1 **Evolutionary constraints shape caste specific gene expression across 15 ant**

2 **species**

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4 **Running title**: Evolution of caste specific gene expression

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43 **Data availability**

44 The raw reads of the transcriptomes are publicly available in the DNA Data Bank of

45 Japan under bioproject ID PRJDB4088, sample accession numbers ID

46 SAMD00035735-SAMD00035834 (Morandin et al. 2016). All transcriptome

47 assemblies can be found on Fourmidable (Wurm et al. 2009). All data used in this

48 study can be found in Table S1.

49

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Abstract

Development of polymorphic phenotypes from similar genomes requires gene expression differences. However, little is known about how morph-specific gene expression patterns vary on a broad phylogenetic scale. We hypothesize that evolution of morph specific gene expression, and consequently morph specific phenotypic evolution, may be constrained by gene essentiality and the amount of pleiotropic constraints. Here, we use comparative transcriptomics of queen and worker morphs, i.e. castes, from 15 ant species to understand the constraints of morph-biased gene expression. In particular, we investigate how measures of evolutionary constraints at the sequence level (expression level, connectivity, and number of gene ontology terms) correlate with morph-biased expression. Our results show that genes indeed vary in their potential to become morph-biased. The existence of genes that are constrained in becoming caste-biased potentially limits the evolutionary decoupling of the caste phenotypes, i.e. it might result in 'caste load' occasioning from antagonistic fitness variation, similarly to sexually antagonistic fitness variation between males and females. On the other hand, we suggest that genes under low constraints are released from antagonistic variation and thus more likely to be co-opted for morph specific use. Overall, our results suggest that the factors that affect sequence evolutionary rates and evolution of plastic expression may largely overlap.

Introduction

Gene expression differences are the key to polyphenic development, such as environmentally determined sex specific phenotypes (e.g. fish (Manousaki et al. 2014), reptiles (Yatsu et al. 2016)), feeding morphs (e.g. toad tadpoles (Leichty et al. 2012)) dispersal morphs (e.g. aphids (Braendle et al. 2006)), or social insect female castes (Wilson 1971)). Given that polyphenic phenotypes are built from similar sets of genes, with the exception of sex chromosomes, differential gene expression is necessary for selection to work in a morph specific manner (Evans and Wheeler 1999; Pereboom et al. 2005; Sumner et al. 2006; Parsch and Ellegren 2013; Perry et al. 2014). Thus, understanding the features of genes that are expressed in a morph-specific manner (hereafter "morph-biased genes") is crucial for understanding evolution of polymorphism.

The increased availability of transcriptomic data has revealed the dual nature of morph-biased genes (Mikheyev and Linksvayer 2015). On the one hand, morph-biased genes are disproportionately likely to be genes that evolve fast, either under relaxed purifying selection (Hunt et al. 2010, 2011; Leichty et al. 2012; Helanterä and Uller 2014; Schrader et al. 2016), or positive selection (Devlin and Nagahama 2002; Guidugli et al. 2005; Ellegren and Parsch 2007; Mank and Ellegren 2009; Harpur et al. 2014; Yang et al. 2016). Fast evolution of morph-biased genes is also suggested by overrepresentation of taxonomically restricted genes in morph-biased genes, especially in social insects (Ferreira et al. 2013; Feldmeyer et al. 2014; Jasper et al. 2014; Sumner 2014). On the other hand, in line with the genetic toolkit hypothesis, genes with conserved functions are thought to represent the shared genomic basis of polyphenism; comparative studies suggest that such functions include core

101 physiological processes such as metabolism and reproduction (Swain and Lovell-

102 badge 1999; Nef et al. 2005; Toth and Robinson 2007; Toth et al. 2010; Fischman et

103 al. 2011; Woodard et al. 2011; Berens et al. 2014). In general, genes with conserved

104 or essential function (Richards et al. 2005; Toth and Robinson 2007; Carroll 2008;

105 Jovelin and Phillips 2009; Bergmiller et al. 2012), or under high pleiotropic

106 constraints due to either ubiquitous expression across tissues (McShea 2000; Mank et

107 al. 2008; Mank 2009; Meisel 2011) or due to high number of interactions with other

108 genes (i.e. high connectivity) (Provero et al. 2002; Carlson et al. 2006; Jasper et al.

109 2014), or displaying a large number of gene ontology (GO) terms (Choi et al. 2007;

110 Jovelin and Phillips 2009) are likely to be constrained in how readily they can be

111 expressed in a morph-biased manner, as their stable expression is necessary in both

112 morphs (Meisel 2011; Purandare et al. 2014).

