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## GENETIC REGULATION OF COLONY SOCIAL ORGANIZATION IN FIRE ANTS: AN INTEGRATIVE OVERVIEW

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### ABSTRACT

*Expression of colony social organization in fire ants appears to be under the control of a single Mendelian factor of large effect. Variation in colony queen number in *Solenopsis invicta* and its relatives is associated with allelic variation at the gene *Gp-9*, but not with variation at other unlinked genes; workers regulate queen identity and number on the basis of *Gp-9* genotypic compatibility. Non-genetic factors, such as prior social experience, queen reproductive status, and local environment, have negligible effects on queen number, which illustrates the nearly complete penetrance of *Gp-9*. As predicted, queen number can be manipulated experimentally by altering worker *Gp-9* genotype frequencies. The *Gp-9* allele lineage associated with polygyny in South American fire ants has been retained across multiple speciation events, which may signal the action of balancing selection to maintain social polymorphism in these species. Moreover, positive selection is implicated in driving the molecular evolution of *Gp-9* in association with the origin of polygyny. The identity of the product of *Gp-9* as an*

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*odorant-binding protein suggests plausible scenarios for its direct involvement in the regulation of queen number via a role in chemical communication. While these and other lines of evidence show that Gp-9 represents a legitimate candidate gene of major effect, studies aimed at determining (i) the biochemical pathways in which GP-9 functions; (ii) the phenotypic effects of molecular variation at Gp-9 and other pathway genes; and (iii) the potential involvement of genes in linkage disequilibrium with Gp-9 are needed to elucidate the genetic architecture underlying social organization in fire ants. Information that reveals the links between molecular variation, individual phenotype, and colony-level behaviors, combined with behavioral models that incorporate details of the chemical communication involved in regulating queen number, will yield a novel integrated view of the evolutionary changes underlying a key social adaptation.*

**A** MAJOR GOAL in evolutionary biology is to understand the genetic architecture of complex adaptations in wild populations (Orr and Coyne 1992; Orr 2005). Comprehensive models of the evolution of significant adaptations can only be developed with information on the numbers and types of genes influencing expression of key phenotypes, spectra of mutational effects at these genes, patterns of epistasis and degree of pleiotropy the genes exhibit, and norms of reaction of the genotypes (e.g., Robinson 1999; Baker et al. 2001; Linksvayer and Wade 2005; Nachman 2005; Vasemägi and Primmer 2005; Greenberg and Wu 2006). In the study of social behavior, in particular, intense interest has centered on the genetic architectures underlying important social adaptations (de Bono and Bargmann 1998; Krieger and Ross 2002; Lim et al. 2005; Linksvayer and Wade 2005; Robinson et al. 2005; Nedelcu and Michod 2006). This interest stems, in large part, from curiosity about the numbers and types of genetic steps that convert a solitary animal into a group-living one, and that, subsequently, can radically transform social structures. Such information is substantially enriched when accompanied by descriptions of the causal biochemical and physiological links between molecular variation, individual phenotype, and social behavior, an integrative view that Robinson (1999) has termed “sociogenomics.”

Increasingly, genetic components to specific behaviors have been documented in social insects. For example, individual reproductive roles in honey bees (Moritz and Hillesheim 1985; Page and Robinson 1994; Montague and Oldroyd 1998), worker task performance in ants, wasps, honey bees, and caterpillars (Snyder 1992; Hunt et al. 1995;

O'Donnell 1996; Fewell and Bertram 2002; Costa and Ross 2003), and components of the dance language in honey bees (Johnson et al. 2002) have been shown to have a heritable basis. At the level of the society, genetic effects on colony social organization have been documented in ants and sweat bees (Cahan et al. 1998; Plateaux-Quénu et al. 2000). Remarkably, a few complex group-level traits have been shown to have an apparently simple genetic architecture featuring one or few factors of major effect (Moritz 1988; Hunt et al. 1995; Ross and Keller 1998; Johnson et al. 2002). Of these, the best characterized system involves the genetic regulation of colony queen number in fire ants.

