RESEARCH ARTICLE

Vitellogenin and vitellogenin-like gene expression patterns in relation to caste and task in the ant *Formica fusca*

C. Morandin[1,2] · A. Hietala[1,3] · H. Helanterä[1,2,4]

Abstract

Social insect colonies are characterized by division of labour, and extensive morphological, physiological and behavioural differences between queens and workers. The storage protein vitellogenin (Vg) affects multiple aspects of social insect life histories, and has been suggested as a key player for caste differentiation and maintenance. Recently, three genes homologous to Vg have been described in the ant *Formica exsecta*. Their role is currently unclear but their structural variation suggests variable functions. We examined the expression patterns of the conventional Vg and the three Vg-like genes using qRT-PCR in the common black ant *Formica fusca* between queens and workers, between nurse and foragers workers, and across social contexts (queenless vs. queenright nests), and sampling time. As expected, we found a significant queen caste and nurse task-related increase for the conventional Vg, while Vg-like-C displayed a consistent forager-biased expression pattern. Task (forager vs. nurse) was the only factor that explained expression variation among workers in any of the studied genes. The removal of the queen did not affect expression, although the proportion of fertile nurses increased in queenless nests. The observed expression biases suggest that in *Formica fusca,* the ancestral duplication has led to alternative social functions for Vg-like genes across castes and tasks. To get a broader picture of the role of gene duplications in social evolution and the roles of Vg-like genes in caste differentiation and maintenance, how these genes achieve these roles at a molecular level need to be investigated further.

Keywords Gene expression · *Formica* · Vitellogenin · Gene duplication · Caste

Introduction

The societies of ants, social bees and wasps with morphologically separated castes are remarkable examples of evolution of reproductive altruism (Hamilton 1964, 1972; Boomsma and Gawne 2018), where the reproductive queens and the non-reproductive workers arise from the same diploid female genome, and exhibit extensive physiological, morphological and behavioural differences (Wheeler 1986, 1991; Hölldobler and Wilson 1990). This extreme instance of phenotypic plasticity, where multiple phenotypes can develop from the same genotype (Pigliucci 2001; West-Eberhard 2003), depends, in most cases, on abiotic cues and social environment (e.g., nutrition, temperature, diapause but see Schwander et al. (2010) for exceptions), and is an important factor of social insect ecological success (Wilson and Hölldobler 2005). In addition to reproductive division of labour between queens and workers, workers also divide labour through task specialization. Task specialization can be related to morphological differences (Wilson 1971; Oster and Wilson 1978; Hölldobler and Wilson 1990), may be partly genetically determined (Fraser et al. 2000; Hughes et al. 2003; Schwander et al. 2005) or may vary with age, with young workers typically specializing on within-nest tasks such as nursing and older workers on outside tasks such as foraging (Wilson 1971).

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Despite long standing research investigating the molecular pathways and genes involved in division of labour, the precise mechanisms remain relatively unknown (but see e.g., Chandra et al. (2018) for recent developments). The so-called ground plan or toolkit hypotheses (West-Eberhard 1989; Toth and Robinson 2007; Toth et al. 2010) predict that social phenotypes such as task regulation are based on co-option of ancient solitary genetic pathways. One of the key examples of a co-opted ground plan gene is vitellogenin (Vg). Vg is a phospholipoglycoprotein involved in egg yolk formation and produced by most oviparous organisms. In insects, Vg is synthesized in the fat body (Bloch and Grozinger 2011; Amdam et al. 2012), and either stored there or transported via hemolymph to oocytes (Pan et al. 1969; Hagedorn and Kunkel 1979; Tufail and Takeda 2008). Because of its primary role in egg formation, in social insects the conventional Vg is commonly upregulated in queens compared to workers (Piulachs et al. 2003; Weil et al. 2009; Wurm et al. 2011; Corona et al. 2013), and is one of the most studied genes involved in division of labour (Amdam and Omholt 2003; Corona et al. 2007, 2013; Morandin et al. 2014; Salmela et al. 2016). Vg is involved in regulation of non-reproductive features of colonies as well. Examples include ageing and queen longevity (Excels 1974; Corona et al. 2007), temporal worker division of labour (Guidugli et al. 2005b; Nelson et al. 2007; Münch and Amdam 2010; Bloch and Grozinger 2011), and royal jelly production (Amdam et al. 2003). In addition to its multiple social roles, Vg has been shown to be involved in antioxidation to enhance stress resistance (Seehuus et al. 2006; Münch and Amdam 2010; Havukainen et al. 2013), defence against inflammation, and transgenerational immune priming (Salmela et al. 2015).

Multiple roles for a single protein are predicted to lead to a gene duplication event, as it is unlikely that the same gene sequence is optimal for several different roles. Social insects exhibit a variable number of copies of the conventional Vg gene (Morandin et al. 2014; Kohlmeier et al. 2018), ranging from one copy in the honey bee (Piulachs et al. 2003), the ant *Formica exsecta* (Morandin et al. 2014) and the ant *Camponotus floridanus* (Bonasio et al. 2010), to two copies in the ant *Pogonomyrmex barbatus* (Smith et al. 2011a, b) and the ant *Atta cephalotes* (Suen et al. 2011), four copies in the ant *Solenopsis invicta* (Wurm et al. 2011), and to five copies [the ant *Linepithema humile* (Smith et al. 2011a, b)]. Variation in copy number of Vg, along with its numerous known functions and its caste-specific expression pattern, imply that Vg duplication events may have played a significant role in the evolution of caste polyphenism in social Hymenoptera (Rueffler et al. 2012; Corona et al. 2013; Morandin et al. 2014). The multiple copies of the conventional Vg seem to have subfunctionalized to acquire caste- and behaviour-specific expression patterns associated with reproductive and non-reproductive functions (Corona et al. 2013; Morandin et al. 2014). For instance, in *P. barbatus,* two copies of the conventional Vg can be found, one preferentially expressed in queens and nurses, while the other one is expressed preferentially in foragers (Corona et al. 2013). Similar patterns were also found in *Solenopsis invicta* (Wurm et al. 2011), where different conventional Vg copies showed subcaste- and task-related expression patterns, potentially linked to the loss of reproductive constraints and evolution of new functions for the duplicated copies. Once the conventional Vg underwent several duplication events, the resulting multiple Vg homologs became responsible for several distinct physiological functions and consequently gene expression differences arose.