113

114 Importantly, such constraints may hinder morph specific evolution, resulting in

115 antagonistic fitness variation, or genetic load. In other words, if selection pressures

116 are different in the different morphs for any given gene, selection cannot reach the

117 optimum for either caste, unless expression of the gene is caste specific (i.e. the gene

118 is downregulated in the morph where the its expression has negative effects). Such

119 constraints on the ability of the genome to respond adaptively to the divergent

120 selective pressures on the two morphs simultaneously have mainly been discussed

121 with respect to sexually antagonistic variation and sex biased gene expression (Rice

122 1998; Ellegren and Parsch 2007; Innocenti and Morrow 2010; Holman 2014) but the

123 same logic applies to castes in social insects, where "caste load" could potentially

124 ensue for genes whose caste specific expression evolution is constrained (Holman

125 2014). These constraints are the direct consequences of antagonistic selective

126 pressures for the caste phenotypes and genetic covariance between the reproductive

127 queens and the non-reproductive workers (Holman 2014).

128

129 In order to understand the constraints and flexibility of morph-biased expression

130 across species, and the potential for genetic load resulting from constraints on

131 expression evolution, comparisons of gene expression in several species that share the

132 same polyphenism are needed (Helanterä and Uller 2014). Although numbers of

133 species compared in studies to date are usually small, flexibility of morph-biased

134 expression patterns among species seems to be a general pattern, and only a very

135 small proportion of genes linked to conserved functions maintain a biased expression

136 pattern across species (Meiklejohn et al. 2003; Ellegren and Parsch 2007; Toth and

137 Robinson 2007; Toth et al. 2010; Fischman et al. 2011; Woodard et al. 2011; Wilkins

138 2013). This suggests that genes whose expression level and sequence are under

139 relatively weak purifying selection, are more likely to be expressed in a morph-biased

140 manner in any particular species, and ultimately even evolve in a morph-specific way

141 under positive selection (Helanterä and Uller 2014).

142

143 Caste differences in eusocial insects, such as ants, are one of the key model systems

144 for studying evolution of morph-biased gene expression (Gadau et al. 2011; Libbrecht

145 et al. 2013; Helanterä and Uller 2014). Originating from a common ancestor ca. 140–

146 168 Ma ago (Gadau et al. 2011), the reproductive division of labor between queen and

147 worker castes has facilitated enormous diversification especially in the worker castes

148 of ants, and dominance of many terrestrial ecosystems. In general, caste

149 differentiation is driven by early environmental differences, and queens and workers

150 arise from similar genetic backgrounds (Wilson 1971; Schwander et al. 2010).

Recently, a 16 species comparison of queen and worker transcriptomes covering the
major ant subfamilies showed that caste-biased expression patterns are highly variable
across species (Morandin et al. 2016). In this study, we investigate the determinants
of the variation in caste biases, and the potential for genetic load ("caste load",
Holman 2014) resulting from constraints on caste-biased expression. We use three
measures of evolutionary constraint. The first one, average expression level, has been
shown to correlate with slow sequence evolution (Pál et al. 2001; Drummond et al.
2005a,b) and gene essentiality (Drummond et al. 2005b; Clark et al. 2007), i.e. the
severity of the harmful effects of gene deletion on fitness (Chen et al. 2012). Second,
connectivity of a gene in a co-expression network measures the number of
interactions a gene has with other genes (Provero et al. 2002). It is thus a correlate of
slow sequence evolution due to pleiotropic constraint, where the evolution of a
sequence is limited by its multiple roles. Third, the number of Gene Ontology (GO)
terms is similarly a measure of genes potentially having many biological functions,
and slow sequence evolution due to pleiotropic constraint.

More specifically, we will test six predictions. First, if the same features that correlate
with conserved sequence evolution also constrain caste-biased expression, we predict
that genes with high expression level, high connectivity and high number of GO terms
should have on average little caste-bias. Second, if these same features constrain caste
specific expression in any given species, we predict that they should be associated
with the correlation of expression levels among castes within species. Third, if these
same features constrain fast changes in expression patterns, the genes with high
expression level, high connectivity and high number of GO terms should show little
evolutionary flexibility in their expression patterns. Fourth, if constraints in the

176 evolution of gene expression patterns apply similarly to expression in both castes, and

177 thus produce "caste load", we predict that expression variability across species should

178 show similar patterns in both queen and worker specific expression. Fifth, given that

179 worker phenotypes, and the selective pressures they face, have across the ant

180 phylogeny diverged more than queen phenotypes, we predict that caste specific

181 expression patterns should be more variable among species in workers. Finally, we

182 predict that genes showing little evolutionary flexibility in their expression patterns

183 would disproportionately include genes involved in conserved functions, such as

184 metabolism or development.

185

186 **Materials and Methods**

187 **Gene expression analysis.** In a previous study, we sequenced the transcriptomes of

188 queens and workers of 16 ant species from three subfamilies, which differed in a

189 variety of key traits (Figure 1) using Illumina Hi-Seq technology (Morandin et al.

