44, 45, 59). For
304 instance, Notch appears to be an essential pathway regulating ovary development in
305 the presence of queen pheromone (59) and it is generally assumed to be a co-opted
306 pathway in solitary animals that facilitates eusociality (59). Although we found Notch to
307 be differentially co-expressed in three of the five treatments, termite, QMP and QMP +

308 brood, differing Notch signalling genes were affected in each treatment. An alternative
309 candidate pathway is the yellow gene family. In Hymenoptera, this gene family is also
310 responsible for regulating aspects of eusociality and includes a clade of genes referred
311 to as the major royal jelly proteins (60, 70). In *D. melanogaster,* there are 14 members
312 of this gene family (60, 71). The yellow gene family (*yellow-h* and *yellow-g2 in D.*
313 *melanogaster*) was differentially co-expressed across all groups except for the brood
314 treatment. Thus, these pathways seemingly provide a potential co-option target but do
315 not appear to be a universal driver of pheromone response. Rather, given the
316 widespread regulatory changes associated with eusociality (72–74), these gene families
317 likely serve as candidate targets in refining eusocial traits.
318 Despite differences in the unpinning transcriptomic response, each treatment
319 yielded smaller ovary size (Figure 1). As previous work demonstrated, pheromone
320 exposure had no effect, even at much higher doses, on adult *D. melanogaster* (75). The
321 ineffectiveness of pheromone-mediated reproductive inhibition on adults is also
322 observed in other eusocial species (75–79). Insect oogenesis is a highly sensitive
323 process disrupted by numerous environmental, developmental and gene-regulatory
324 factors (80, 81). This includes the fact that cell death in the ovary is distinct from other
325 tissues and mediated independently (80, 81). Furthermore, pheromone-mediated
326 reproductive inhibition is not observed in primitively eusocial species like *Polistes satan*
327 (paper wasp) or species with high reproductive plasticity like *Euglossa dilemma* (orchid
328 bee) (41, 82). However, orchid bees form small 2–3 individual social groups wherein an
329 abundance of specific cuticular hydrocarbons correlated with social dominance and
330 ovary size (83). Therefore, oogenesis is a key candidate for pheromone-mediated
331 disruption. Moreover, influencing oogenesis provides a potential mechanism for the
332 monopolisation of colony reproduction and the evolution of eusociality.
333 Despite arising independently, several eusocial species share phenotypic and
334 transcriptomic similarities (41–43). However, consistent with a growing body of
335 comparative genomics (20, 84), we suggest this is not due to the co-option of identical
336 or similar pathways in solitary ancestors. Rather, positive selection drives the
337 convergence of characteristically eusocial phenotypes with diverse genomic origins. An
338 example of this arises in the evolution of intra-colony communication. Insects rely on a

339 host of olfactory signals and cues with the majority of these being detected by
340 chemosensory receptors in the odorant receptor (OR) or ionotropic receptor (IR) gene
341 families (85). These chemosensory pathways provide the core mechanism for intra-
342 colony communication in several eusocial species (19, 20, 86, 87). Genomic analysis
343 revealed that although termites and honey bees have expanded receptor gene families,
344 termites had greater IRs and bees greater ORs (49, 86, 88–91). This means that
345 although high-level analysis, such as GO, may detect these two gene family expansions
346 as similar, closer examination demonstrated their independence.
347 *D. melanogaster* accurately depicts the effect of queen pheromone in eusocial
348 species. When comparing our results with the transcriptome of honey bees and
349 bumblebees exposed to queen pheromone (44), we found strong conservation of
350 differentially expressed genes in pheromone treatments. Demonstrating the reliability of
351 our results and the power of comparing the effect of distinct components (i.e.
352 pheromones) in a single species.
353 Our work demonstrates the potential origins of eusociality evolution. The first
354 steps of eusocial evolution likely began with a localised reproductive impediment that,
355 over time, resulted in quasi-eusocial living. The absence of a core pheromone-induced
356 transcriptomic response and yet conserved phenotypic response, suggests female
357 fertility suppression can rapidly evolve using numerous compounds that induce
358 regulatory changes. This is evident as eusociality has evolved at least eight times
359 independently with limited overlap between the specific pheromones meditating this (67,
360 92).
361 The primer pheromonal signal in insects originated at least 100 Mya and evolved
362 independently in multiple species. Their evolution and signal can be well traced across
363 three groups: Blatoidea, Hymenoptera and Coleoptera, causing virtually a similar
364 phenotype in all groups. The independent evolution of pheromones, the high
365 conservation with their eusocial counterpart, combined with our results (i.e. pheromone
366 treatment causing a similar ovary reaction in flies with no overlapping gene network and
367 pathways) suggests pheromones are not a product of gene co-option, but the result of
368 convergent phenotypic evolution. Producing a similar phenotype (inhibition of ovary
369 development) through a different pathway in the species on which it evolved.