The fire ant *Solenopsis invicta* exists in two distinct social forms that differ in the number of reproductive queens per colony. Monogyne colonies are always headed by a single reproductive (wingless, egg-laying) queen, whereas polygyne colonies contain multiple such queens (sometimes hundreds). The two social forms differ not only in colony queen number, but also in many other life-history and reproductive traits (Ross and Keller 1995; Tschinkel 2006). For instance, colony founding in the monogyne form occurs when young virgin queens undertake nuptial flights, when they mate and disperse widely, then attempt to start a colony without the help of workers (independently) and without foraging (claustrally). In preparation, young monogyne queens accumulate extensive fat reserves during a two-week maturation period in their natal nest (Keller and Ross 1993a, 1999; DeHeer 2002). Young polygyne queens, in contrast, often make only very limited nuptial flights or forgo them altogether and mate within their nest (Porter 1991; Ross et al. 1996a; DeHeer et al. 1999;

Goodisman et al. 2000a). Queens of this form rarely try to found nests independently, instead opting to enter existing polygyne nests in attempts to gain admission as new reproductives (Glancey and Lofgren 1988; Porter 1991; Goodisman and Ross 1999). Associated with these alternative reproductive strategies, most young polygyne queens acquire far less fat reserves than their monogyne counterparts (Keller and Ross 1993a; DeHeer 2002).

The contrasting dispersal and reproductive strategies in the two social forms lead to different colony and population structures. Mature monogyne colonies (with their single reproductive queens) are simple families that are highly territorial and aggressive toward nonnestmate conspecifics (Ross and Fletcher 1985; Ross et al. 1997; Vander Meer and Alonso 2002), which leads to a highly overdispersed distribution of nests (Tschinkel 2006). Polygyne colonies contain multiple queens and families, and both nestmate relatedness and aggression between nonnestmates is reduced compared to the monogyne form (Ross and Fletcher 1985; Ross et al. 1997; Vander Meer and Alonso 2002). Consequently, polygyne ants move between nests, including newly budded and parent nests (Vargo and Porter 1989). Nests are frequently clustered, and individual ants (as well as nests) are present at far higher densities than in the monogyne form (Porter 1992). Thus, a seemingly simple difference between the forms, colony queen number, affects population attributes such as colonizing potential, as well as higher order ecological attributes such as energy flow through food webs (Tschinkel 2006). Although some differences exist between polygyne populations in the native South American range and in the United States, where the species has been introduced, the fundamental dichotomy in colony and population structure associated with the two social forms is universal in *S. invicta* (Ross et al. 1996a, 1997; Porter et al. 1997).

The existence of a strong heritable component to the expression of colony queen number and allied individual traits, featuring a single Mendelian factor of large effect, has been well documented in *S. invicta* and its close relatives over the past two decades. In this paper, we review the evidence for this ge-

netic architecture, discuss the influence of nongenetic factors on social organization, and describe a candidate gene implicated in regulation of this complex social syndrome. We then discuss the remaining important gaps in our knowledge of this system, emphasizing the most promising avenues for future research to fill them. We conclude by presenting a new behavioral model for the regulation of colony queen number in fire ants that is consistent with available data. Future refinement of our model, pending the advent of information linking molecular variation, individual phenotype, and colony-level behaviors, promises to provide new insights into the evolution of a key social adaptation.

#### WHAT HAS BEEN LEARNED ABOUT *Gp-9*

##### A SINGLE MENDELIAN FACTOR REGULATES SOCIAL ORGANIZATION IN *S. INVICTA*

Variation in colony queen number in introduced *S. invicta* in the United States appears to be determined by allelic variation at a single Mendelian factor. This has been established by virtue of the fact that variation in social organization is completely associated with allelic variation at a single nuclear, protein-coding gene. This gene, designated *general protein-9* (*Gp-9*), encodes electrophoretically distinct protein products that have been shown to be inherited as allelic variants (Ross 1997; see Keller 2007 for an account of the discovery of *Gp-9*). The nature of the association between social organization and *Gp-9* variation is remarkably simple (Ross 1997; Ross and Keller 1998; Shoemaker et al. 2006); without exception, the *b* allele of the gene (*Gp-9<sup>b</sup>*) is represented at relatively high frequency among the workers and queens in polygyne colonies, however, this allele is never found among the inhabitants of monogyne colonies. Colonies of the monogyne form instead contain only individuals bearing the alternate, *B* allele (*Gp-9<sup>B</sup>*), which also segregates along with allele *b* in the polygyne form. An important related difference in *Gp-9* composition between the forms concerns the genotypes of reproductive queens. While all monogyne colonies are headed by a single homozygous *BB* queen, effectively all

reproductive queens in polygyne colonies are *Bb* heterozygotes. (Reasons for the near exclusive occurrence of these heterozygotes are discussed below.) Thus, there is no overlap in the genotypes of reproductive queens of the two social forms. Importantly, DNA sequence data have confirmed the allelic nature of the *B* and *b* variants of *Gp-9* initially characterized by protein electrophoresis (Krieger and Ross 2002).