190 2016). One species (*Lasius turcicus*) was not used in this study due to the lack of

191 queen samples. Three independent replicates for queens and workers, using whole-

192 body, were used in this study and variable number of samples were pooled per species

193 to obtain sufficient amount of RNA (Table S2). In total, we sequenced 100 libraries

194 from whole-body queen and worker samples, representing biological replicates of

195 each caste. In the absence of a reference genome for most species, we used a *de novo*

196 assembly methods to construct reference transcriptomes for each species separately

197 for use in read mapping for gene expression profiling using Trinity software (release

198 2013-02-25, default settings (Haas et al. 2013)). Paired-end reads were mapped to the

199 *de novo* transcriptomes using RSEM (Li and Dewey 2011) to obtain raw expression

200 values that were subsequently used in the Generalised Linear Models below.

201

Weighted Gene Co-expression network to estimate Gene Connectivity. Weighted

gene co-expression networks were constructed on the entire gene set using the mean

of normalized expression counts for each gene. A total of 9,859 gene expression data

were initially analyzed with the WGCNA package (Langfelder and Horvath 2008).

The input dataset consists of a table with each row representing one of the 9,859 gene

and each column one of the 100 samples (Table S2). Prior to the analysis, genes with

too many missing samples or zero variance were removed from the calculation as they

may affect our ability to detect gene co-expression. After this cleaning step, we

calculated the connectivity between each gene using the *softConnectivity* function

from the package WGCNA. More details on sample collection, cDNA synthesis,

library preparation, and weighted gene co-expression network analysis are described

by Morandin et al. (2016).

214

Influence of constraints on the caste-biased expression patterns. To test our first

hypothesis that genes with high expression level, high connectivity and high number

of GO terms are less likely to show caste-biased gene expression patterns on average,

we investigated the correlates of biased expression level using generalized linear

model (GLM) analysis (R Core Team 2012, version 3.1.1, glm function in the

package *stats*). First of all, in addition to the WGCNA cleaning steps described above,

genes with expression data from less than nine species were omitted from our study as

they would weaken our ability to detect a phylogenetic signal. Then we used each

gene as a sample ($n = 6608$), and quantified caste-bias for each gene with a non-

directional measure of the lower expression level (either queen or worker) divided by

the higher, using the average expression value across pools for each caste. This

226   produced a range of expression ratio values ranging from 0 to 1, where 0 indicates

227   extreme caste-bias with no expression in one caste and 1 represents equal expression

228   levels in the two castes. This value represents the average caste-bias in expression

229   level of each gene and was set as the response variable. As explanatory variables, we

230   used putative correlates of evolutionary constraints (obtained from Morandin et al.

231   2016), i.e. expression levels (a correlate of gene essentiality (Pál et al. 2003; Rocha

232   and Danchin 2004), shown to correlate with evolutionary rates in an earlier analysis,

233   Morandin et al. 2016), gene connectivity (a measure of pleiotropic constraint, shown

234   to correlate with evolutionary rates in Morandin et al. 2016) and the number of GO

235   terms (a potential correlate of pleiotropic constraint). All variables were log-

236   transformed to normalize the distribution of residuals before processing. In order to

237   find the best fitting model, we used the function *step* from the R software (R

238   developmental Core Team 2008) and the MASS package (Venables and Ripley 2002)

239   to choose a model using Akaike information criterion (AIC) with a stepwise

240   algorithm using the default values. Similar processes were used for all GLM analyseis

241   below.

242

243   **Influence of constraints on the correlation in gene expression between queen and**

244   **worker**. Second, we tested the hypothesis that genes with high expression level, high

245   connectivity, and high numbers of GO terms would tend to be similar in their

246   expression among castes within species. The correlation of expression among castes

247   (similar to C_{mf} used in Dean and Mank 2016 for analyzing sex biased expression) for

248   each gene was calculated using the average queen and average worker gene

249   expression across all fifteen species. Only genes where expression levels were

250   available for both queen and worker samples within a species were used in this

251 analysis. The correlation coefficient for each gene was used as a response variable in

252 a GLM with gene connectivity, expression level and number of GO terms set as the

253 explanatory variables. Model selection was carried out as described above, and to

254 normalize the distribution of model residuals, the correlations of caste gene

255 expression were transformed prior to the analysis using the 'powerTransformation'

256 function with Yeo-Johnson family in the 'car' library (Fox and Weisberg 2011) and a

257 subsequent Yeo-Johnson transformation using the 'VGAM' library (Yee 2016).

258

259 **Influence of constraints on the evolutionary flexibility of gene expression**

260 **patterns.** Third, we tested the hypothesis that genes with high expression level, high

261 connectivity, and high numbers of GO terms would vary little in their expression

262 values among closely related species. To do so, we investigated the influence of these

263 correlates of evolutionary constraints i.e. expression levels, gene connectivity and the

264 number of GO terms on the phylogenetic signal in expression levels using GLM

265 analysis. Phylogenetic signal is defined by Blomberg and Garland (2002) as the

266 "tendency of related species to resemble each other more than species drawn at

267 random from the same tree". We used the Blomberg's K values (Blomberg et al.