370 # Materials & Methods

371 We used the fruit fly *D. melanogaster* to investigate the hypothesis that
372 eusociality evolved multiple times by co-opting existing regulatory pathways present in a
373 solitary ancestor. We achieved this by studying the pheromone genetic signature of fruit
374 flies exposed to pheromones from three different eusocial species: honey bee queen
375 mandibular pheromone (QMP), bumblebee queen pheromone (Bumblebee), termite
376 queen pheromone (Termite) and a pheromone produced by honey bee larvae, the
377 brood pheromone (Brood). To simulate the conditions present in honey bee colonies,
378 when they are exposed to both brood and queen pheromone, we also added a group
379 with a mix of queen and brood pheromone (QMP + Brood).

380 Fly husbandry

381 Flies were raised on standard Bloomington media at 24 °C under a 16h light / 8h
382 dark cycle in 50 mL vials. The *D. melanogaster* strain Canton-S, a generous gift from
383 the Van Vactor Unit, was used in all experiments. Each experimental vial contained ten
384 males and females, of approximately 5-day-old. Each treatment group consisted of 10
385 vial replicates.

386 Pheromone treatment

387 Bloomington fly food was made and cooled to >50°C before mixing in each
388 phenomenon treatment (Table 2) at a 1 in 150 ml concentration. Ten millilitres of
389 supplemented food were added to each vial and left to dry for 24 hours. Vials were kept
390 at 25°C and under a powerful extractor to prevent cross-contamination due to
391 pheromone volatility. Adult flies were left on the vial for four days to lay eggs and then
392 removed and discarded. Virgin females were collected and allowed to mature for five
393 days in vials containing the aforementioned food with the corresponding treatment
394 pheromone.

395 **Table 2 – Pheromone Treatments, suppliers, and dilution.**

Treatment	Pheromone Formula	Supplier (ID)	Dilution
Control	-	-	50 µl isopropanol
QMP (*Apis mellifera*)	9-ODA, two isomers of 9-HDA, HOB and HVA	TempQueen (DC-705)	20 µl of synthetic QMP mix diluted in 30 µl isopropanol
Brood pheromone (*Apis mellifera*)	Methyl palmitate, Ethyl palmitate, Methyl stearate, Ethyl stearate, Methyl oleate, Ethyl oleate, Methyl linoleate, Ethyl linoleate, Methyl linolenate, Ethyl linolenate	Contech (SuperBoost)	10 µl SuperBoost diluted in 40 µl isopropanol
QMP and Brood pheromone mix (*Apis mellifera*)	9-ODA, two isomers of 9-HDA, HOB and HVA; Methyl palmitate, Ethyl palmitate, Methyl stearate, Ethyl stearate, Methyl oleate, Ethyl oleate, Methyl linoleate, Ethyl linoleate, Methyl linolenate, Ethyl linolenate	TempQueen (DC-705) and Contech (SuperBoost)	- 20µL of synthetic QMP, 10µL Supperboost and 20µL isopropanol
Bumblebee queen pheromone (*Bombus terrestris*)	CHC pentacosane (C25)	Sigma-Aldrich (286931)	1 µg C25 diluted in 50 µl isopropanol
Termite queen pheromone (*Reticulitermes speratus*)	n-butyl-n-butyrate and 2-methyl-1-butanol	n-butyl-n-butyrate Sigma-Aldrich (281964); 2-methyl-1-butanol Wako Chemicals (133-08385)	15 µl n-butyl-n-butyrate and 7.5 µl 2-methyl-1-butanol diluted in 27.5 µl isopropanol

396

397 Ovary measurements

398 Ten biological replicates were used for each treatment or control. For each
399 replicate, ovary collection for measurement was performed in triplicate and on the same
400 day to prevent temporal changes in physiological measurements of ovaries. Ovary
401 image identities were encoded such that they could be measured blind to reduce bias.
402 Measurements were performed using ImageJ2 (93). We used normal QQplot and
403 Shapiro-Wilk test to identify deviations from normality. Although termites had two

404 outliers, all the points fall approximately along the reference line, and thus we assumed
405 normality. We compared differences in ovary size between different treatment groups
406 using One-way repeated-measures ANOVA.