Allelic variation at a second nuclear gene, *phosphoglucosyltransferase-3* (*Pgm-3*), has also been associated with social organization in *S. invicta* (Ross 1992; Keller and Ross 1993b; Ross et al. 1996b), although the association is not complete as it is for *Gp-9*. Indeed, variation at *Pgm-3* does not remain significantly associated with traits diagnostic for the two forms, such as queen weight and queen acceptability to workers, once the effects of *Gp-9* genotype are taken into account (Keller and Ross 1999). The cause of the association of *Pgm-3* variation with social organization when the gene is considered in isolation presumably is its tight linkage to and strong gametic disequilibrium with *Gp-9* and/or other genes linked to *Gp-9* that affect social behavior (Ross 1997; Keller and Ross 1999).

Other nuclear genes surveyed in *S. invicta* do not exhibit meaningful differentiation associated with social organization. Indeed, allele frequencies at a variety of polymorphic loci typically are highly similar between the two social forms where they occur in sympatry in the introduced (United States) and native (Argentina) ranges (Figure 1), consistent with extensive interform gene flow (Ross and Shoemaker 1993; Shoemaker and Ross 1996; Goodisman et al. 2000b). Evident conclusions to be drawn from these data are: (i) most of the nuclear genome is shared between the two social forms by virtue of their recent ancestry and ongoing gene flow; (ii) elements of the genome marked by *Gp-9* and *Pgm-3* are not shared as extensively between the forms, presumably because of incompatibilities they induce between the forms in their social and breeding habits (Ross and Shoemaker 1993; Shoemaker and Ross 1996; Ross and Keller 1998); and (iii) these elements do not cover a large portion of the nuclear genome. This last conclusion is also

consistent with the existence of a single Mendelian factor with a major effect on social organization.

The defining feature of fire ant social organization, that is, the number and identity of the reproductive queens heading a colony, is under the collective control of workers, which tolerate and nurture queens judged to be acceptable as new reproductives and execute those deemed not to be so (Fletcher and Blum 1983a; Keller and Ross 1993b; Keller and Ross 1998). A corollary of the observation that colony *Gp-9* composition is completely predictive of social organization is that workers of each social form discriminate against queens of inappropriate genotype when they attempt to become supernumerary or replacement reproductives. Indeed, a series of laboratory experiments have confirmed that (i) colonies with workers bearing the *b* allele (typical polygyne colonies) accept multiple queens also bearing this allele but do not tolerate *BB* queens, and (ii) colonies with only *BB* workers (typical monogyne colonies) accept single *BB* replacement queens but not queens bearing the *b* allele (Keller and Ross 1998, 1999; Ross and Keller 1998). These results are important in explaining the characteristic genotype compositions of colonies in the wild.

Rarely, *Gp-9* genotype composition is not fully consistent with other evidence regarding the form of social organization of a colony. In a study of 1060 colonies sampled widely across the USA range, Shoemaker et al. (2006) found that 0.4% of colonies lacked the *b* allele despite evidence from other markers that they comprised multiple families, whereas 2.3% of colonies contained only one detectable matriline despite the presence of some females bearing the *b* allele. The former most likely were monogyne colonies in which queen turnover had recently occurred (DeHeer and Tschinkel 1998), while the latter most likely were polygyne colonies with very low effective queen numbers. Significantly, every polygyne *S. invicta* colony confirmed as such on the basis of recovery of multiple reproductive queens has been shown to contain individuals bearing the *b* allele (Ross 1997; Ross and Keller 1998, 2002; Ross et al. 1999; Fritz et al. 2006).