268 2003) to quantify the phylogenetic signal of expression levels across species with the

269 function phylosig from the R package *phytools* (Revell 2012), based on the phylogeny

270 in Morandin et al. (2016). The Blomberg's test was chosen as it takes the

271 phylogenetic tree branch length into consideration in the analysis. Blomberg's K

272 values close to 0 indicates phylogenetic independence, while high values indicate a

273 relationship between the phylogeny and the studied trait (Münkemüller et al. 2012).

274 Blomberg's K values, calculated separately for expression level in queens and

275 workers, were set as the response variables in two separate GLM analyses, and gene

connectivity, number of GO terms, expression levels, and coefficient of variation in expression among species were set as explanatory variables. The coefficient of variation in expression level across species for each gene was added as an additional explanatory variable to investigate the effect of overall variability on the phylogenetic signal (Blomberg's K). For example, an extremely conserved gene could show zero variation in its expression level, and consequently would not show any phylogenetic signal either. The GLM analyses were conducted independently for expression levels in queens and workers as the response variable. Transformations and model selection were carried out as described above.

Analysis of potential caste load and caste differences in expression variability. If the evolution of expression levels is similarly limited by general evolutionary constraints in both castes, queens and workers should show correlated values of the phylogenetic signals (Blomberg's K) and the overall levels of variation (measured as coefficient of variation over species). We tested this prediction using Spearman's rank correlation (R Core Team 2012, version 3.1.1. Because queens have a similar reproductive function across species, while workers perform a comparatively more diverse range of non-reproductive tasks, and face variable environments, in our sample of species, we predicted that queen expression patterns would show stronger phylogenetic signal than worker expression patterns (higher Blomberg's K values), i.e. that caste specific expression patterns should be more variable among closely related species in workers. Blomberg's K values for each gene (one value per gene calculated using all species) were calculated separately using expression level in queens and workers, and were compared between queen and worker expression patterns using a paired t-test.

Analysis of GO term enrichment for genes displaying a significant phylogenetic

signal. Finally, we used GO term enrichment analysis to describe the functions of the

genes with significant phylogenetic signals ($p < 0.05$) to test the prediction that genes

with strong phylogenetic signals (high Blomberg's K values) have conserved

functions. Gene ontology (GO) terms for all genes were determined using

BLAST2GO (using BLASTp with an e-value cut-off $\leq 10^{-3}$) (Conesa et al. 2005).

Additionally, the GOstats package for R (Beissbarth and Speed 2004) was used to

conduct GO term enrichment analysis on genes presenting a significant phylogenetic

signal for queen or worker expression patterns ($p < 0.05$), as well as genes having a

phylogenetic signal common to both castes. We used the set of all genes for which

GO terms were available as the universe. Enriched GO terms (FDR < 0.05) were

subsequently clustered using Revigo (Supek et al. 2011) to avoid redundancy.

Results

Influences of constraints on the caste-biased expression patterns. After quality

filtering, 6608 genes comprising expression data from at least nine of the studied

species were retained for further analysis (mean number of species = 14, s.d. = 1.4).

The average caste biases for these genes are summarized in Figure S1, and the

frequency distribution of genes with significant expression bias for a given number of

species is given in Figure S2, showing that a majority of genes are never, or only very

rarely caste biased.

As predicted, genes with high average expression levels showed relatively little

average caste-bias (i.e. these genes had higher expression ratios, GLM, $p < 0.001$;

Table 1, Figure S3). However, and contrary to our initial predictions, neither

326   connectivity, nor the number of GO terms (Provero et al. 2002; Carlson et al. 2006;

327   Choi et al. 2007; Jovelin and Phillips 2009), had any significant relationship with the

328   ability of a gene to display a caste-biased expression patterns (Connectivity, GLM, p

329   = 0.65; Number of GO terms, GLM, p = 0.07, Table 1).

330

331   **Influence of constraints on the correlation of gene expression patterns among**

332   **castes**. According to our expectations, genes with high expression levels and also

333   genes that were highly connected showed a positive association with the correlation in

334   gene expression between queen and worker across the broad phylogenetic scale

335   (GLM, $p < 0.001$; Table 2 and Figure S4). However, there was also a negative

336   interaction among expression level and connectivity, such that the positive overall

337   effect of connectivity was the smaller, the higher the expression value of the gene was

338   (GLM, $p < 0.001$; Figure S4). The correlation between sequence evolutionary rates

339   (*dN/dS*, as calculated for each gene in Morandin et al 2016) and the among caste

340   correlation in expression was weakly negative ($p < 0.001$; $r_s = -0.11$; Figure S5,

341   supporting our interpretation that conserved genes are less likely to become caste

342   biased (Figure S2).

