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Expression of *foraging* and *Gp-9* are associated with social organization in the fire ant *Solenopsis invicta*

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Abstract

The aim of this study was to investigate levels of expression of two major genes, the odorant binding protein *Gp-9* (*general protein-9*) and *foraging*, that have been shown to be associated with behavioural polymorphisms in ants. We analysed workers and young nonreproductive queens collected from nests of the monogyne (single reproductive queen per nest) and polygyne (multiple reproductive queens) social forms of *Solenopsis invicta*. In workers but not young queens, the level of *foraging* expression was significantly associated with social form and the task performed (ie localization in the nest or foraging area). The level of expression of *Gp-9* was also associated with social form and worker localization. In addition there was a higher level of expression of the *Gp-9^b^* allele compared with the *Gp-9^B^* allele in the heterozygote workers and the young nonreproductive queens. Finally, in the polygyne colonies the level of expression of *foraging* was not significantly associated with the *Gp-9* genotype for either workers or young nonreproductive queens, suggesting that both genes have independent non-epistatic effects on behaviour in *S. invicta*.

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Introduction

Explaining how interactions between genes and environment influence social behaviour is a fundamental research goal, yet there is still only limited information for species exhibiting complex social behaviour (Linksvayer & Wade, 2005; Robinson *et al.*, 2005; Linksvayer, 2006; Nedelcu & Michod, 2006). For example, in ants, which have played a central role in the study of the mechanisms regulating social behaviour, two major genes have been shown to be associated with behavioural polymorphisms.

The first example was discovered in the fire ant *Solenopsis invicta*, in which a genomic region containing the odorant-binding protein (OBP) *Gp-9* (*general protein-9*) determines whether colonies contain one queen (monogyne social form) or several queens (polygyne social form) (Ross, 1997; Ross & Keller, 1998; Krieger & Ross, 2002; Gotzek & Ross, 2007). Recently, *Gp-9* was found to be located on a large (13.8 Mb, 55% of the chromosome) genomic region that does not recombine between the two variants [ie the social b (Sb) chromosome contains the *Gp-9^b^* allele and the social B (SB) chromosome contains the *Gp-9^B^* allele (Wang *et al.*, 2013)]. Monogyne colonies invariably contain one SB/SB queen and only SB/SB workers. By contrast, polygyne colonies contain several SB/Sb queens and both SB/SB and SB/Sb workers. This very unusual genotypic distribution stems from the Sb chromosome containing one or more lethal recessive genes, leading to the death of all females (ie queens and workers) with two copies of this chromosome soon after they hatch from the pupae (Ross, 1997; Hallar *et al.*, 2007). Moreover, there is a complete lack of SB/SB queens in polygyne colonies because the Sb chromosome behaves as a selfish genetic element by inducing workers with one copy of this chromosome to eliminate all the reproductive queens lacking a copy of

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this chromosome (ie SB/SB queens) when they initiate reproduction (Keller & Ross, 1998). The nonrecombining region on the pair of social chromosomes contains slightly more than 600 genes. It is likely that several of these genes in the nonrecombining region are implicated in the many other traits [eg queen fecundity, queen tendency to accumulate fat during sexual maturation, the odour of mature queens, the quantity of sperm produced by males and worker size (Keller & Ross, 1998; Goodisman *et al*., 1999; DeHeer, 2002; Ross & Keller, 2002; Vander Meer & Alonso, 2002; Krieger, 2005; Lawson *et al*., 2012)] that differentiate the two social forms.

The other identified genetic component influencing behaviour in ants is the *foraging* gene (*for*). This gene was first described in *Drosophila melanogaster*, in which allelic variations result in differences in the level of foraging behaviour of larvae (Sokolowski, 1980; de Belle & Sokolowski, 1987; de Belle *et al*., 1989). The *for* gene, also known as *dg2*, encodes a cyclic guanosine monophosphate (cGMP)-dependent kinase (PKG) expressed from the *dg2* locus (Osborne *et al*., 1997) and affects hundreds of downstream genes (Kent *et al*., 2009). Genetic manipulations have shown that behavioural polymorphism is related to allelic variation at the *for* locus in *Drosophila melanogaster* (Osborne *et al*., 1997). The *for* gene is also associated with behavioural polymorphisms in food related behaviours in the worm *Caenorhabditis elegans* (Fujiwara *et al*., 2002) and swarming polymorphism in the locust *Schistocerca gregaria* (Lucas *et al*., 2010b). In social insects, variation in the level of expression of *for* regulates temporal division of labour in the honey bee *Apis mellifera* and the ant *Pogonomyrmex barbatus* (Ben-Shahar *et al*., 2002; Ingram *et al*., 2005), as well as caste polyethism and aggressive behaviour in the ant *Pheidole pallidula* (Lucas & Sokolowski, 2009). Thus, the *for*-PKG molecule is emerging as a major player in regulating behavioural polyethism and the observed effect in a wide variety of organisms suggests the possibility that the *for* gene is a general behavioural modifier in the animal kingdom (Sokolowski, 2010; Székely *et al*., 2010; Lucas *et al*., 2010a).

The aim of this study was to investigate the interplay between the level of expression of the *for* gene and the presence or absence of the Sb chromosome in queens and workers of the fire ant *S. invicta*. To this end we collected young winged queens that had not yet departed on a mating flight as well as workers both within the nest and in the foraging area. Monogyne and polygyne colonies were both used and reared under laboratory competition to address four main questions.