407 RNA Sequencing

408 For RNA sequencing, we randomly selected four vials and pulled five ovaries per
409 vial. RNA extraction was performed with an in-house modified protocol (Supporting
410 Text). Libraries were prepared using NEBNext® Ultra™ II Directional RNA Library Prep
411 Kit (E7760L) for Illumina as instructed by the manufacturer.

412 Data Analysis

413 The complete analysis pipeline and associated results are publicly available at
414 https://github.com/marivelasque/eusociality_evolution. This pipeline is a modified
415 version of our previously developed pipeline (94). RNA data were trimmed with
416 Trimmomatic 0.38 (95) and quality was assessed using FASTQC (96). Transcript
417 abundance was calculated with RSEM/bowtie2 (97, 98). Mapping and abundance
418 calculations were performed against the *D. melanogaster* genome assembly BDGP6
419 (release 89). Differential expression analysis was performed using edgeR (99). We
420 adjusted p-values to control the false discovery rate using the Benjamini-Hochberg
421 method. The threshold of significance was defined by $p<0.05$. Visualisation relied on
422 packages venn (100), ggplot2 (101), igraph (102) and ggraph (103). Analysis, figures
423 and HTML files were generated using R in the RStudio environment.

424 Gene co-expression network analysis

425 To investigate changes in gene networks mediated by eusocial pheromones we
426 used Weighted Gene Co-Expression Network Analysis WGCNA (62). We used the
427 WGCNA R package to construct the co-expression network using gene counts obtained
428 from RSEM. Prior to the analysis, we removed gene outliers using the
429 “goodSamplesGenes” (62) function and accessed overall sample clustering in the
430 expression matrix using “hclust” (104). We used the lowest threshold power that
431 resulted in approximate scale-free topology to construct the gene co-expression
432 network using the blockwiseModules function (62). Then, we estimated the gene

433 expression profile, module eigengene, and the module-treatment relationship,
434 calculating the correlation between the module eigengene and treatment group. We
435 implemented the analysis in two steps (i.e. lowest threshold power and differential co-
436 expression) using the functions SoftPower and wgcna.est from (94). The threshold of
437 significance was defined as $p<0.05$.

438 Enrichment and pathway analysis

439 We obtained Gene Ontology (GO) and KEGG (Kyoto Encyclopedia of Genes and
440 Genomes) annotations using the package biomaRt (105) clusterProfiler (104)
441 respectively. We used Fisher's exact test to identify significantly enriched genes using
442 the package topGO (106) with the threshold of significance defined by $P<0.05$. We
443 identified enriched pathways using Pathway-Express, a package part of the Onto-Tools
444 (107). The significant pathways for predicting target genes were identified according to
445 the Kyoto Encyclopedia of Genes and Genomes (KEGG) database obtained using the
446 package clusterProfiler (104, 108). A minimal size of 40 genes of each geneSet was
447 used, and the significance threshold was defined by $p<0.05$. Although there was an
448 overlap on "Ribosome biogenesis in eukaryotes", the enrichment showed a different
449 directionality, overrepresented in Bumblebee, Termites, QMP, a mix of QMP and Brood
450 over-represented in Brood pheromone.
451 The pathway analysis was implemented using the Pathway-Express package
452 part of the Onto-Tools (107). Signalling pathways were identified using only differentially
453 expressed genes. The relationship between each gene was inferred based on their
454 KEGG pathway and magnitude of expression change (logFC,) and the visualisation of
455 significantly enriched pathways was performed using Igraph (102).

456 Cross-species comparison

457 To ensure pheromonal-induced changes in the fruit fly transcriptome are
458 comparable to eusocial insects, we compared the network conservation of eusocial
459 insects and fruit flies exposed to pheromone. Holman et al. (44) compared the whole
460 transcriptome of several species, including *Apis mellifera* and *Bombus terrestris,* treated
461 with either solvent-only control or their own species' queen pheromone. To determine