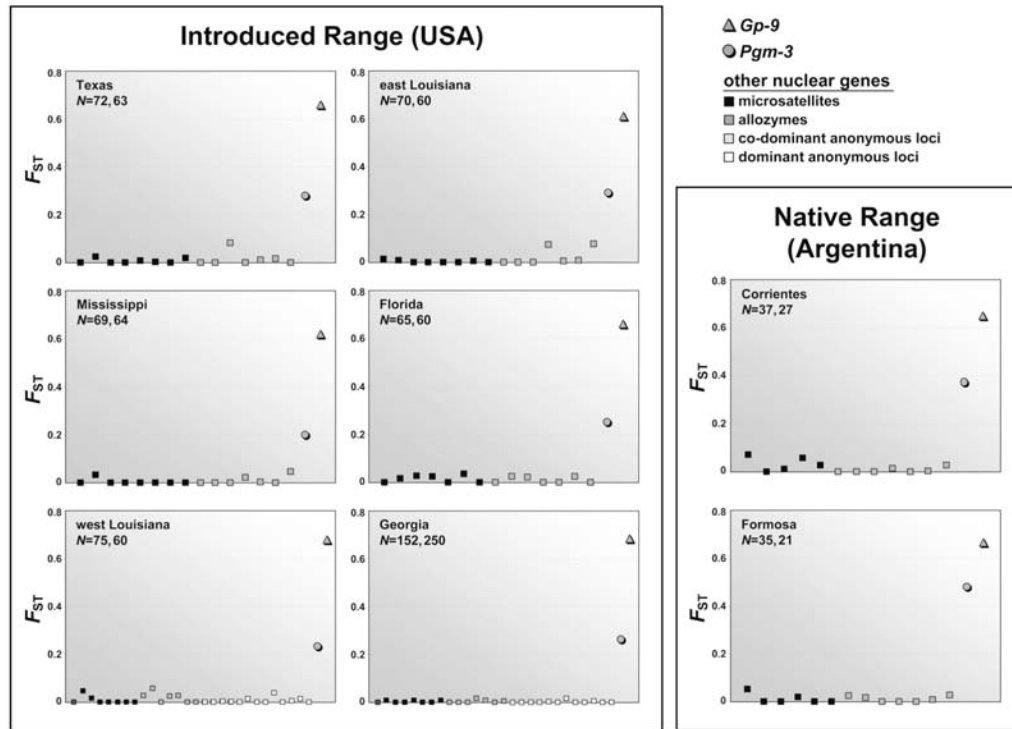


FIGURE 1. GENETIC DIFFERENTIATION BETWEEN SYMPATRIC MONOGYNE AND POLYGYNE *S. INVICTA* POPULATIONS

Estimates of  $F_{ST}$ , a standard measure of the extent of allele frequency differentiation between populations, are shown for *Gp-9* and the linked gene *Pgm-3*, as well as for other nuclear genes of various classes (microsatellites and allozymes were surveyed in all populations; two classes of anonymous nuclear loci were surveyed as well in two introduced populations).  $F_{ST}$  values around zero indicate no meaningful allele frequency differences between populations.  $N$  indicates the number of nests (monogyne, polygyne) from which genetic data were obtained for each population (one genotype per nest was used for the  $F_{ST}$  estimates). Data from Ross et al. (1996b, 1997, 1999, forthcoming), Ross (1997), Shoemaker et al. (2006).

NONGENETIC FACTORS HAVE  
LITTLE INFLUENCE ON SOCIAL  
ORGANIZATION IN *S. INVICTA*

Although population genetic data from *S. invicta* clearly implicate a single genetic factor in the expression of social organization, nongenetic factors conceivably could still play some role. Therefore, the influence of factors such as prior worker social experience and queen reproductive phenotype in overriding the effects of colony *Gp-9* composition were tested in controlled laboratory trials (Ross and Keller 1998). Effectively, such trials assess the penetrance of *Gp-9* in controlling the essential features of social organization across environments. (For colony-level traits such as

social organization, penetrance can be defined as the proportion of colonies of a given genotype composition that display the expected colony phenotype.) The trials employed in these and related studies tested the willingness of temporarily queenless colonies of varying history and genetic composition to accept multiple introduced queens bearing the *b* allele, a hallmark of polygyny in this system. Alternatively, they tested the willingness of colonies to accept a single *BB* queen, a hallmark of monogyny.

Ross and Keller (1998) speculated that workers that were reared and spent their entire adult lives in a colony with a single reproductive queen might be inclined to remain

tolerant of only a single replacement queen. However, trials using a variety of such effectively monogyne test colonies revealed that only the presence or absence of the *b* allele in the worker force influences queen number. That is, effectively monogyne colonies with workers bearing allele *b* expressed the polygyne social phenotype by accepting multiple *Bb* queens but rejecting *BB* queens, whereas colonies lacking this allele expressed the monogyne social phenotype by accepting single replacement *BB* queens but rejecting *Bb* queens.