First, we compared the level of expression of *for* between SB/SB and SB/Sb young winged queens that had not yet initiated reproduction (gynes). Previous work has shown that SB/SB gynes produced by monogyne colonies disperse on the wing to initiate a new colony by feeding the first brood produced from their body reserves (DeHeer, 2002). By contrast, SB/Sb gynes from polygyne colonies disperse much shorter distances and usually infiltrate an existing polygyne colony immediately after the mating flight (Porter *et al*., 1991; Gotzek & Ross, 2007). Given that the level of *for* expression and *for*-PKG activity have been shown to differ in the brains of singly vs. multiply mated queens (Richard *et al*., 2007) and affect search trajectories, foraging strategies, energy homeostasis and lipid storage in *Drosophila* (Sokolowski, 2001; Kaun & Sokolowski, 2009), as well as behavioural polymorphisms in ants (Lucas & Sokolowski, 2009; Lucas *et al*., 2010a), we tested whether the differential tendency of SB/SB and SB/Sb queens to disperse was associated with differences in the level of expression of *for*.

Second, we compared the level of expression of *for* between SB/SB and SB/Sb workers from the nest and the foraging area. In the honey bee *Apis mellifera* and in the ant *P. barbatus*, *for* gene function is not only linked to food search behaviour but also to temporal polyethism (Ben-Shahar *et al*., 2002; Ingram *et al*., 2005). In these species, inside-nest workers involved in tasks such as nest cleaning and brood care have different *for* gene expression levels than workers involved in foraging. We therefore hypothesized that there might be a difference in *for* expression between *S. invicta* workers collected inside and outside the nest as they exhibit marked differences in behaviour. To test this we compared the *for* expression of SB/SB and SB/Sb workers collected in the nest and from the foraging area.

Third, we tested whether there was an association between the level of expression of *Gp-9* and the *Gp-9* genotype. *Gp-9* is not expressed in the larval or pupae stages and is undetectable in newly emerged workers and queens. It becomes abundant in females once they are more than 8–14 days old (Ross, 1997; Liu & Zhang, 2004). Despite the proposed role of *Gp-9* in social organization, there is only limited information on the level of expression of this gene in monogyne and polygyne queens and workers (Gotzek & Ross, 2007). Two microarray studies failed to detect variations in expression between SB/SB and SB/Sb workers (Wang *et al*., 2008; Nipitwattanaphon *et al*., 2013), but these studies were not specifically designed for this purpose and there is currently no information for gynes of alternate genotypes. We therefore compared the level of expression of *Gp-9* between SB/SB and SB/Sb queens as well as SB/SB and SB/Sb workers. Moreover, we compared the allelic-specific gene expression of *Gp-9*^{B} and *Gp-9*^{b} in both SB/Sb queens and workers.

Finally, we investigated whether there were differences in the proportion of SB/SB and SB/Sb workers and gynes

in the nest and in the foraging area in polygyne colonies. Previous studies have shown differences between the expected and observed proportions of SB/SB and SB/Sb adults (Ross, 1997; Goodisman *et al.*, 2000; Fritz *et al.*, 2006). For example, in colonies headed by a SB/Sb queen fertilized by a single SB male, there were significant deviations from the expected proportion of 50% SB/SB and 50% SB/Sb workers. However, these studies sampled individuals only from inside the nest, raising the possibility that discrepancies between the expected and observed proportions arose from a nonrandom distribution of workers for each genotype between the nest and foraging area. We therefore compared, for both workers and gynes, the *Gp-9* genotype frequencies between individuals collected in the nest and individuals collected in the foraging area.

Results

foraging *gene expression*

In polygyne colonies, the level of expression of *for* was not significantly associated with the *Gp-9* genotype for workers ($F_{1,126} = 1.19$, $P = 0.28$) and gynes ($F_{1,98} = 0.82$, $P = 0.37$; Fig. 1). Gene expression data from polygyne SB/SB and SB/Sb individuals were therefore pooled together in later analyses. By contrast, the level of expression of *for* was associated with social form, the level of expression being higher in monogyne than polygyne workers ($F_{1,12} = 5.16$, $P < 0.05$) and lower in monogyne than polygyne gynes ($F_{1,9} = 6.32$, $P < 0.05$; Fig. 1).

In colonies of both social forms, there was a large difference in the level of *for* expression between workers