462 whether the QMP network signature was well conserved between *D. melanogaster*, *A.*
463 *mellifera* and *B. terrestris,* we used a WGCNA integrated function (modulePreservation)
464 (62) to calculate module preservation statistics. Orthologs between these two groups
465 were obtained using BLAST-based on the *D. melanogaster* genome. Holman et al. data
466 were obtained from BioProject DRP004516 (44). A total of 5507 unique *D.*
467 *melanogaster* orthologs were identified in *A. mellifera* and 2744 in *B. terrestris*. To
468 ensure that the conserved genes between the two data sets correspond to pheromone-
469 sensitive genes, we compared the differential expression between each QMP treatment
470 and control using exactTest function in edgeR (99). The threshold of significance was
471 defined by $P<0.05$. No publically available pheromone termite treatment data were
472 identified.
473
474 Evolutionary Origins
475 We performed a protein-based homology search across the tree of life to
476 determine the evolutionary age of pheromone-sensitive genes in *D.melanogaster* using
477 Phylostratr (109). We compared *D. melanogaster* gene sequences against 182 spp.
478 (NCBI proteins database) by the blastp algorithm at the e-value cutoff of 1e-03 and
479 selected the best match for pheromone-sensitive ortholog in each species. Following,
480 we compared the phylogenetic age of the pheromone-sensitive genes within each
481 treatment using the Transcriptome age index (TAI)(110, 111). To estimate TAI, we
482 retrieved the phylostrata (15 out of 27 phylostrata) of all genes that were differentially
483 expressed across all pheromone treatments (all pheromone-sensitive genes) and their
484 respective normalised expression. We used the pheromone-sensitive phylostrata to
485 reconstruct phylogeny using ape and ggtree (112–115).

486 Declarations

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Availability of Data & Materials

All code and compiled markdown file showing will be available on GitHub prior to publication https://github.com/marivelasque/eusociality_evolution. A permanent copy of the final analysis markdown file is available at XXX <DOI provided prior to final publication>. All raw sequencing data are available under the BioProject XXX <provided prior to final publication>.

Author Contributions

MV and JAD conceived the project, designed and performed the experiments, analysed the results and wrote the paper. YT, MV and AL prepared the sequencing libraries. All authors edited the manuscript and interpreted the results.

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Supporting Text & Materials

Materials & Methods

RNA Extraction.

A modified TRIzol RNA extraction protocol used for this work.

Materials

- 1 ml TRIzol
- 200 µl RNase-free chloroform
- 1 µl Glycogen
- 1 ml Isopropanol 100% w/w – chilled to -80°C
- 3 ml Ethanol 75% w/w – prepared fresh
- 500 µl Back Extraction Buffer (BEB)
- 20 µl Nuclease-free water

Sample homogenization and RNA extraction

1. Flash freeze samples (samples must be kept at -80oC) and transfer to dry ice (for processing) or -80oC freezer.
2. Add 500µL of TRIzol to each Eppendorf tube (keep samples in dry ice)
3. Homogenise sample with a pestle
4. Add 500µL of TRIzol
5. Mix by pipetting samples
6. Let it sit at room temperature for 5 min
7. Add 200µL of RNase-free chloroform (make sure it is a new vial or that it is only used on RNA work)
8. Shake vigorously by hand for 15s DO NOT VORTEX SAMPLES (vortex degrades the DNA)
9. Let it sit at room temperature for 3 min
10. Centrifuge at 4oC < 12,000g for 15 min
11. Transfer the upper aqueous phase to a new tube (leave 8-10µL in the bottom of the Eppendorf tube to prevent TRIzol contamination from getting into the next phase
12. Transfer the upper aqueous phase to a new tube (leave 8-10µL in the bottom of the tube to prevent TRIzol contamination from getting into the next phase. DO NOT DISCARD LOWER PHASE
13. Keep both upper and lower phases in separate tubes and proceed to 1- RNA and siRNA purification (upper aqueous phase) or 2- DNA purification (lower phase)

RNA and siRNA purification (upper aqueous phase)

1. Add 1µL of glycogen (can be skipped)
2. Add 500µL of RNase-free 100% isopropanol/ethanol (chilled to -80oC)
3. Invert by hand 10-20 times to mix (or very gently with the pipet)
4. Move samples to the fridge and let them rest overnight at -80 fridge
5. Centrifuge at 4oC < 12,000g for 10 min to precipitate the RNA (move samples very carefully not to agitate the mix, if disturbed, leave them overnight again).
6. Remove the supernatant and discard the rest.
7. Add 1µL of 75% Ethanol to pellet