Recently, we discovered three formerly unknown copies of Vg-like genes in the ant *Formica exsecta* transcriptome. Two of these homologs, Vg-like-A and -B can be found in all insects, while the third, Vg-like-C can only be found in Hymenoptera (Morandin et al. 2014; Kohlmeier et al. 2018). These homologs partly differ in their conserved protein domains and have undergone rapid evolution after duplications. The gene duplication events seem older (Morandin et al. 2014) than the species-specific Vg duplications identified in the conventional Vg (Wurm et al. 2011). The function of these novel genes remains unknown, however due to their structural similarities to the conventional Vg, we expect these diverged Vg-like gene to retain some of the conventional Vg functions. In this light, recent studies have shown that Vg-like-A (and to some extend Vg-like-B) responded more strongly than the conventional Vg to inflammatory and oxidative conditions in the honey bee (Salmela et al. 2016), and that Vg-like-A is linked to the regulation of nursing behaviours in the ant *Temnothorax longispinosus* (Kohlmeier et al. 2018). Additionally, in the honey bee, Vg-like-A may play an important role in over-wintering worker longevity due to its strong temporal expression variation, similarly to the conventional Vg (Ricigliano et al. 2018). All these findings support the idea of neo- or subfunctionalization, but whether the Vg-like genes share these functions in several social insects, and play a role in division labour widely, remain unclear.

The goals of this study were to investigate the caste- and task-specific expression patterns of the conventional Vg, and Vg-like-A, -B and -C, in the ant *Formica fusca*. More precisely, to begin to comprehend the role of Vg homologs and their potential involvement in division of labour, we aimed to investigate the expression patterns relationships: (1) between castes (queens vs. workers), (2) between workers performing different tasks (nursing *vs.* foraging), (3) between workers in colonies with (queenright) and without (queenless) a queen, (4) and between multiple time points (days) during the egg laying period. To do so, we experimentally manipulated the presence of

queens in *Formica fusca* colonies to induce egg laying in the workers. We then collected whole-body gene expression data to investigate the expression patterns of Vg and Vg-like genes using RT-qPCR.

We expect the conventional Vg to be upregulated in queens compared to workers (both foragers and nurses) due to its necessary role in egg laying, but also upregulated in nurses compared to foragers, a well-established pattern (Amdam et al. 2004; Nelson et al. 2007; Ricigliano et al. 2018). If regulation of reproduction and behaviour are linked in *Formica fusca* as they are in the honeybee (Guidugli et al. 2005a; Amdam et al. 2008), the expression of the conventional Vg should be higher in nurses compared to foragers. Given the increased amounts of worker-laid eggs in queenless nests of *F. fusca* (Helanterä and Sundström 2005), workers, especially nurses, of queenless colonies are expected to show higher expression of conventional Vg compared to queenright ones. Conventional Vg levels should respond positively to the onset of egg laying in queens and in egg-laying workers in queenless colonies. Regarding the Vg-like genes, and following the recent finding that Vg-like-A knockdown leads to a decrease in brood care (Kohlmeier et al. 2018), we predict that Vg-like-A expression levels would be significantly higher in nurses compared to queens and foragers. Furthermore, following the results of Morandin et al. (2014), we also predict that Vg-like-A and Vg-like-C would be upregulated in workers compared to queens and Vg-like-B expressed equally in both. Upregulation of Vg-like-C in non-reproductive bumble bee workers (Harrison et al. 2015) suggests that Vg-like-C might be upregulated in *F. fusca* foragers compared to other female castes. We hypothesize that caste-specific expression pattern of Vg-like-C will differ clearly from the conventional Vg, as it most likely has a completely distinct function, indicated by its distinct protein structure (Morandin et al. 2014).

Materials and methods

Sample collection

The study species, *Formica fusca,* is a Palearctic ant that nests underground and in rotten tree stumps (Collingwood 1979). It is a facultatively polygynous species, and colonies usually contain between 1 and 20, but occasionally even more than a 100 queens (Hannonen 2003; Hannonen et al. 2004) with the number of workers per nest usually reaching up to 1500 (Savolainen 1990). Workers are fertile and lay male eggs, especially in the absence of a queen (Helanterä and Sundström 2005). We collected *F. fusca* colonies around Tvärminne Zoological Station in Hanko peninsula in southwestern Finland in spring 2013, after the snow had melted and ants had resumed their activity after hibernation, but before the onset of egg laying. Colonies were brought back to the laboratory, where the queens were counted, and colonies with several queens were selected for the experiment. Each colony was split into two equal sized parts, one queenright (QR) nest consisting of queens and workers and a queenless (QL) nest consisting of workers only. A total of five colonies were included with a minimum of 400 workers per nest, and a variable number of queens (see Table 1). Laboratory nests were established in plastic trays ($40 \times 30 \times 15$ cm) supplied with some of the original nest material, *Sphagnum* moss, peat and a ceramic plate (15×15 cm). Throughout the experiment, the ants were fed with standard Bhatkar–Whitcomb diet (Bhatkar and Whitcomb 1970), placed on top of a feeding platform to allow detection of active foragers. The onset of egg laying was monitored every 5 days. The experiment was run for 20 days, during which both the queenright and the queenless nests started to produce eggs (see Table 1 for details). Sampling for gene expression analyses was done every five days, starting on day 5. Five foragers and five

Table 1 Initial number of queens per colony (when colonies were collected), number of queens and workers collected every 5 days, and initial appearance of eggs in the nest boxes

Colony	Treatment	Initial number of queens	Number of queens collected per sampling day	Number of workers (nurse + forager) collected per sampling day	Sampling days					Found at the disposal of the colonies
					0	5	10	15	20	
P70	Queenright	17	3	5 + 5					Eggs	Eggs, pupae
	Queenless			5 + 5			Eggs	Eggs	Eggs	Larvae, pupae
S161	Queenright	10	2	5 + 5				Eggs	Eggs	Larvae, pupae
	Queenless			5 + 5			Eggs	Eggs	Eggs	Larvae, pupae
S169	Queenright	69	4	5 + 5				Eggs	Eggs	Larvae, pupae
	Queenless			5 + 5				Eggs	Eggs	Larvae, pupae
S157	Queenright	39	4	5 + 5				Eggs	Eggs	Larvae, pupae
	Queenless			5 + 5			Eggs	Eggs	Eggs	Larvae, pupae
P126	Queenright	28	4	5 + 5				Eggs	Eggs	Larvae, pupae
	Queenless			5 + 5			Eggs	Eggs	Eggs	Larvae, pupae

nurses were sampled from each nest and two–four queens were sampled from the queenright nests each sampling day (Table 1). The workers found under the ceramic plate with the queens and the brood were considered as nurses, while the individuals gathering food on the platforms were considered as foragers. The samples were stored individually in TRIsure tubes at −80 °C for RNA extraction.