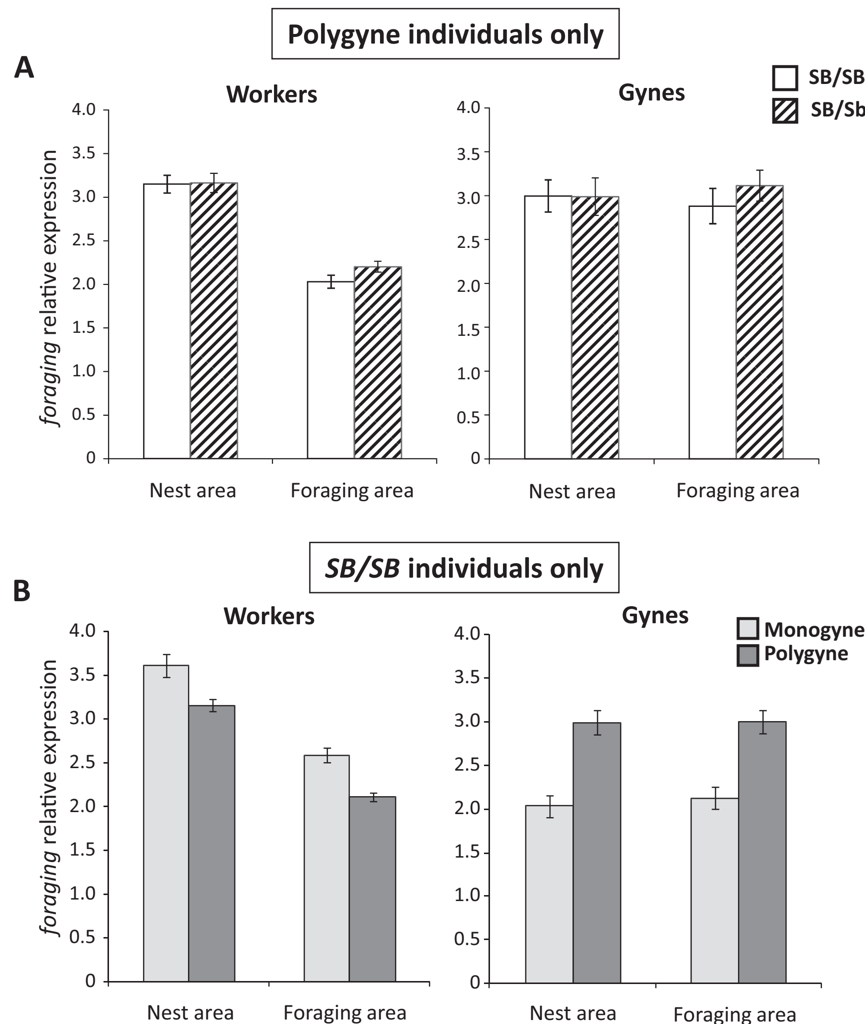

Figure 1. Relative expression of the *foraging* gene. (A) Polygyne individuals only [statistics for workers (social form: NS; location: $P < 0.001$; social form × location: NS); statistics for gynes (social form: NS; location: NS; social form × location: NS)]. (B) SB/SB individuals only [statistics for workers (social form: $P < 0.05$; location: $P < 0.001$; social form × location: NS); statistics for gynes (social form: $P < 0.05$; location: NS; social form × location: NS)]. SB, social B chromosome; Sb, social b chromosome.

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collected from the nest and workers collected from the foraging area ($F_{1,175} = 216.21$, $P < 0.001$) but no significant interaction between location and social form ($F_{1,175} = 0.001$, $P = 0.97$). The level of expression was always higher in workers from the nest than those from the foraging area. By contrast, there was no significant association between the location where gynes were collected and the level of expression of *for* ($F_{1,133} = 0.13$, $P = 0.72$).

Gp-9 *gene expression*

In polygyne colonies, there was a significant association between the *Gp-9* genotype and level of expression of *Gp-9*. The level of expression was higher for *Gp-9^{Bb}* than *Gp-9^{BB}* individuals for both workers ($F_{1,126} = 33.17$, $P < 0.001$) and gynes ($F_{1,98} = 12.90$, $P < 0.001$; Fig. 2A). There was also an effect of the location of workers ($F_{1,126} = 50.85$, $P < 0.001$). For workers of both genotypes and both social forms the level of expression was greater in individuals from the nest compared with individuals outside the nest (Fig. 2A, B). When considering SB/SB workers only (Fig. 2B), the level of expression was higher in monogyne than polygyne individuals ($F_{1,12} = 8.40$, $P = 0.013$). An analysis of SB/Sb individuals (Fig. 2C) revealed a higher level of expression of the *Gp-9^{b}* than the *Gp-9^{B}* allele in both workers ($F_{1,121} = 9.59$, $P = 0.002$) and gynes ($F_{1,95} = 12.82$, $P < 0.001$).

Area repartitions of polygyne individuals

The meta-analyses of the *P*-values (Pearson's Chi^2 tests) revealed that the spatial distribution of workers was different from random with SB/SB individuals being more common in the foraging area than inside the nest ($P = 0.002$; Fig. 3, Table 1). By contrast, there was no such effect for gynes ($P = 0.106$; Table 1).

Discussion

foraging *gene expression in workers and gynes*

This study revealed that the level of expression of *for* is lower in fire ant workers collected from the foraging area than individuals collected inside the nest. This finding is in line with the finding that *for* expression is lower in foragers than nurses in the ant *P. barbatus* (Ingram *et al.*, 2005) and that pharmacological activation of the cGMP-dependent protein kinase encoded by *for* reduces foraging behaviour in the ant *P. pallidula* (Lucas & Sokolowski, 2009). Interestingly, these results in ants differ from the pattern observed in honey bees (Ben-Shahar *et al.*, 2002) and bumble bees (Tobback *et al.*, 2011), in which foragers have a higher expression of *for* than nurses. In nonsocial insects, contrasting results have also been reported, with *for* expression being up-regulated with foraging behaviour in *D. melanogaster* or *Schistocerca gregaria*, but down-regulated in *C. elegans* (Osborne *et al.*, 1997; Fujiwara *et al.*, 2002; Lucas *et al.*, 2010b). In *D. melanogaster*, the precise DNA polymorphism responsible for rover/sitter behavioural polymorphism is still not known (Reaume *et al.*, 2011). In social insects, in all seven ant species sequenced as well as *A. mellifera*, there is only one-to-one orthologue for the *for* gene (unpublished part of the fourmidable database, Wurm *et al.*, 2009). In *S. invicta*, the *for* gene is located on the linkage group LG1, which is not on the social chromosome (Wurm *et al.*, 2011) and no information is available on sequence polymorphisms.

Our data also revealed that the expression of *for* is lower in workers from polygyne colonies than those from monogyne colonies. This finding is interesting with regards to the difference in the level of aggression between the two types of workers. Several experiments have shown that monogyne workers are more aggressive than polygyne workers (Chirino *et al.*, 2012), whereas studies in the ant *P. pallidula* found that the soldiers, which are involved in colony defence, have higher *for*-PKG activity than workers less involved in colony defence. Moreover, pharmacological treatments using a specific activator of *for*-PKG did increase aggressive behaviour (Lucas & Sokolowski, 2009), raising the possibility that differences in the expression of *for* may mediate the differences in aggressiveness between monogyne and polygyne fire ant workers. By contrast, the opposite pattern was observed in gynes in which *for* expression was lower for monogyne individuals than polygyne individuals. This social form effect on *for* expression may be correlated to the higher capacity to disperse of the monogyne gynes (DeHeer, 2002). However, because there is no genotypic effect, more experiments are needed to determine whether the level of *for* expression has effects on patterns of dispersal. Importantly, the difference in expression of *for* in gynes and workers of the two social forms is unlikely to be explained by genotypic differences because, in polygyne colonies, there was no significant difference in the level of expression of *for* between SB/SB and SB/Sb individuals (both for workers and gynes).