343

344   **Influence of constraints on evolutionary flexibility of gene expression patterns.**

345   Contrary to our expectations, genes with high expression levels had low Blomberg's

346   K values (e.g. low phylogenetic signals) (GLM, $p < 0.001$; Table 3, i.e. their

347   expression levels tended to be highly variable among closely related species).

348   However, highly connected genes showed high Blomberg's K values (i.e. low

349   variation among closely related species) for both castes as predicted (GLM, $p < 0.001$;

350   Table 3), especially if the genes also had high average expression (GLM, $p < 0.001$;

Table 3, Figure 2a and b). Finally, the coefficient of variation was negatively correlated with the Blomberg's K values for both castes (GLM, $p < 0.001$; Table 3) showing that high phylogenetic signals are not just artefacts of high variability overall. The number of GO terms was not correlated with the Blomberg's K values in either caste ($p = 0.50$, Table 3). Against our predictions, the Blomberg's K values were not correlated with evolutionary rates (dN/dS) (Worker, $p = 0.08$; Queen, $p = 0.77$), however.

Analysis of potential caste load and caste differences in expression variability.
A total of 21 % of the genes showed a significant phylogenetic signal (Blomberg's K, $p < 0.05$) for worker expression patterns (1379 genes), and 25 % for the queen expression patterns (1636 genes). Among these genes, 754 genes (11 %) displayed a significant phylogenetic signal for both queens and workers simultaneously, which is significantly higher than expected if Blomberg's K values in queens and workers were independent from each other (Fisher's test, $p < 0.001$). As predicted, queen and worker Blomberg's K values were positively correlated (Spearman correlation, $r_s = 0.49$, $n = 6608$, $p < 0.001$; Figure 3a), suggesting that for most genes, expression patterns do not change independently between queens and workers in a species-specific manner across the ant phylogeny. A similar pattern was also found for the coefficient of variation (Spearman correlation, $r_s = 0.72$, $n = 6608$, $p < 0.001$; Figure 3b), suggesting that genes that are highly variable in expression levels across species are likely to be variable for both queens and workers. As we further predicted, the expression patterns in queens had stronger phylogenetic signals (higher Blomberg's K values) than expression patterns in workers (mean $_{worker} = 0.39$, s.d. $_{worker} = 0.50$, mean $_{queen} = 0.45$, s.d. $_{queen} = 0.63$, paired student t-test, $n = 6608$, $p < 0.001$, Figure S6,

376 Table S3), indicating that expression levels in queens are more likely to be similar

377 among closely related species than those in workers.

378

379 **Analysis of GO term enrichment for genes displaying a significant phylogenetic**

380 **signal.** Genes showing a significant phylogenetic signal in workers were enriched for

381 GO terms linked to "Mitotic DNA Damage Checkpoint" and "Metabolic Processes".

382 For queens, terms such as "Metabolic Processes" and "Gene Expression Processes"

383 were enriched. Finally, genes that showed a phylogenetic signal in expression pattern

384 for both castes were enriched in terms related to "Development" and "Meiosis". A

385 simplified figure of the GO terms and the complete list of enriched terms can be

386 found in the additional materials Figure S7 and Table S4.

387

388 **Discussion**

389 This study shows that not all genes are equally likely to be expressed in a morph-

390 biased manner in the queen-worker caste polyphenism of ants. Instead, features like

391 gene connectivity and average expression levels explain variation and average of

392 caste-biased expression, respectively, suggesting potential evolutionary constraints on

393 evolution of expression patterns. Patterns of variation in queen and worker

394 expression were correlated, suggesting that constraints of expression patterns in the

395 two castes are tightly associated in many genes, possibly leading to "caste load" that

396 limits independent evolution of the two morphs (Holman 2014). Our results highlight

397 the importance of analysing evolutionary constraints, such as those arising from gene

398 network context and conserved function, on the evolution of morph-biased gene

399 expression, and ultimately the decoupled evolution of polyphenic phenotypes.

400

Influences of constraints on the caste-biased expression patterns. The finding that

highly expressed genes are less likely to be caste-biased when averaged across species

fits our general argument about the shared factors constraining sequence and

expression evolution. Levels of expression are consistently negatively correlated with

rates of gene sequence evolution in multiple organisms, including the set of ant

species studied here (Krylov et al. 2003; Hahn and Kern 2005; Jovelin and Phillips

2009; Mikheyev and Linksvayer 2015; Morandin et al. 2016). Thus, evolutionary

constraints that play a role in sequence evolution are also likely to affect the evolution

of morph-biased expression patterns, and weakly constrained genes are likely to be

more easily co-opted for morph-specific use (Hunt et al. 2011; Leichty et al. 2012;

Helanterä and Uller 2014).