8. Centrifuge at <7,500g for 5 min at 4oC
9. Remove supernatant and discard
10. Add 1µL of 75% Ethanol to pellet
11. Centrifuge at <7,500g for 5 min at 4oC
12. Remove supernatant and discard
13. Pulse spin samples at room temperature
14. Carefully remove the remaining supernatant with a pipette without disturbing the RNA pellet (use gel loading tips)
15. Air-dry the pellet at room temperature for 2-5 min to evaporate Ethanol
16. If necessary, heat tubes at 42°C to evaporate remaining Ethanol
17. Add 20µL of nuclease-free water to the pellet (over-dried RNA might take longer to dissolve)
18. Gentle mix the sample to solubilise the RNA
19. Place the solubilised RNA on ice immediately
20. Aliquot 8µL of the sample for quantification and cDNA synthesis (keep the remaining 12µL in -80oC)
21. Quantify RNA concentration and purity.

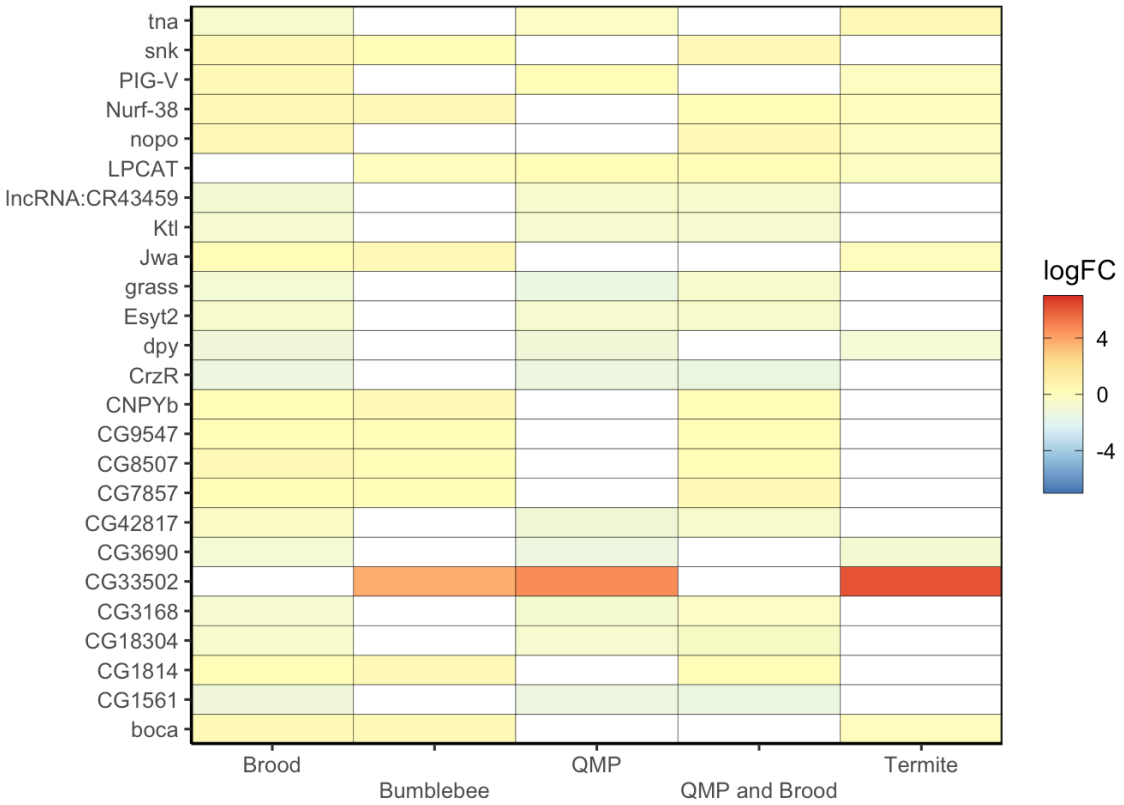

Figure S1. Log_2–fold change heatmap of the top 25 differentially expressed genes. Differential gene expression was determined using deseq. Both up- and down-regulated genes were considered. The colour scale reflects the fold change in gene expression in pheromone compared to the controls, ranging from down-regulated (blue) to up-regulated (red). Threshold of significance was defined as $p<0.05$.