Gp-9 *gene expression in workers and gynes*

Our analyses also revealed that the expression of *Gp-9* is differentially regulated between monogyne and polygyne workers. When considering only SB/SB workers, there was a higher level of expression in monogyne than polygyne individuals. By contrast, a previous study performed on whole bodies showed no significant difference between the two social forms (Wang *et al.*, 2008). A possible explanation for this discrepancy lies in the type of tissues analysed, as we used only brains in this study. Nevertheless, consistent with Wang *et al.* (2008), we

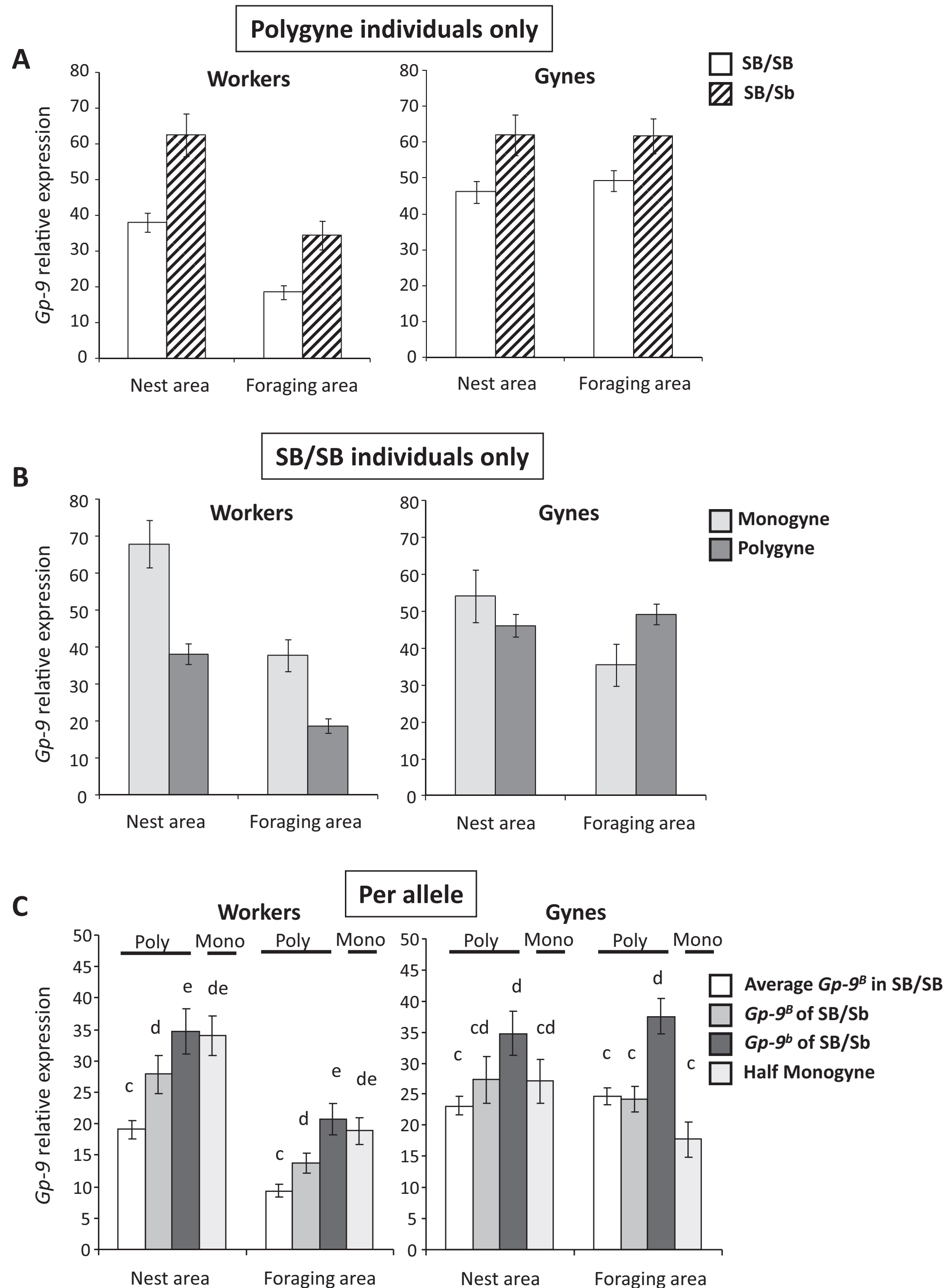

Figure 2. Relative expression of *Gp-9*. (A) Polygyne individuals only [statistics for workers (social form: $P < 0.001$; location: $P < 0.001$; social form × location: NS); statistics for gynes (social form: $P < 0.001$; location: NS; social form × location: NS)]. (B) S*B/SB* individuals only [statistics for workers (social form: $P < 0.05$; location: $P < 0.001$; social form × location: NS); statistics for gynes (social form: NS; location: NS; social form × location: $P < 0.01$)]. (C) Per allele [significant differences between pairwise tests ($P < 0.05$) within the same location are represented with different letters]. Statistical comparisons between locations are not shown but each genotype comparison was significant for workers ($P < 0.05$) and only one was significant for gynes (half monogyne vs. *Gp-9^B* allele, $P < 0.01$). SB, social B; Sb, social b.

found that the level of *Gp-9* expression was associated with the genotype, with higher expression in SB/Sb than SB/SB workers. For gynes, the expression of *Gp-9* was also higher in SB/Sb than SB/SB individuals. This finding also contrasts with a previous study that showed no differences in the level of expression of *Gp-9* between SB/Sb and SB/SB queens within the age classes investigated (1-day-old, 11-day-old and reproductive queens; Nipitwattanaphon *et al*., 2013). There are two possible explanations for this discrepancy. The first again relates to