Against our predictions, gene connectivity did not significantly correlate with the

average tendency of a gene to be expressed in a caste-biased manner, even if

connectivity has been shown to correlate with pleiotropic interactions (Promislow

2004; Camps et al. 2007; Razeto-barry et al. 2011) and slow sequence evolution

(Hahn and Kern 2005; Jovelin and Phillips 2009; Mikheyev and Linksvayer 2015;

Morandin et al. 2016) in many studies. Since patterns of caste-bias are shared among

species for only a small minority of genes (this study, Berens et al. 2014; Feldmeyer

et al. 2014; Morandin et al. 2015, 2016), it seems that measuring average caste-bias

over species across the phylogeny is likely to conceal many of the interesting patterns.

Indeed, the correlation between connectivity and lack of caste bias was demonstrated

in the analysis where the correlation among castes within each species was

investigated. This suggests that highly connected genes, that are presumable highly

426   pleiotropically constrained, are less likely to vary among castes within species, but

427   their expression levels are coupled within species. This, together with positive

428   correlation among expression levels and the correlation among castes within species

429   suggests that understanding caste biases needs to take into account both the functional

430   context of the genes, and their variation across species (Morandin et al. 2016).

431

432   **Influence of constraints on evolutionary flexibility of gene expression patterns.**

433   Highly connected genes were more likely to have high phylogenetic signal in their

434   expression variation, i.e. tended to vary little among closely related species. The

435   observed positive statistical interactions suggest that this effect was particularly high

436   for genes with high average expression, even if gene expression level alone was

437   negatively correlated with the phylogenetic signals. In this context connectivity can

438   be seen as a measure of pleiotropic constraint (Hahn and Kern 2005; Kim et al. 2007);

439   for example, Carlson et al. (2006) found a relationship between gene connectivity and

440   sequence conservation in yeast. Also in previous studies looking at caste-biased gene

441   evolution in social insects, including earlier analyses of this same data set (Mikheyev

442   and Linksvayer 2015; Morandin et al. 2016), connectivity has been shown to be

443   negatively correlated with evolutionary rates. Similarly, high expression levels have

444   been shown to correlate with slow sequence evolution in this data set and others (Pál

445   et al. 2001; Drummond et al. 2005a; Morandin et al. 2016), and the correlation

446   between gene essentiality and high expression level in model organisms lends further

447   support to the use of expression level as a proxy for evolutionary constraint (Fraser et

448   al. 2004). Thus overall, these patterns suggest that factors such as connectivity, and to

449   a smaller effect also expression level, do affect both sequence evolutionary rates and

450   evolution of expression levels. For instance, stabilizing selection may maintain both

stable gene expression levels and sequence evolutionary rates across phylogeny

(Lemos et al. 2005; Laarits et al. 2016). However, causes and consequences are

difficult to tease apart, given the likely evolutionary (adaptive and neutral) feedbacks

between evolutionary rates, expression bias, average expression, and connectivity

(Helanterä and Uller 2014).

Analysis of potential caste load and caste differences in expression variability.

We found a strong positive correlation between phylogenetic signals and overall

variability observed in queen and worker gene expression patterns, suggesting that

genes with conserved expression pattern in both castes are unlikely to evolve caste

specific expression patterns. This result reinforces the concept of a "caste load"

suggested by Holman (2014), and the principle of morph antagonistic fitness variation

(Rice and Chippindale 2001; Morrow et al. 2008; Innocenti and Morrow 2010) and

antagonistic fitness variation in general (Immler et al. 2011). That is, genes whose

expression pattern is conserved in one caste, also typically retain a stable expression

pattern for both castes across the ant phylogeny due to e.g. pleiotropic constraint or

conserved function, impeding decoupling of caste phenotypes through differential

expression. We suggest that analysis of phylogenetic signals is a valuable tool for

understanding antagonistic fitness variation (see e.g. Dean and Mank 2016), and

should become more widely used as more and more phylogenetically informative

datasets become available. To date, antagonistic fitness variation has been thoroughly

assessed in relation to sex antagonistic variation and gender load, but we stress that it

is predicted to be a pervasive phenomenon applying to any polymorphism (Van

Dyken and Wade 2010; Snell-Rood et al. 2011; Holman 2014).

Queen-worker differences in phylogenetic stability of expression pattern. Lower phylogenetic signal in gene expression patterns was found when looking at expression patterns in workers, compared to queens. This result is following our expectations because worker morphology, physiology and behavior have diverged more rapidly than those of queens (e.g. evolution of several worker castes (Wheeler 1991)). Moreover, queens have similar reproductive function across species and experience less fluctuating environmental conditions due to the buffering provided by the nest surroundings and the worker force. Furthermore, while workers undertake various tasks within the colony, they also experience environmental changes faster than the queens, which live in relatively well-buffered environments for most of their lives. Thus, the higher variation in workers could reflect the effect of diverse environments (e.g. *Formica* in diverse environments in the boreal zone, and *Monomorium chinense* in the subtropical zone) on expression patterns being more pronounced in workers than queens. Within a genus the environment may also dramatically vary (e.g. *Formica aquilonia* in closed forest and *Formica cinerea* on open sand dunes).