Nurse ovary development

At the start of the experiment (day 0), 30 workers were collected from each colony for ovary inspection; 30 workers were collected from each queenright and queenless nest on day 15, by which all nests had produced eggs. The workers were collected in proximity of queens and the brood and were hence considered as nurses. Their abdomen was dissected and workers with oocytes larger than 90% of the size of an egg in their ovaries were classified as fertile (Helanterä and Sundström 2007). To find out whether queen removal influenced nurses' fertility, we used a generalized linear mixed model with a binomial distribution [using package lme4, function glmer (Bates et al. 2015)] to examine the effect of treatment [start (day 0), queenright (day 15) and queenless (day 15) colonies] on the ovary sizes, with colony as a random effect.

Vg expression using rt-qPCR

Evidence for a single copy of the conventional Vg in *Formica fusca*

We used the basic local alignment search tool (BLAST) to search for evidence of a single or multiple copies of Vg in the de novo transcriptome assembly of *Formica fusca* (Morandin et al. 2016). We found only one putative Vg contig in the transcriptome with high similarity to Vgs published in other ants (e.g., *Formica exsecta*, Morandin et al. 2014). Thus, only one conventional Vg copy was found expressed at the adult stage.

RNA extraction and cDNA preparation

RNA was extracted from whole body for each individual separately using a standard Trizol protocol (TRIsure, Bioline) and contaminating genomic DNA was removed by DNAse I digestion (Fermentas), both steps following the instructions of the manufacturer. The final elution volume was 50 μL and the final RNA concentration varied between 40.2 and 4299.8 ng/μL (average 397.3 ng/μL, S.D. 562.9).

The yield and the quality of the RNA were assessed using the A280/A260 ratio (NanoDrop, Thermo Scientific). The RNA was converted into single-stranded cDNA using the RevertAid First Strand cDNA synthesis kit reagents using random hexamer primers (Thermo Scientific) and 500 ng of total RNA as the starting amount (within a standardized 11 μL volume).

We characterized whole-body gene expression of Vg and Vg-like genes in queens and workers. This approach approximates the proportional role of each gene in each caste. For example, queens have proportionally larger ovaries, and we expect that higher levels of ovary-expressed genes in queen bodies overall as a result reflect the relative importance of ovaries to the queen phenotype. Differences in tissue size, including extreme cases such as the presence or absence of functional ovaries, are manifestations of the overall phenotype. So, to characterize the roles of genes in the social phenotype of the colony in total, and division of labour, gene expression patterns of whole bodies of queens vs. workers (as adults) are a key comparison.

Selection of reference genes and primer design

The qPCR values of the target genes were normalized using two internal reference genes Rp48 and RpS9 chosen from Morandin et al. (2014) (Table 2). We used the software geNorm (Vandesompele et al. 2002) to assess the expression stability of the two reference genes under our experimental conditions gene, using the M value (e.g., a measure of expression stability). A gene with $M < 1.5$ is usually treated as a stable reference gene (Guo et al. 2010; Han et al. 2012). We analysed the melt curve output to confirm that no primer

Table 2 List of primer sequences used for real-time PCR, annealing temperatures and efficiencies

Gene	Forward	Reverse	Annealing temperature (°C)	Efficiency
Conventional Vg	AGGTTGTCATCGTCAACGAA	CGTTGCTTGTAGGGGATAGG	58	107
Vg-like-A	GAAGAACATCTTCCTGCGAATG	AAGAATGCTGGCTTTCCTGA	56	100
Vg-like-B	ACACATATTAACCACTTATCTTTGTA	GGACGCACAGCACTGTTTTT	60	98
Vg-like-C	GAACTTTACATGGATATGACTACTT	ATTTGAGAAGGCGAATAGAC	58	95
RpS9	CCAACGGCATATTCGAGTAC	CAGTTTAATCCTCCTCTTCTTC	58	95
Rp48/RpL32	CAAGGGCCAATACTTGATGC	TTAAGACTTCAAGTTCCTTCAC	60	104

dimer or DNA amplification was produced during the qPCR cycles. The qPCR efficiency (*E*) for the primer pairs was calculated from the slopes of a dilution series of 1, 1:2, 1:4, 1:8, 1:16, using the software BioRad CFX manager v. 1.6 and according to the following equation: $E = 10[-1/\text{slope}]$ (Table 2).

qPCR run conditions

Real-time PCR was performed using the following thermal cycling programme: initial denaturation at 95 ℃ for 3 min, 45 cycles with denaturation at 95 ℃ for 10 s, primer pair-specific annealing temperature for 10 s (Table 2), elongation at 72 ℃ for 30 s, and final elongation at 72 C for 3 min. Reactions were performed with BioRad iQ qPCR SYBR Green Master Mix using a BioRad CFX 384 qPCR Cycler. Two technical replicates were run for each reaction to ensure the accuracy and repeatability of RT-PCR. New duplicates were run for a sample if the Cq value difference between the first two duplicates was more than one. Averages of the duplicate Cq values were used in further calculations of the relative expression level. Negative controls (no template control and no-reverse transcription control) were used to confirm that no contaminations, primer dimers, or traces of genomic DNA were amplified. If the amplification curve showed no sign of amplification for the two replicates, we set the final Cq to 45, the highest Cq value for the study that we considered as null expression level. Finally, if the expression level of a sample could not be assayed in any of the reference and target genes, this sample was removed from further analysis.