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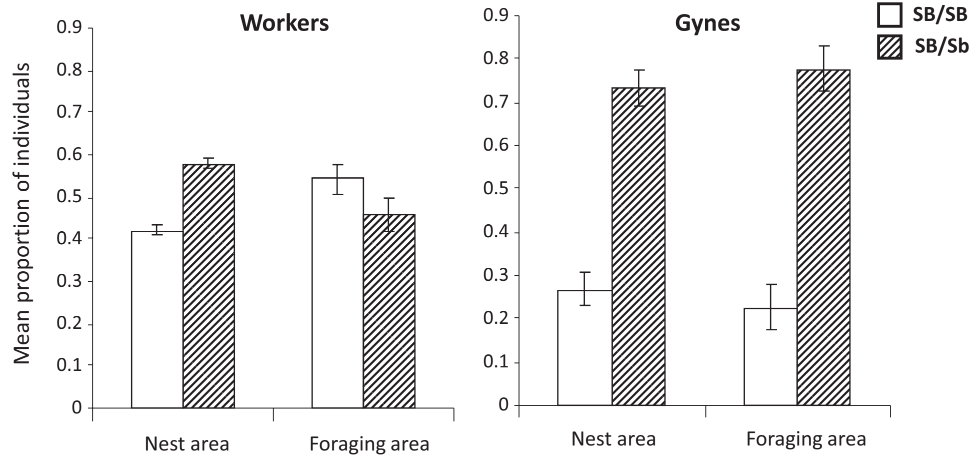

Figure 3. Mean proportion (± SE) of polygyne individuals in each location according to their *Gp-9* genotypes. Worker values were calculated from eight colonies with 166 to 189 individuals per colony; gyne values were calculated from six colonies with 107 to 154 individuals per colony. See detailed statistical results in Table 1. SB, social B; Sb, social b.

the type of tissue analysed (brains for this study vs. whole bodies for previous studies). Alternatively, it could also be that the difference observed in our study reflects age differences between the gynes analysed. The level of expression of *Gp-9* seems to be age-related in queens, as suggested by microarray data showing that the level of expression is highest in reproductive queens, intermediate in 11-day-old gynes and the lowest in 1-day-old gynes (Nipitwattanaphon *et al.*, 2013). Thus, the difference between genotypes in our study could possibly have arisen if the SB/Sb gynes were older than the SB/SB gynes (although there is no reason for such a bias to occur in our design). Finally, it is important to note that the difference in Gp-9 expression observed in this study was quite small and less likely to be detected with a cDNA-probes microarray approach [as used in the studies of Wang *et al.* (2008) and Nipitwattanaphon *et al.* (2013)] than a quantitative PCR (qPCR) approach (used in this study), which is more sensitive (see review, Chuaqui *et al.*, 2002).

Gp-9 has been shown to encode for an OBP (Krieger & Ross, 2002; Gotzek & Ross, 2007). OBPs play a role in insect chemoreception by transporting hydrophobic odorant molecules to the odorant receptors of the sensory neurones (Blomquist & Vogt, 2003). Thereby, variations in OBP expression may interfere with pheromone

Table 1. Total number of polygyne individuals in each location according to their *Gp-9* genotypes. The column 'SB/SB proportions' represents the proportions of individuals in the nest or foraging area. Positive correlation values are in bold as well as significant *P*-values. The *P*-values were calculated with Pearson's Chi² test (meta-analysis *P*-values: workers $P = 0.002$; gynes $P = 0.106$)

		Nest area			Foraging area			SB/SB proportions				
Caste	Colony	SB/SB	SB/Sb	Total	SB/SB	SB/Sb	Total	Nest	Foraging	Chi²	*P*-value	r
Workers	191Pg8	36	50	86	51	40	91	0.42	0.56	3.559	0.059	**0.142**
Workers	208Pg8	43	51	94	61	34	95	0.46	0.64	6.510	**0.011**	**0.186**
Workers	210Pg8	43	46	89	63	30	93	0.48	0.68	7.058	**0.008**	**0.197**
Workers	212Pg8	38	53	91	36	56	92	0.42	0.39	0.131	0.717	–0.027
Workers	213Pg8	36	54	90	38	49	87	0.40	0.44	0.246	0.620	**0.037**
Workers	216Pg8	36	53	89	37	49	86	0.40	0.43	0.119	0.730	**0.026**
Workers	241Pg8	34	48	82	50	34	84	0.41	0.60	5.415	**0.020**	**0.181**
Workers	244Pg8	34	57	91	54	37	91	0.37	0.59	8.801	**0.003**	**0.220**
Gynes	197Pg8	25	63	88	15	51	66	0.28	0.23	0.633	0.426	–0.064
Gynes	215Pg8	11	48	59	4	44	48	0.19	0.08	2.334	0.127	–0.148
Gynes	218Pg8	23	63	86	14	70	84	0.27	0.17	2.534	0.111	–0.122
Gynes	219Pg8	16	40	56	32	43	75	0.29	0.43	2.744	0.098	**0.145**
Gynes	222Pg8	14	81	95	16	34	50	0.15	0.32	5.950	**0.015**	**0.203**
Gynes	244Pg8	41	53	94	7	49	56	0.44	0.13	15.616	**0.000**	–0.323

SB, social B chromosome; Sb, social b chromosome.