Functional annotation of genes displaying a phylogenetic signal showed that gene sets with conserved expression patterns were enriched in genes with metabolic and developmental process functions, suggesting it is these genes that underlie large parts of the possible caste load. Genes involved in these processes are also predominantly under the control of purifying selection, showing low *dN/dS* ratios across species (e.g. yeast (Hahn and Kern 2005; Vitkup et al. 2006; Lu et al. 2007); mammals (Vinogradov 2015)). This finding is thus also in line with our assertion that factors correlated to conservation at the sequence level are correlated with conservation at the gene expression patterns as well. Our study is the first, to our knowledge, to study

501   conserved expression patterns in particular functional sets of genes across such a large

502   number of species. Metabolic and developmental processes include many conserved

503   functions making it possible that genes with such functions have universally relatively

504   stable expression patterns.

505

506   We used phylogenetic signal as a proxy of how likely a gene is to show variable

507   expression patterns among closely related species, thus reflecting the ease with which

508   expression patterns change. Given that a phylogenetic signal possibly reflects

509   outcomes of several different evolutionary processes, i.e. that variation in the

510   phylogenetic signal can emerge from variation in e.g. mutation rates and type or

511   strength of selection, (e.g. Revell et al. 2008; Kamilar et al. 2013), we have to remain

512   cautious in inferring the kind of evolutionary processes underlying variable

513   expression patterns, especially when we observed no correlation between

514   evolutionary rates and phylogenetic signals in expression levels. In other words, not

515   all genes showing low phylogenetic signal are necessarily ones where expression

516   patterns are evolving under strong positive or divergent selection, or unconstrained.

517   Such caution is especially important given the lack of a clear neutral baseline

518   expectation (comparable to neutral expectation in sequence evolution) for expression

519   evolution (Helanterä and Uller 2014), and our modest understanding of the prevalent

520   evolutionary processes underlying expression evolution (Khaitovich et al. 2006;

521   Warnefors and Kaessmann 2013; Rohlfs et al. 2014; Hodgins-Davis et al. 2015),

522   including the role of neutral drift in expression evolution. Nevertheless, the observed

523   associations of connectivity, expression levels, total variation in expression levels,

524   and phylogenetic signals give us some confidence in interpreting low phylogenetic

525   signal as a measure of how flexible expression patterns of a given gene are.

Evolution of caste biased gene expression in social insects has interesting similarities

to evolution of tissue-specific gene expression. Indeed, if an insect society is seen as a

superorganism, queens and workers are analogous to reproductive and somatic

tissues, respectively (Helanterä 2016). As with social insect castes, it seems that genes

with a tissue-specific expression profile are prone to fast evolution due to low

pleiotropic constraint (Duret et al. 1999), but it remains an open question whether

genes with little constraint have been co-opted into tissue-specific expression pattern

or whether the evolutionary rates have increased after the expression patterns have

changed. Tissue specificity is also an important determinant of morph biased

expression, as genes with high specificity are more likely expressed in a sex biased

manner (Yang et al. 2006; Ellegren and Parsch 2007; Dean and Mank 2016).

Conclusion. Our study broadly suggests that constrained genes are less likely to be

involved in caste-biased expression. Conversely, genes evolving under lower

constraint are also likely to evolve morph-biased expression, and especially variable

morph-biased expression. Furthermore, relatively unconstrained genes are likely to

have expression patterns that are decoupled in the two castes, whereas constrained

genes are likely to share their expression patterns between castes. In other words,

genes that tend to operate under relatively little evolutionary constraints are more

likely to exhibit caste-biased expression patterns, and thus be co-opted for caste

specific use, in turn potentially leading to positive morph specific selection that may

resolve antagonistic selection pressures. Our results emphasize that large-scale

phylogenetic comparisons of morph-specific expression patterns and their correlates

550 are an essential tool in eventually understanding the roles of adaptive and non-

551 adaptive processes in polyphenic evolution.

552

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832   **Figures**

833   **Figure 1. Phylogenetic relationships of 15 ant species** (including two social forms

834   of *Solenopsis invicta*)**.** The phylogenetic tree was constructed using gene alignments

835   with the software RAxML (v. 8), using 1427 genes and 3.59 Mb of sequence, and the

836   analysis was partitioned by gene and conducted under a GTRGAMMAI model

837   (Morandin et al. 2016). This tree was used to infer phylogenetic signals using the

838   Blomberg test.

839

840   **Figure 2.** Plots showing the relationship between the phylogenetic signals

841   (Blomberg's K values) and gene connectivity in relation to the average gene

842   expression for a) worker b) queen. Values were log-transformed.