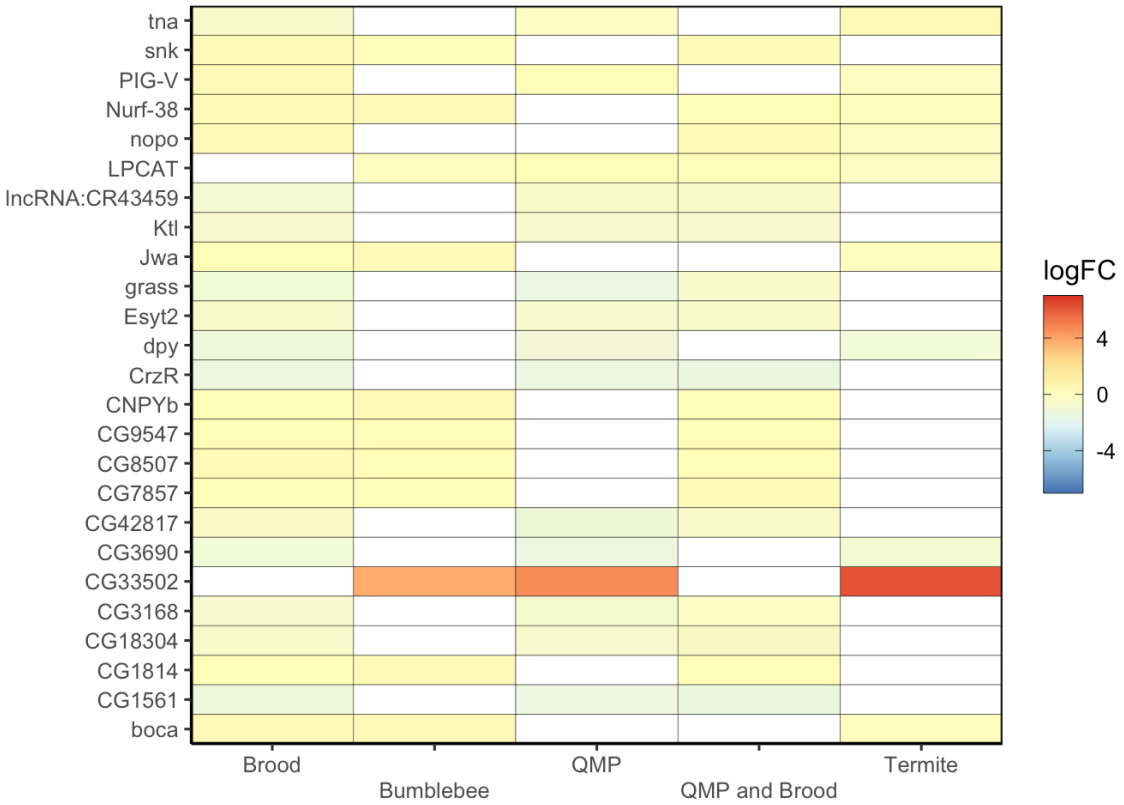

Figure S2. Log_2–fold change heatmap of the top 25 differentially expressed genes. Differential gene expression was determined using WGCNA. Both up- and down-regulated genes were considered. The colour scale reflects the fold change in gene expression in pheromone compared to the controls, ranging from down-regulated (blue) to up-regulated (red). Threshold of significance was defined as $p<0.05$.