transduction but there is a complex functional mosaic of combinatorial recognition patterns between OBPs and odorant receptors, making straightforward interpretations difficult (Swarup *et al.*, 2011). In insects, some OBPs are also expressed in nonchemosensory tissues such as the male accessory gland (Paesen & Happ, 1995), the abdomen (He *et al.*, 2011), and the haemolymph (Graham *et al.*, 2001; Paskewitz & Shi, 2005) raising question about what their function is (Vogt, 2005; Forêt & Maleszka, 2006; Gotzek & Ross, 2007). In female ants, OBPs are only present in small quantities in the antennae (Ishida *et al.*, 2002; Ozaki *et al.*, 2005) and it has been suggested that *Gp-9* might not be involved in pheromone detection within chemosensilla but may act as a transporter of pheromone into the haemolymph (Calvello *et al.*, 2003; Pelosi *et al.*, 2005; Gotzek & Ross, 2007). Regardless of the specificity of *Gp-9* compared with 'standard' OBPs, the regulation of colony queen number is clearly linked to specific chemical signals emanating from the queens (Keller & Ross, 1998; Ross & Keller, 1998). There has been considerable controversy over the role of *Gp-9* in mediating odour differences amongst individuals (Leal & Ishida, 2008; Gotzek & Ross, 2009). A recent study showed that *Gp-9* is in a large (*c.* 13.8 Mb), nonrecombining region, raising the possibility that one or several of the *c.* 600 linked genes may be involved in odour differences amongst individuals with alternate genotypes (Wang *et al.*, 2013). Further support for the view that other genes may be involved in odour differences comes from microarray analyses showing that 18 genes involved in chemical signalling are differentially expressed between $Gp\text{-}9^{BB}$ and $Gp\text{-}9^{Bb}$ queens (Wang *et al.*, 2008; Nipitwattanaphon *et al.*, 2013). Thus, variations in *Gp-9* expression such as those observed in this study call for future experiments to disentangle the causes and consequences of the observed variations of *Gp-9* expression and their potential role in the regulation of colony queen numbers.

Interestingly, our study revealed higher expression of the $Gp\text{-}9^{b}$ allele than the $Gp\text{-}9^{B}$ allele in both heterozygote workers and gynes. The significance of the higher expression of the $Gp\text{-}9^{b}$ than the $Gp\text{-}9^{B}$ allele in both heterozygote workers and gynes remains unclear but may also be associated with the selective elimination of $Gp\text{-}9^{BB}$ queens by $Gp\text{-}9^{Bb}$ workers (Keller & Ross, 1998). The finding of higher expression of the $Gp\text{-}9^{b}$ than the $Gp\text{-}9^{B}$ allele in both heterozygote workers and gynes is interesting because previous work on sex chromosomes has revealed that genes in the Y or Z nonrecombining chromosome usually have lower levels of expression than their homologues on the X or W recombining chromosomes (Hahn & Lanzaro, 2005; Xiong *et al.*, 2010; Deng *et al.*, 2011; Larschan *et al.*, 2011). An analysis of the level of expression of 288 genes in the nonrecombining region of SB/Sb queens revealed significant allele-specific expression differences for about 11% of the genes but there was no evidence for a pattern of higher expression of alleles on the SB haplotype compared with the Sb haplotype (Wang *et al.*, 2013). The lack of a consistent difference in the level of expression of genes located on the SB and Sb chromosomes may reflect the fact that there has been only limited degeneration of the Sb chromosome, possibly because it recently evolved and also because there is selection at the haploid stage on Sb males (Correns, 1908; Bachtrog *et al.*, 2011; Bergero & Charlesworth, 2011; Wang *et al.*, 2013). It remains to be investigated whether the higher expression of the $Gp\text{-}9^{b}$ than $Gp\text{-}9^{B}$ allele is because of selection or just random changes in the level of expression as has been documented for the neo-Y chromosome in *Drosophila miranda* (Bachtrog, 2006). It may also imply differences in the underlying regulatory machinery amongst individuals of alternative genotypes or possible some genomic rearrangement in the nonrecombining region.

Area repartitions of polygyne individuals

The *Gp-9* genotypic distribution of workers was found to be associated with their location in the colony. Although significant, the effect was small and not found in all colonies. In polygyne colonies, there was an overrepresentation of SB/SB workers in the foraging area compared with the nest. Given that $Gp\text{-}9^{b}$ is linked to more than 600 other genes in the nonrecombining region (Wang *et al.*, 2013), it is currently not possible to determine why there is an association between genotype and spatial distribution. Previous work has shown that complex interactions between genetic backgrounds can affect division of labour through task efficiency (Oldroyd & Fewell, 2007; Libbrecht & Keller, 2013). It is thus possible that one or several genes in the nonrecombining region may affect worker propensity to move from one within-nest task to foraging. Those genes may also affect lifespan thus leading to different genotypic representation in the nest and outside. Whatever the mechanism, the finding of differential genotypic representation inside and outside the nest calls for special care when collecting individuals in studies aimed at determining behavioural and other phenotypic differences associated with genotype. In that respect it is notable that several previous studies showed discrepancies between the expected and observed $Gp\text{-}9^{BB}$ genotype proportions (Ross, 1997; Goodisman *et al.*, 2000; Fritz *et al.*, 2006). Detailed studies on the differential distribution of workers of alternative genotypes remain to be conducted to elucidate the reasons underlying these discrepancies between the expected and observed differences in genotypic distribution.

In contrast to workers, there was no significant association between genotype and location in the nest for gynes.

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However, there was a low proportion of SB/SB gynes (0.25 ± 0.03 mean proportion ± SE) amongst the gynes collected, similarly to previous studies (Ross, 1997; Goodisman *et al.*, 2000). This low proportion reflects the fact that they are selectively eliminated by SB/Sb workers when they initiate reproduction in polygyne colonies (Keller & Ross, 1998, 1999).

Conclusions

This study revealed that two genes associated with behavioural polymorphisms, *for* and *Gp-9*, are related to social organization in the fire ant *S. invicta*. The level of *for* and *Gp-9* expression was associated with social form and there was a large difference in the level of expression between workers collected from the nest and workers collected from the foraging area. It is likely that other factors such as worker size and age (foragers tend to be older than nurses) could also have an impact on expression of these genes and it would be interesting to investigate the exact relationship amongst all of these variables. In addition, there was also a higher level of expression of the $Gp\text{-}9^b$ allele compared with the $Gp\text{-}9^B$ allele for both workers and gynes. Finally, the level of expression of *for* was not significantly associated with the *Gp-9* genotype for either workers or gynes, suggesting that both genes have independent non-epistatic effects on behaviour in *S. invicta*.