Statistical analysis

The relative expression of the conventional Vg and the three Vg-like genes was analysed based on standardized Ct values (ΔCt) (Livak and Schmittgen 2001), normalized to the geometric average expression of the two reference genes (RPS9 and RP48, Morandin et al. 2014). The normalized ΔCq was analysed in R (version 3.3.2 https://www.cran.r-project.org/) using the function lme from the nlme package (Pinheiro et al. 2017). We used generalized linear mixed model (GLMM) to examine the effect of caste, treatment and time of sampling on the expression of Vg and Vg-like genes. ΔCq values were exponentially transformed to achieve normality. Colony was included in all models as a random effect to manage the non-independence of observations from the same colony. Two different analyses were done separately for each Vg copy: (1) we compared the expression levels in foragers, nurses and queens in the queenright nests, while taking into account the effect of caste and time of sampling, using a GLMM. ΔCq values were set as a response variable, while day and caste were set as fixed explanatory variables, and colony as

a random factor. (2) We compared expression levels between treatments, time of sampling and worker castes. ΔCq values were set as response variable, while treatment, day and caste (forager/nurse) were set as fixed explanatory variables, and colony as a random factor. For both analyses, we selected the best fitting statistical model by Akaike's information criterion (AIC) and Bayesian information criterion (BIC) values of models with and without interactions of explanatory variables. The parameters of the linear mixed models were estimated by maximum likelihood (Müller et al. 2013). The models with interactions were discarded, as the simpler models with only the main effects showed lower AIC.

Results

All GLMM assumptions were met prior to further analysis. The expression stability value of both reference genes was under the acceptable threshold (0.5 and 1.3, respectively). The analysis of expression patterns using qualitative RT–PCR showed that all studied Vg copies were expressed in all sample classes, but levels of expression varied considerably across the social conditions examined. As expected, the overall relative expression level was the highest for the conventional Vg (mean expression level: 38.9 S.D. 103.5) and lowest for Vg-like-C (mean 0.03, S.D. 0.04), and between these extremes, Vg-like-A (mean 1.2, S.D. 2.6) being somewhat more strongly expressed than Vg-like-B (mean 0.6, S.D. 0.5).

Nurse ovary size

The proportion of fertile nurses was higher in queenless nests by day 15 (QL_{15}: mean 0.49, S.D. 0.50) than it was at the start of the experiment (Table 3; Fig. 1: GLMM $p \leq 0.001$). The proportion of fertile nurses was also higher in queenless nests by day 15 than in queenright nests on day 15 (QR_{15}: mean 0.37 S.D. 0.48, GLMM $p = 0.03$). The fertility in queenright nests on day 15 was not significantly higher compared to the start of the experiment (GLMM $p = 0.16$).

Table 3 Comparison of nurses' fertility (ovary indices 0 and 1) between the start of the experiment (day 0), queenright (day 15) and queenless (day 15) colonies, $n = 445$ individuals

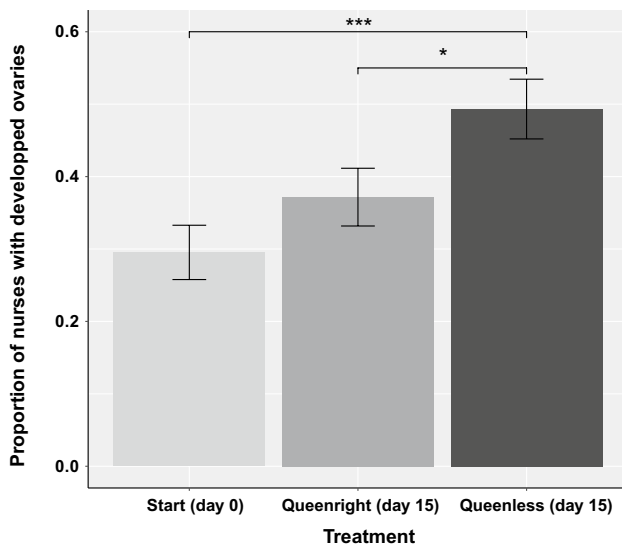| Response	Estimate	Std. error	*p* value
Start vs. queenright	0.35	0.25	0.16
Start vs. queenless	0.86	0.25	<0.001***
Queenright vs. queenless	0.50	0.24	0.03*

***$p < 0.001$, **$p < 0.01$, *$p < 0.05$

Fig. 1 Proportion of fertile nurses at the start of the experiment (day 0), in queenright colonies (day 15) and queenless colonies (day 15). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$

Expression patterns

Conventional Vg

As expected, the conventional Vg showed the highest expression level in all castes regardless of the treatment and time of sampling. In QR nests (Fig. 2), the conventional Vg was overexpressed in the queens (mean of expression 126.9, S.D. 192.7) compared to the nurses (mean 31.1, S.D. 70.6; Table 4; GLMM $p_{\text{nurse–queen}} < 0.001$, $t_{\text{nurse–queen}} = -6.4$) and to the foragers (mean 14.2, S.D. 74.3, GLMM, $p_{\text{forager–queen}} < 0.001$, $t_{\text{forager–queen}} = -11$). There was also a statistically significantly higher expression level in nurses compared to foragers ($p_{\text{forager–nurse}} < 0.001$, $t_{\text{forager–nurse}} = 4.9$). In workers (from both QR and QL nests), there was no statistically significant association between Vg gene expression and removal of the queen (Table 5; $p_{\text{treatment}} = 0.7$, $t_{\text{treatment}} = -0.3$), or day of sampling (Table 5; $p_{\text{day}} = 0.6$, $t_{\text{day}} = -0.5$).