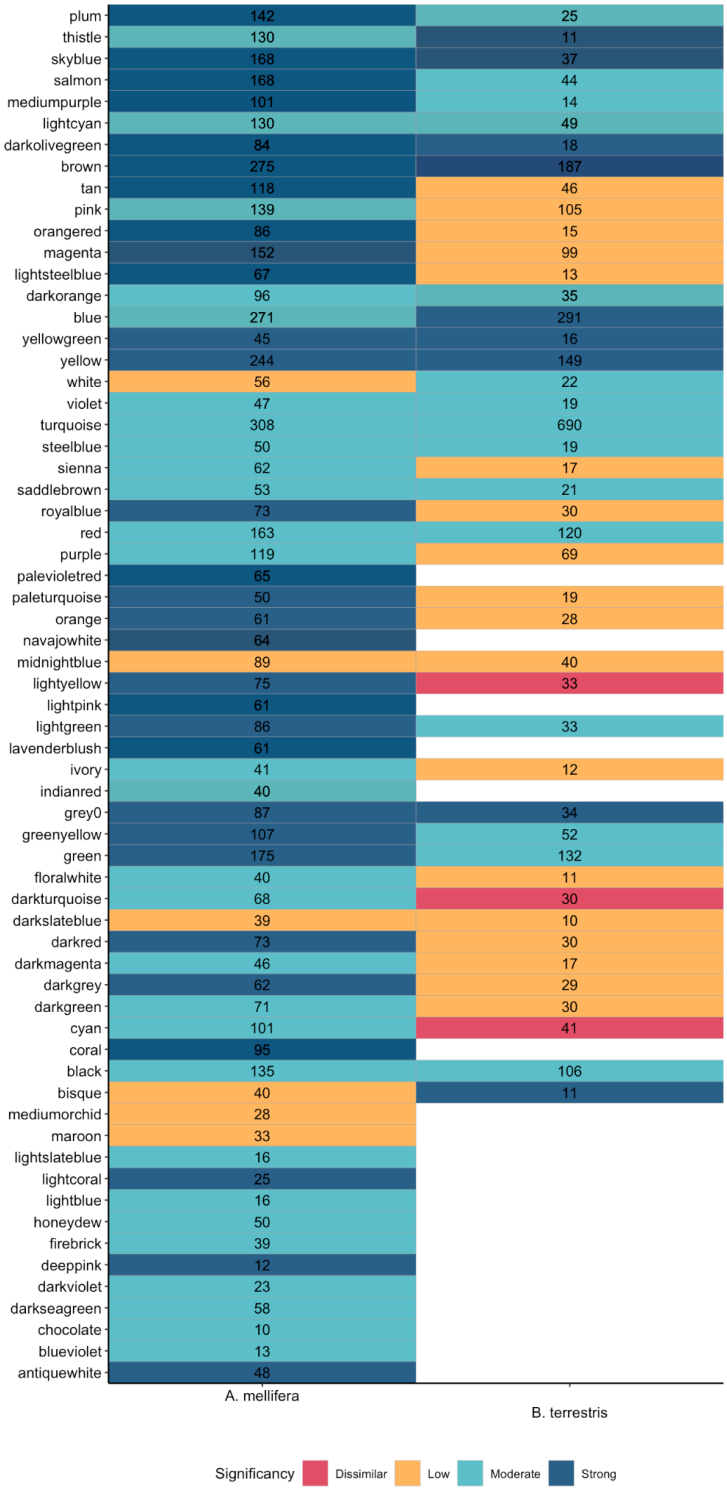

Figure S3. All pheromone-responsive modules preserved in cross-species comparison between *Drosophila melanogaster* and *Apis mellifera* or *Bombus terrestris.* The WGCNA module-preservation function facilitated comparisons between *Drosophila melanogaster* and *Apis mellifera* pheromone-response to *A. mellifera* queen pheromone, and *D. melanogaster* and *Bombus terrestris* pheromone-response to *B. terrestris* queen pheromone. Number indicates the number of genes associated with each module.

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853 **Table S1**. ***Drosophila melanogaster* ovary size by pheromone treatment.** Flies were exposed to
854 treatment pheromone from egg until collection. Virgin female flies were collected and dissected five days
855 after emergence. SD denotes standard deviation.

Treatment	Replicates	Mean size (mm^2)	SD
Bumblebee	10	0.32	0.064
Termite	10	0.35	0.145
QMP	10	0.51	0.085
QMP and Brood	10	0.59	0.156
Brood	10	0.65	0.123
Control (2nd)	10	0.80	0.146
Control (1st)	10	0.88	0.136

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858 **Table S2**. **Multiple Comparisons of Means: Tukey Contrasts for ovary sizes of each pheromone**
859 **treatment.** Highlighted comparisons are statistically significantly different. All treatments are significantly
860 different to the controls. The controls are not significantly different.

Group 1	Group 2	Estimate	p.Val
Termite	Bumblebee	0.027	1.000000
QMP	Bumblebee	0.189	0.014997
QMP and Brood	Bumblebee	0.270	0.000026
Brood	Bumblebee	0.328	0.000000
Control (2nd)	Bumblebee	0.483	0.000000
QMP	Termite	0.162	0.076808
QMP and Brood	Termite	0.244	0.000262
Brood	Termite	0.301	0.000002
Control (2nd)	Termite	0.456	0.000000
QMP and Brood	QMP	0.082	1.000000
Brood	QMP	0.139	0.262967
Control (1st)	QMP	0.371	0.000000
Brood	QMP and Brood	0.058	1.000000
Control (1st)	QMP and Brood	0.289	0.000005
Control (1st)	Brood	0.231	0.000823
Control (1st)	Control (2nd)	0.076	1.000000

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