Experimental procedures

Colonies collection and rearing conditions

Monogyne and polygyne colonies of *S. invicta* were collected near Athens (Georgia, USA) in April 2008 and reared under standard laboratory conditions to minimize environmental effects (Jouvenaz *et al.*, 1977). Social forms were determined after collection by genotyping several individuals per nest and queens were morphologically checked (Ross & Keller, 1995; Ross, 1997). Colonies were acclimatized for 3 months in the laboratory before sampling. Ants were reared into a plaster nest localized in a plastic box covered with Teflon (Whitford LTD., Runcorn Cheshire, UK) on the top. Feeding occurred on the same days twice a week. Food and water were deposited only into a second plastic box connected with the first box by a 1-m plastic tube (outside surface was covered with Teflon). To get access to the food, ants had to get out of the plaster nest (nest area) and move through the tube from the first to the second box and reach the food in a cup (foraging area).

Sampling of individuals

Individuals were sampled with a vacuum aspirator 2 days after feeding between 13:30 and 17:00 h in order to minimize variations in gene expression. To avoid DNA/RNA sample bias owing to size or age, we collected only winged queens and medium-sized workers with dark cuticles to avoid very young individuals, which are yellowish. The first group of individuals was collected in the plaster nest near the brood and another group in the foraging area near the food cup. All individuals collected in the foraging arena were foragers whereas the vast majority of individuals collected near the brood were nurses [as for other ants (Hölldobler & Wilson, 1990)]. There is a clear spatial separation between nurses and foragers in *S. invicta* and, like other social insects that have been studied (Mersch *et al.*, 2013), foragers tend to be older than nurses (Mirenda & Vinson, 1981). All collected individuals were immediately anaesthetized with CO_2 and flash-frozen in liquid nitrogen. Ants were genotyped using DNA from the body (thorax and abdomen) and qPCR analyses were carried out using heads only. To do so, heads were removed from the body using a scalpel blade on a cooled sterile pod. To avoid DNA and RNA contamination, blades and forceps were cleaned in ethanol then flamed and cooled down in liquid nitrogen for the dissection of each individual. Bodies and heads were then flash-frozen in liquid nitrogen and stored at –80 °C until extraction.

Table 2. Primer sequences [final concentrations: 500 nM for genotyping and pyrosequencing; 200 nM for quantitative PCR (qPCR)]

Name	Sequence 5′–3′	Experiment
GP9CLF2	TCT CGA TTG GTG AAG TAT CAA GT	Genotyping
GP9CLR2	CAT GTC AAT ACA AAA GAA AGC TG	Genotyping
GP9CLF1	GAT ACC GAA CTA CAC AAA AAT GGT TGC	Genotyping
GP9CLR1	TTA GAA TCG GCG AGC ACA GCT T	Genotyping
Eef1a1_F2	CAG CCG ATG TAG CTC ACC CT	qPCR
Eef1a1_R2	TGT CAA ATT CGT CTC CCG TG	qPCR
qGAPDHP1_F1	GCT ACA CCG AGG ACG AAG TTG	qPCR
qGAPDHP1_R1	CCA CGA GAT CAG TTT CAC GAA G	qPCR
RPS-3_F3	GTG ACG ATC TTT CGG CAT GG	qPCR
RPS-3_R3	TTG GAG AAG ACC GAC GGA AT	qPCR
qforP1_F1	CTG CCC GAG GAA ACT CTA ATC	qPCR
qforP1_R1	CAC CTC TCG CTC CTT GTC TTA T	qPCR
GP9-41F	TGG CTT TCG CTT CTG CAT C	qPCR
GP9-141R	GTC ATC CTC TGT TAG ACT ATG TTC GG	qPCR
GP9SNIP_F1	AGG ATC CCA ATA TGA CAA TTA CGC	Pyrosequencing
GP9SNIP_R1	Biotinilated-TTT TGT GCT GGC CAC TTG ATA CT	Pyrosequencing
GP9SNIP_S1	TAC GCG ACT TGC TTA	Pyrosequencing

Gp-9 *genotyping*

Bodies (thorax and abdomen) were ground by using 3 mm Tungsten Carbide Beads (Qiagen, Hombrechtikon, Switzerland) in a TissueLyser (Qiagen). DNA was extracted using a DNeasy 96 Blood & Tissue Kit with the Biosprint 96 extraction robot following the manufacturer's protocol (extraction of animal tissues, Qiagen). DNA was suspended in 150 μl Milli-Q water (Millipore, Billerica, MA, USA) after extraction and then concentrated to 10 μl with a Vacufuge speedvac (Eppendorf, Montesson, France). The *Gp-9* gene was amplified using a nested PCR amplification method. Two μl of DNA were amplified by a first PCR reaction with primers GP9CLF2 and R2 (Table 2) with 0.5 units of Taq DNA polymerase (Qiagen, 201203) in 20 μl PCR mix [94 °C, 5 min; 30× (94 °C, 30 s; 57 °C, 30 s; 72 °C, 45 s); 72 °C, 10 min; 4 °C until stored). Two μl of the first PCR were used for the second nested PCR amplification using primers GP9CLF1 and R1 with 0.5 units of Taq polymerase in 20 μl mix [94 °C, 5 min; 35× (94 °C, 30 s; 64 °C, 30 s; 72 °C, 45 s); 72 °C, 10 min; 4 °C until stored].

The *Gp-9* genotype was determined by performing two independent allele-specific restriction fragment length polymorphism

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analyses using two different enzymes specific to either *GP-9*^{B} and *GP-9*^{b} allele (on amino acid position 117). First the *Gp-9*^{B} allele band was cut by the *Bsm*I restriction enzyme (New England BioLabs, Beverly, MA, USA; R0134L), which gave rise to a 470-bp and a 298-bp product (this enzyme does not cut the *b* allele). Three μl of PCR products were used in a total mix of 15 μl using 10 units of *Bsm*I following the conditions given by the manufacturer. For additional verification, we used the *Bve*I (*Bsp*MI) restriction enzyme (New England Biolabs, R0502L). This enzyme only cuts the *Gp-9*^{b} allele, giving rise to a 481-bp and a 289-bp product. Three μl of PCR products were used in a total mix of 15 μl using 1 unit of *Bve*I. Digestion products were then electrophoresed in a 2% TAE (Tris-acetate-EDTA buffer) agarose gel and analysed for band patterns.