Vg-like-A

In QR nests (Fig. 3), we found that the expression levels of Vg-like-A was significantly higher in queens (mean 1.6, S.D. 2.6) compared to foragers (mean 0.6, S.D. 1.3; Table 4; GLMM, $p_{\text{queen–forager}} < 0.001$, $t_{\text{queen–forager}} = -3.7$), but there were no differences between queens and nurses (mean 2, S.D. 4.2, GLMM, $p_{\text{queen–nurse}} = 0.8$, $t_{\text{queen–nurse}} = 0.3$). There was a significantly lower expression level in foragers compared to nurses ($p_{\text{forager–nurse}} < 0.001$, $t_{\text{forager–nurse}} = 4.2$). In workers (from both QR and QL nests), there was also no statistically significant association between Vg gene
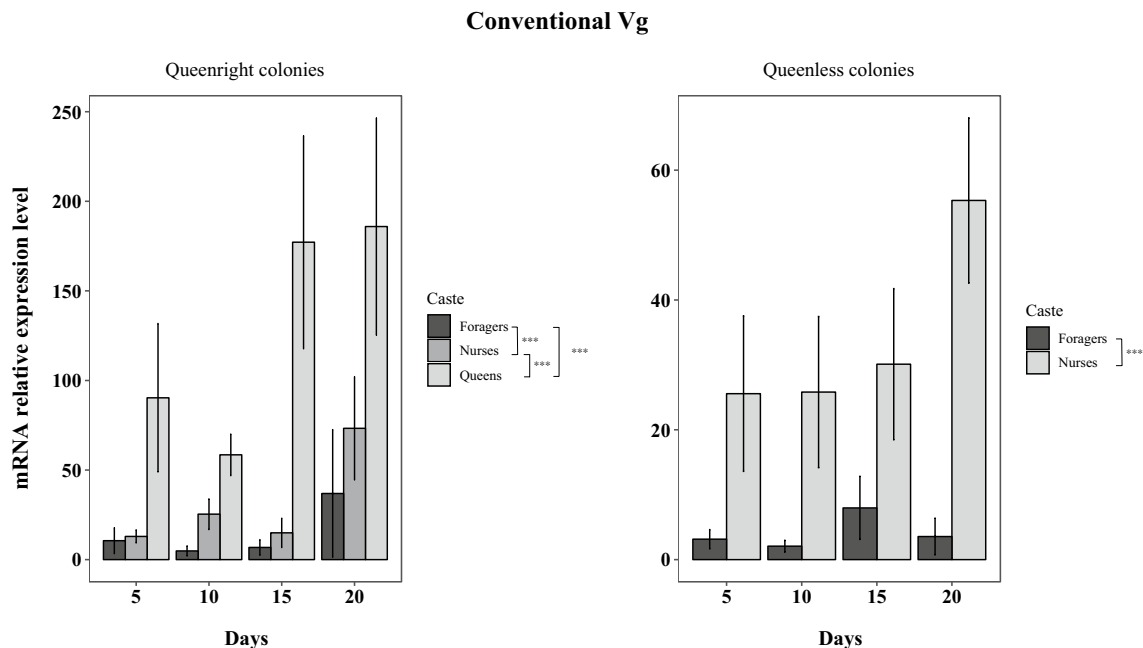
Conventional Vg

Fig. 2 Relative expression of the conventional Vg in five *F. fusca* colonies from queenright and queenless treatments over 5 sampling days. Barplot represents the mean expression values normalized to the reference genes, error bars indicate the standard error. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$

Table 4 Detailed results of the mixed effect models looking at the effects of sampling days (day) and caste (nurse, forager, queen) on the conventional Vg and the three Vg-like genes mRNA relative expression levels in the queenright nests

	Value	Std. error	DF	T value	p value
Conventional Vg					
Day	−0.001	0.003	216	−0.4	0.7
Forager vs. queen	−0.5	0.05	216	−11	<0.001***
Nurse vs. queen	−0.3	0.05	216	−6.4	<0.001***
Nurse vs. forager	0.2	0.04	216	4.9	<0.001***
Vg-like-A					
Day	−0.0004	0.001	216	−0.3	0.8
Forager vs. queen	−0.07	0.02	216	−3.7	<0.001***
Nurse vs. queen	0.006	0.02	216	0.3	0.8
Nurse vs. forager	−0.08	0.02	216	4.2	<0.001***
Vg-like-B					
Day	0.002	0.0008	216	2.6	<0.01**
Forager vs. queen	0.06	0.01	216	5.5	<0.001***
Nurse vs. queen	0.04	0.01	216	3.6	<0.001***
Nurse vs. forager	−0.02	0.01	216	−2	<0.05*
Vg-like-C					
Day	0	0	216	−0.5	0.6
Forager vs. queen	0.2	0.01	216	16	<0.001***
Nurse vs. queen	0.2	0.01	216	13.6	<0.001***
Nurse vs. forager	−0.03	0.01	216	−2.4	<0.05*

ΔCq values were set as a response variable, while day and caste were set as fixed explanatory variables, and colony as a random factor. The pairwise comparisons of treatment levels are obtained from separate models with different treatments as the intercept levels

***$p < 0.001$, **$p < 0.01$, *$p < 0.05$

Table 5 Detailed results of the mixed effect models looking at the expression levels of the conventional Vg and the three Vg-like genes between treatments, time of sampling and worker caste

	Value	Std. error	DF	t value	p value
Conventional Vg					
Day	−0.001	0.002	311	−0.5	0.6
Caste	0.2	0.03	311	8.3	<0.001***
Treatment	−0.01	0.03	311	−0.3	0.7
Vg-like-A					
Day	−0.0009	0.001	311	−0.8	0.4
Caste	0.07	0.01	311	5.5	<0.001***
Treatment	0.02	0.01	311	1.4	0.2
Vg-like-B					
Day	0.002	0.0007	311	3	<0.01**
Caste	−0.03	0.008	311	−4.3	<0.001***
Treatment	−0.0006	0.008	311	−0.1	0.9
Vg-like-C					
Day	0.0002	0.0007	311	0.4	0.7
Caste	−0.03	0.008	311	−4	<0.001***
Treatment	0.008	0.008	311	1	0.3