843

844   **Figure 3. a) Relationship between phylogenetic signals (Blomberg's K values) for**

845   **queen and worker expression patterns.** Phylogenetic signals (i.e. the tendency of

846   related species to resemble each other more than species drawn at random from the

847   same tree (Blomberg and Garland 2002)) of female caste gene expression patterns are

848   significantly positively associated, and are thus unlikely to change independently in

849   queens and workers. Values were log-transformed. **b) Relationship between**

850   **coefficient of variation for queen and worker expression patterns.** Coefficient of

851   variation of female caste gene expression patterns are significantly positively

852   associated, and are thus unlikely to change independently from each other.

853

854   **Tables**

855   **Table 1. Influences of constraints on the caste-biased expression patterns.** Results

856   of the **GLM** analyses (see text for details) of the average caste bias in expression

857   (represented as the ratio of queen and worker expression level) across the 15 study

858   species.

859

860   **Table 2. Influence of constraints on correlation of caste gene expression.** Results

861   of the GLM analyses (see text for details) of the correlation of gene expression

862   between queens and workers across the 15 study species.

863

864   **Table 3. Influence of constraints on evolutionary flexibility of gene expression**

865   **patterns.** Results of the **GLM** analyses (see text for details) of the phylogenetic signal

866   (Blomberg's K) in expression patterns of queens and workers across the 15 study

867   species.

868

869

870   **Supplementary Materials**

871   **Figure S1. Frequency distribution of average caste-bias across species in**

872   **expression level of each gene.** We quantified caste-bias as expression ratio, i.e. the

873   lower expression level (either queen or worker) divided by the higher, using the

874   average expression value per caste. This produced a non-directional measure of

875   expression ratio with values ranging from 0 to 1, where 0 represents extreme caste-

876   bias with no expression in one caste and 1 represents equal expression levels in the

877   two castes.

878

879   **Figure S2. Barplot representing the number of genes found commonly**

880   **differentially expressed across species.** There are 2300 genes that are never found

881   differentially expressed (0 species) and only one gene found differentially expressed

882   across all 15 species (including two social forms of *Solenopsis invicta*).

883

884   **Figure S3. Boxplot depicting the distribution of caste-biased expression through**

885   **the genes expression levels.** The black line in the box represents the second quantile

886   (median) of caste biases and the upper and lower boundary of box means third

887   quantile and first quantile, respectively for a) Queen b) Worker.

888

889   **Figure S4. The relationship between gene connectivity and the correlation in**

890   **gene expression between queen and worker**, in relation to the average caste

891   expression levels. Values were log-transformed.

892

893   **Figure S5. The negative relationship between the rates of molecular evolution**

894   **(*dN/dS*) and the correlation in gene expression between queen and worker within**

895   **species**. Values were log-transformed.

896

897   **Figure S6. Barplot representing the mean Blomberg's K (phylogenetic signal)**

898   value for expression levels of queen and worker. Errors bars indicate the standard

899   errors.

900

901   **Figure S7. Plots of functional enrichment analysis.** Pie chart from enriched GO

902   terms in the set of genes showing a significant ($p < 0.05$) phylogenetic signal of gene

903   expression patterns in queen, worker, and both. Enrichment analysis was performed

904   using GOstats package for R (Beissbarth and Speed 2004), and enriched GO terms

905   were subsequently summarized to meaningful clusters for visualization using Revigo

906   (Supek et al. 2011).

907

908   **Table S1. Data frame used as input for the GLM analysis.** 6608 genes were

909   considered for the analysis. BLAST annotations and number of GO terms were

910   computed using Blast2GO (Conesa et al. 2005) and the BLASTp function. Gene

911   connectivity was calculated using WGCNA (Morandin et al. 2016). Phylogenetic

912   signals were computed using the Blomberg test.

913

914   Table S2. **Number of queens and workers used for RNA pooling before library**

915   **preparation**. Three replicates per species per caste were sequenced using an equal

916   number of samples in each of them. Number of pooled *Formica exsecta* samples can

917   be found in Morandin et al. (2015).

918

919   **Table S3. Numbers of genes with significant phylogenetic signal for queen and**

920   **worker expression patterns.** The phylogenetic signal was computed using the

921   Blomberg test.

922

923   **Table S4. GO terms.** List of enriched Gene Ontology annotation for genes with

924   significant ($p < 0.05$) phylogenetic signal observed for queen, worker expression

925   patterns and both (represented by the caste column). Phylogenetic signals were

926   computed using the Blomberg test. The GOstats package for R (Beissbarth and Speed

927   2004) was used to conduct GO term enrichment analysis.

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