RNA extraction

Pools of five worker heads with the same genotype, or individual head for gynes, were ground using 1.4 mm zirconium silicate beads (QuackenBush, Crystal Lake, IL, USA) in a FastPrep24 tissue lyser (MP, 116004500) at 4 °C for two periods of 60 s with a 30-s gap. Between three and five samples were processed per colony. *Gp-9*^{bb} individuals were too rare to permit meaningful analyses and were thus discarded from the study. RNAs were extracted using the RNeasy Mini kit (Qiagen, 74106) with DNase treatment then eluted in 50 μl Milli-Q water (Millipore). Samples were concentrated with a Vacufuge speedvac (Eppendorf), resuspended in 11 μl of Milli-Q water and stored at −80 °C. Owing to the low RNA concentration, a Quant-iT RiboGreen kit (Invitrogen, Carlsbad, CA, USA; R11490) was used for quantification using 1 μl of each sample. Three technical replicates were measured using a black-coloured F96 MicroWell plate (Nunc, Thermo Fisher Scientific, Villebon sur Yevette, France; 137103) in a SpectraFluor Plus fluorometer (Tecan). RNA quality was controlled using an Agilent RNA 6000 Pico Kit (Agilent, Palo Alto, CA, USA; 5067-1513) with a Pico Chip in a 2100 Bioanalyser (Agilent).

cDNA synthesis and qPCR

The same cDNA samples were used for qPCR analyses of *Gp-9* and of *for*. Worker and gyne samples were treated separately in two independent experiments but under the same conditions. Six monogyne and eight polygyne colonies were used for worker analyses; four monogyne and six polygyne colonies for gyne analyses. cDNA synthesis was performed using 56.5 ng of total RNA for all samples with DNA polymerase Superscript III (Invitrogen, 12574-018), random hexamers (Invitrogen 48190-011) and RNasin Plus RNase Inhibitor (Promega, Madison, WI, USA; N2611). Reverse tanscriptase reactions were diluted 10 times before performing quantitative expression analyses (qPCR) with an ABI 7900 thermocycler (Applied Biosystems, Foster City, CA, USA) at the DNA array facility of Lausanne (DAFL). Pipetting was carried out with a robot (Tecan) on a 384-well plate. Amplification was monitored by Power SYBR Green detection (Applied Biosystems, 4309155). Three technical replicates were performed per sample and a unique reference sample was used for all plates to ensure direct normalization and comparison between plates. cDNA of the reference sample was synthesized from 950 ng of RNA, extracted from 100 whole body monogyne workers. It was then conserved in aliquots at −80 °C and the stability (relative abundance) was validated by qPCR. Specific primers for both genes of interest and for the reference genes were designed with PRIMER3 software (Rozen & Skaletsky, 2000) using the fourmidable database (Wurm *et al.*, 2009; Table 2). *Gp-9* primers were designed from a consensus sequence of all *GP-9* alleles (*B* or *b*). The *for* gene sequence is available with the accession number KJ874960 (GenBank). The three reference genes used to normalize expression [eukaryotic translation elongation factor 1A (EeF1A), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ribosomal protein S3 (RPS-3)] underwent preliminary validation by checking their dissociation curves and using sample dilutions. We also tested the efficiency and specificity of the primers (efficiency tests showed a dissociation curve with only one peak).

Pyrosequencing

Because of the genotype effects within polygyne individuals we sequenced *Gp-9*^{Bb} individuals using pyrosequencing with the samples used for qPCR and estimated the relative expression of the *Gp-9*^{B} or *Gp-9*^{b} alleles. Single nucleotide polymorphism (SNP) analyses were performed on a PSQ96 machine (Qiagen) with specific primers designed with PSQ ASSAY DESIGN software from Qiagen (Table 2). Primers amplified two SNPs (amino acid positions 39 and 42) specific for each *Gp-9* allele. The use of two SNPs allow to perform a double control. The primer GP9SNIP_S1 was used for sequencing purposes. Two μl of DNA was amplified by PCR reaction with the primers GP9SNIP_F1 and R1 [94 °C, 5 min; 45× (94 °C, 15 s; 57 °C, 30 s; 72 °C, 15 s); 72 °C, 5 min; 4 °C until stored] with 1.5 units of Taq DNA polymerase (Qiagen) in 50 μl PCR mix.

Statistical analyses

Relative expression of target genes were calculated using ΔΔCt with QBASE software v. 1.3.5 (Hellemans *et al.*, 2007). Normalized gene expression means were tested using generalized linear mixed-effect models (GLMMs) within workers and gynes with JMP 7 software (SAS Institute, Cary, NC, USA). Social forms and locations were processed as fixed factors and colony of origin as a random factor. Pairwise comparisons were carried out with GLMMs with colony as a random effect and the normality of residuals was checked (Bolker *et al.*, 2009).

We used a meta-analysis method to test whether there was an effect of genotype on the repartition of individuals in the nest vs. foraging area in polygyne colonies. The number of individuals of each genotype in each area was counted, transformed into proportions and used as a response variable (repartition) in a global analysis (Rosenthal, 1991; Rosenberg *et al.*, 2000). Repartition effect is a correlation coefficient (r) that gives an estimate of how individuals are distributed between the two areas depending on their genotypes. We defined a positive effect when SB/SB individuals were more present in the foraging area than the nest area and a negative effect when they were more present in the nest area than the foraging area. The *P*-values were calculated with Pearson's Chi^2 test using R 2.7 software (http://www.r-project.org/).

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