ΔCq values were set as response variable, while treatment, day and caste (forager/nurse) were set as fixed explanatory variables, and colony as a random factor
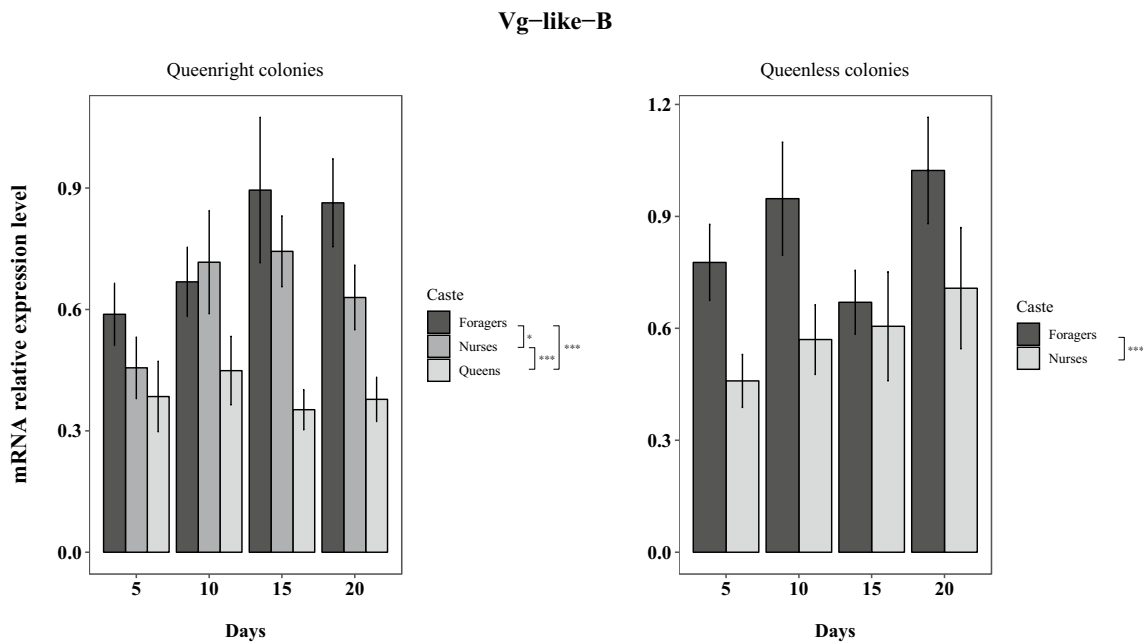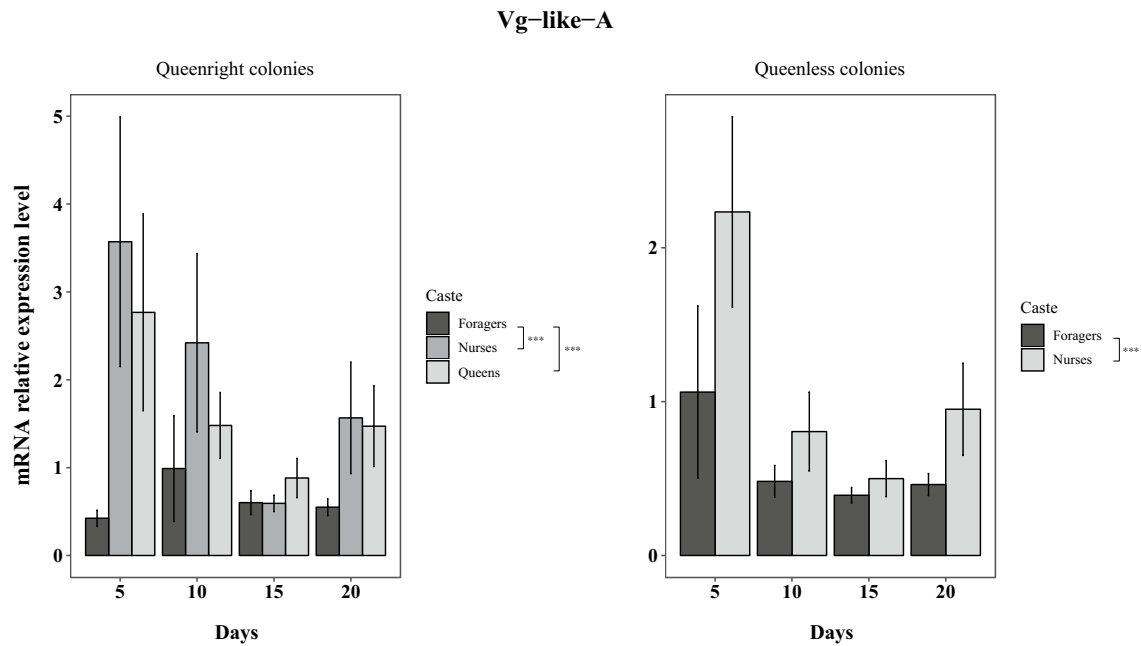
***$p < 0.001$, **$p < 0.01$, *$p < 0.05$

association between Vg gene expression and removal of the queen ($p_{treatment} = 0.9$, $t_{treatment} = −0.1$).

Vg-like-C

As expected, Vg-like-C had very low overall expression levels (mean 0.03, S.D. 0.03) and a clear bias towards workers (Fig. 5). In QR nests, we found that the expression level of Vg-like-C was higher in foragers (mean 0.05, S.D. 0.04) compared to nurses (Table 4; mean 0.03, S.D. 0.03, GLMM, $p_{forager–nurse} < 0.05$, $t_{forager–nurse} = −2.4$) or queens (mean 0.004, S.D. 0.01, $p_{forager–queen} < 0.001$, $t_{forager–queen} = −16$). There was also a statistically significantly higher expression in nurses compared to queens ($p_{nurse–queen} < 0.001$, $t_{nurse–queen} = 13.6$). In workers (from both QR and QL nests), there was no statistically significant association between Vg gene expression and day of sampling (Table 5; $p_{day} = 0.4$, $t_{day} = 0.6$), and removal of the queen ($p_{treatment} = 0.3$, $t_{treatment} = 1$).

Discussion

This study reveals a unique caste-specific expression pattern for each Vg copy in *F. fusca*. As predicted, the conventional Vg was upregulated in queens and nurses compared to foragers, Vg-like-A in nurses and queens. Vg-like-B

expression and removal of the queen (Table 5; $p_{treatment} = 0.2$, $t_{treatment} = 1.4$), or time of sampling ($p_{day} = 0.4$, $t_{day} = −0.8$).

Vg-like-B

In QR nests (Fig. 4), we found that the expression levels of Vg-like-B was higher in foragers (mean 0.7, S.D. 0.5) compared to queens (Table 4; mean 0.4, S.D. 0.3, GLMM, $p_{queen–foragr} < 0.001$, $t_{queen–forager} = −5.5$) or nurses (mean 0.6, S.D. 0.4, $p_{forager–nurse} = <0.001$, $t_{forager–nurse} = −2$). There was also a statistically significantly higher expression in nurses compared to queens ($p_{nurse–queen} < 0.01$, $t_{nurse} = 3.6$). In workers (from both QR and QL nests), there was a statistically significant association between Vg gene expression and day of sampling (Table 5; $p_{day} < 0.01$, $t_{day} = 3$), showing that the expression of Vg-like-B increased over time during egg-laying period. There was no statistically significant

Vg−like−A

Fig. 3 Relative expression of Vg-like-A in five *F. fusca* colonies from queenright and queenless treatments over 5 sampling days. Barplot represents the mean expression values normalized to the reference genes, error bars indicate the standard error. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$

Vg−like−B

Fig. 4 Relative expression of Vg-like-B in five *F. fusca* colonies from queenright and queenless treatments over 5 sampling days. Barplot represents the mean expression values normalized to the reference genes, error bars indicate the standard error. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$

and Vg-like-C expressions were biased toward foragers as expected from previous studies (Morandin et al. 2014; Kohlmeier et al. 2018). Contrary to our initial predictions, manipulation of social environment (i.e., queen presence) did not seem to affect the expression levels of the conventional

Vg or any of the three Vg-like genes, even though queenless nests had more fertile nurses than queenright nests. The within-individual functions of Vg-like genes are largely unknown, but their expression patterns are consistent with roles in division of labour separate from the conventional
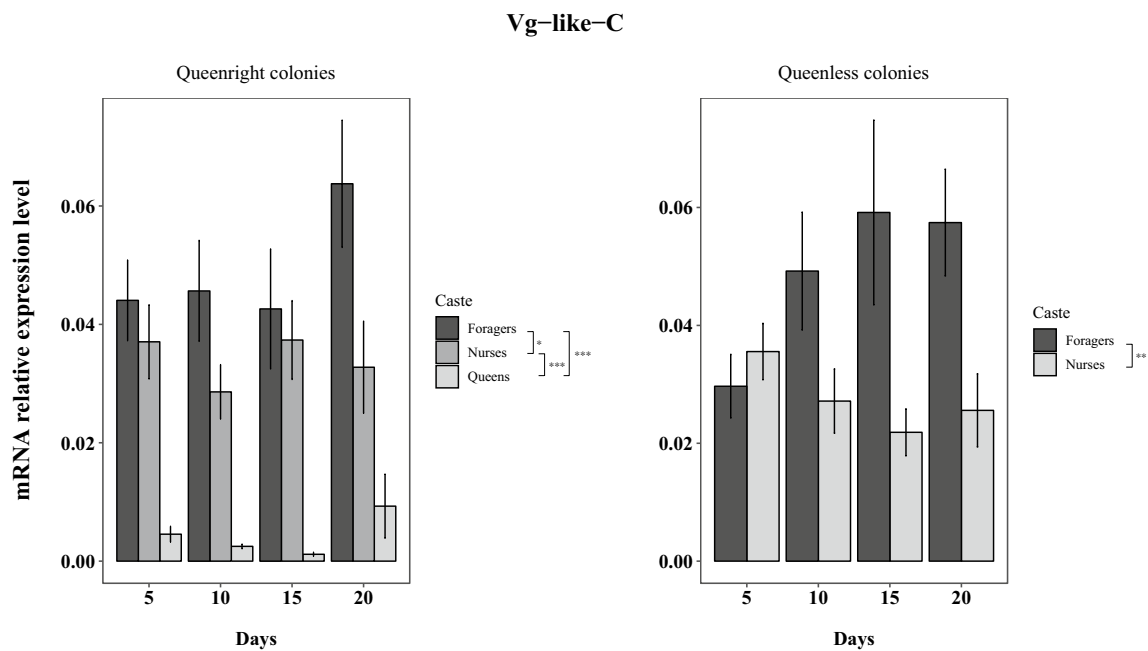
Vg−like−C

Fig. 5 Relative expression of Vg-like-B in five *F. fusca* colonies from queenright and queenless treatments over 5 sampling days. Barplot represents the mean expression values normalized to the reference genes, error bars indicate the standard error. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$

Vg. The results give broad support to the idea that ancient genes may be co-opted to new roles during social evolution, that genes originally involved in reproductive regulation can gain new roles in non-reproductive coordination (the reproductive ground plan hypothesis), and the role of subfunctionalization of gene copies after duplications.

Vg and Vg-like genes expression patterns and expectations

In contrast to our earlier study (Morandin et al. 2014), we observed a clear queen bias in the expression of the conventional Vg in *Formica fusca*, consistent with its role in oogenesis. This is likely explained by our longer sampling period, including dates after the onset of egg laying and an increased sample size. The lack of queen overexpression in the earlier study could moreover be due to the elevated individual variation in queen conventional Vg expression levels, presumably caused by intrinsic differences in fecundity and the timing of oviposition (Ozan et al. 2013). Furthermore, *F. fusca* queens lay eggs only during a short period after recovering from hibernation, and it is not certain when the actual peak in conventional Vg expression occurs during their reproductive cycle.

The reproductive ground plan hypothesis states that mechanisms originally underlying physiological regulation of reproduction can be dissociated from their reproductive roles to regulate other social traits such as worker behaviour (Amdam et al. 2004, 2006). The fact that Vg expression

was higher in nurses than foragers for the conventional Vg and Vg-like-A is compatible with this idea, and thus adds to the growing support of RGPH from ants (Dolezal et al. 2012; Pamminger and Hughes 2017). There is no detailed research about regulation of task allocation in *F. fusca*, but in the ecologically similar and closely related species *Formica selysi*, the worker task allocation is influenced by multiple factors such as body size, genetic lineage and queen number (Schwander et al. 2005). How these factors interact with gene expression pattern remains to be studied.

Vg-like-A expression was lowest in foragers, compared to both queens and nurses, which had relatively similar expression levels. This contrasts to our earlier study looking at an early sample (before any brood was present in the spring) of workers and queens, where Vg-like-A expression was biased towards workers in general (Morandin et al. 2014). In two recent studies, Vg-like-A expression levels showed distinct spatiotemporal expression pattern and tended to increase in long-lived winter bees compared to summer bees (Salmela et al. 2016; Ricigliano et al. 2018). The workers we analysed were all overwintered and relatively old (i.e., eclosed at the latest in the previous summer, > 6 months earlier) but still have several months left in their typical life span. To fully unravel the association between ageing and Vg-like-A in *F. fusca*, data collection over longer time periods is required.

Our current result is in line with a recent RNA-interference study in *Temnothorax* ants (Kohlmeier et al. 2018), where brood caring workers (e.g., nurses) exhibited higher expression of Vg-like-A than foragers. Their study suggests

a key role for Vg-like-A in regulating responses to brood odours and influencing the task choice of ant workers, hence impacting worker behaviour and division of labour, a role that may also occur in other ant species such as *F. fusca*. Given recent comparative and experimental data (Libbrecht et al. 2013; Chandra et al. 2018), it is possible that this responsiveness is linked to insulin and JH signalling, so that conventional Vg and Vg-like-A share regulatory processes. It remains to be studied whether the conventional Vg is similarly connected responses to brood odours.

In contrast to Vg-like-A, Vg-like-B was forager biased in its expression. In our earlier study, Vg-like-B was equally expressed in queens and workers. Our results, however, are not in conflict with this study, considering that the workers in Morandin et al. (2014) were collected from inside the nests and were therefore most probably nurses (as considered in the study). In addition to separate forager and nurse samples, the effect of longer sampling period and increased statistical power may explain the differing results for Vg-like-B between the current study and Morandin et al. (2014). It is more difficult to explain why Vg-like-A was worker biased in the earlier study where probable nurses were sampled, while in the current study queens and nurses did not differ in their expression levels. This could be due to temporal variation in the expression levels, and the small number of queen samples in the earlier study. Furthermore, if Vg-like-A and B are involved in stress resistance in ants, as they may be in honey bees (Salmela et al. 2016), some of the variation observed could reflect responses to immune related or oxidative stress.

We found a very low and consistently worker-biased expression of Vg-like-C in line with the pattern observed earlier in seven species of *Formica* ants (Morandin et al. 2014) and other studies (Harrison et al. 2015; Salmela et al. 2016). Among workers, Vg-like-C was upregulated in foragers, similarly to bumblebees where the highest expression levels were seen in non-reproductive workers compared to males and virgin queens (Harrison et al. 2015). The absence of most conventional Vg structural elements in Vg-like-C protein structure and its very low expression level in multiple social insect species suggest that Vg-like-C might have subfunctionalized to a completely different role. The similarity of expression patterns in ants and bees is compatible with a convergent evolution of a common function in social evolution of separate lineages, as predicted by genetic toolkit hypothesis (Toth and Robinson 2007). Data on Vg-like-C in solitary insects would be very interesting for further investigations of this hypothesis, i.e., whether the role in workers is a completely novel one, or whether it is an ancestral role, absent from the queens.

Effect of social context and time

Contrary to our initial expectations, neither the advancing of the reproductive season, nor the time since colony orphaning explained variation in expression levels. Of all target genes, only Vg-like-B showed a consistent increasing overall temporal trend (Table 5; Fig. 4), in others no clear patterns were found. Queen removal did not trigger gene expression changes, despite this and earlier studies showing a slight increase in worker ovary development, and abundant rearing of worker-laid eggs after queen removal (Helanterä and Sundström 2005, 2007), (Table 3). In contrast, in the sterile worker caste of fire ant *Solenopsis invicta,* transcriptomic differences between foragers and non-foragers faded after the queen was removed (Manfredini et al. 2014). Similarly in the honeybee, conventional Vg levels were higher in queenless workers than in queenright workers, and in both groups, higher in non-foragers than in foragers (Peso et al. 2016).

There may be several reasons for the lack of difference in the expression of conventional Vg between the treatments. According to the ovary dissections, about half (49%) of the nurses in QL nests were fertile, while in QR nests the proportion was lower (37%). The difference is significant, but relatively small (Table 3; Fig. 1). In this study, the reproductive status of the workers sampled for expression analysis was not investigated in detail. It is possible that reproductive individuals were incorrectly represented by chance due to relatively small RNA sample sizes (five nurses/foragers per nest per day). It is also possible that foragers not currently foraging outside were sampled as nurses that would tend to equalize any differences observed, and make our analyses more conservative. Moreover, whole-body samples may blur interesting differences in gene expression between body parts. The possible effect of social environment on conventional Vg expression in workers, and its link to worker egg laying will be better understood by comparing tissue-specific data from workers with known reproductive status.

In general, variation in sizes of tissues where the gene of interest is expressed, especially ovaries that are known to vary (Helanterä and Sundström 2005) and fat bodies, will affect individual variation when the RNA is extracted from whole bodies. More generally, variation in tissue sizes could also partly underlie differences in whole-body expression between queens and workers, given the potentially substantial variation in expression among tissues (Liu et al. 2008; Sonawane et al. 2018). While correlational inference about the roles of differential gene expression in division of labour within the nest can be made from whole-body samples, data on tissue-specific expression will be required for inferring the functional and molecular routes how these genes achieve their social roles.

Functions of the duplicates

The caste-specific expression patterns of Vg copies, together with the earlier observations of variation in expression and protein structure and signatures of fast evolution after duplications suggests subfunctionalization or neofunctionalization (Morandin et al. 2014; Salmela et al. 2016). Considering the generally low but worker-biased expression compared to the conventional Vg, it is unlikely that Vg-like genes B and C would work as storage proteins in egg yolk formation. Furthermore, it seems unlikely that Vg-like-C would play any role in reproduction, not only because of its negligible expression in queens but also for the lack of lipid cavity in the protein structure (Morandin et al. 2014) that precludes a key role in vitellogenesis. Duplicated genes and gene family expansions have generally been hypothesized to be important in the evolution of caste-specific traits (Simola et al. 2013; Rehan and Toth 2015; Chau and Goodisman 2017). Corona et al. (2013), for example, showed that *Pogonomyrmex barbatus* harbors two duplicates of the conventional Vg and suggested they have gone under subfunctionalization to acquire caste-specific functions. The Vg duplicates in this study, however, are not species specific, but very old, with the first duplication event preceding the evolution of sociality in Hymenoptera (Morandin et al. 2014). Our results add to the literature showing how a gene originally regulating reproduction and oogenesis is co-opted to non-reproductive coordination, as predicted by the ovarian ground plan (West-Eberhard 1996), and the more general reproductive ground plan (Amdam et al. 2004) hypotheses. Furthermore, the different roles played by different copies complement the original ground plan hypotheses, as copy-specific functions allow an escape from pleiotropic constraints necessarily faced by multi-functional singe copy genes.

To understand the specific role that Vg-like genes play in social evolution, their co-option into new roles, and their functions within individuals, these genes need to be studied in both social and non-social taxa. Studying their roles across separate evolutionary origins of eusociality would also reveal whether such co-option has occurred convergently in different lineages, according to the logic of genetic toolkit hypothesis (Toth and Robinson 2007). In addition, genome-wide studies in species carrying both primal and more recent Vg duplicates could bring interesting insights on how conserved and novel genes of the same family interact with others to produce alternative phenotypes and whether duplications of the conventional Vg have released possible constraints limiting its caste-specific evolution. A similar examination of not just Vg but also other gene families could shed light on whether duplication events followed by subfunctionalization have played a more general role in social evolution.

Conclusion

This study shows interesting genetic plasticity inside a morphologically monomorphic worker caste with potentially fertile workers, unrelated to age or social context. The upregulation of the conventional Vg in nurses compared to foragers suggests a link between reproductive physiology and worker behaviour, supporting RGPH in ants. The combination of factors that produce the distinct expression profiles corresponding with nursing and foraging behaviour in *F. fusca* still requires closer inspection, as do the functions of the different copies.

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