The social environment in which introduced queens were reared was also shown to have no effect on worker acceptance of queens (Keller and Ross 1998; Ross and Keller 1998). Polygyne or effectively monogyne test colonies with workers bearing the *b* allele accepted multiple *Bb* queens but rejected *BB* queens, regardless of whether the latter originated from polygyne or monogyne colonies. Conversely, effectively monogyne test colonies lacking allele *b* accepted single *BB* queens originating from colonies of either social form. The social environment in which the reproductive queens heading each test colony were reared similarly was found to be unimportant. Altogether, these results indicate that the prior social experience of both workers and queens plays a minimal role in the expression of social organization.

The reproductive status of sexually mature queens (whether they are virgin or mated, egg-layers or not) also seems to have a limited effect on their acceptability to workers in polygyne test colonies, although *Bb* queens collected immediately after their mating flights are not as readily accepted as other types of queens with this genotype (Ross and Keller 1998; Vander Meer and Porter 2001; Goodisman et al. 2000a). Moreover, the degree of physogastry (fecundity) of egg-laying queens plays no role once *Gp-9* genotype is accounted for. This was tested by experimentally restricting the diets of monogyne *BB* queens and supplementing the diets of isolated polygyne *Bb* queens, thus reversing the normal phenotype/genotype associations found in the wild by producing low-fecundity *BB* queens and high-fecundity *Bb* queens (Ross and Keller 1998). Such queens were accepted or rejected

by polygyne colonies solely on the basis of their *Gp-9* genotypes; all *Bb* queens were accepted, but no *BB* queens were accepted (see also Ross 1988 for evidence that multiple highly physogastric *Bb* queens are tolerated in polygyne colonies). This finding is perhaps surprising because fecundity is an important trait affecting acceptability of replacement reproductive queens within the monogyne form (Fletcher and Blum 1983a) in which the *BB* genotype constitutes a uniform *Gp-9* background. Evidently, queen-derived cues associated with *Gp-9* genotype (presumably chemical) occupy a high position in the hierarchy of cues used by workers to regulate colony queen composition in *S. invicta*.

One nongenetic factor that interacts with *Gp-9* genotype to play a role in the acceptability of prereproductive queens is their age (degree of sexual maturity) (Keller and Ross 1998, 1999). Virgin queens possessing the *BB* genotype increasingly become targets of worker aggression in polygyne colonies as they mature sexually following emergence from the pupa. This aggression toward queens lacking a *b* allele is perpetrated mainly by workers that possess it, suggesting that the *b* allele is a selfish genetic element that causes its bearers to act in ways that bias its transmission (Keller and Ross 1998; Mescher 2001). The time period during which aggression escalates (between a few days and two weeks of adult age) coincides with a period of profound physiological change of queens in anticipation of the onset of reproduction (e.g., Goodisman and Ross 1999; Brent and Vargo 2003). Keller and Ross (1999) suggested that the selective elimination of *BB* queens by polygyne workers is mediated by two chemical cues, one signaling a queen's maturity, and the other her *Gp-9* genotype.

Chemical cues that make reproductive queens individually recognizable to workers are well known for monogyne *S. invicta*. Once imprinted on the chemical signature of a single queen, monogyne workers generally will not accept a replacement queen unless they have been held queenless for at least one to several days (Fletcher 1986). Such imprinting on single queens, which apparently also occurs to some extent with polygyne workers (Ross and Keller 1998), can override the

effects of *Gp-9* genotype in queen introduction experiments conducted within 24 hours of dequeening—hence, the need to use temporarily dequeened colonies in the trials described above. Conversely, colonies of either form that have been held queenless for prolonged periods (more than one week) become increasingly tolerant of any conspecific reproductive queens encountered (Ross and Keller 2002; Vander Meer and Alonso 2002).

Evidence that the local physical environment exerts no influence on social organization comes from the fact that the two social forms of *S. invicta* are broadly distributed in both the native and introduced ranges, and that they often occur in close proximity within single microhabitats (Porter et al. 1991; Porter 1992; Ross et al. 1997; Shoemaker et al. 2006). Moreover, polygyne colonies have been observed to displace monogyne colonies over relatively short time spans (Porter et al. 1991; Kenneth Ross, personal observation). This lack of social plasticity in response to local conditions contrasts with the apparent situation in some ants in which the form of social organization is associated with, and presumably induced by, the type of habitat occupied (Elmes and Keller 1993; Herbers 1993; Bourke and Franks 1995).

The combined results from the studies summarized above show that the interaction of queen and worker *Gp-9* genotypes is the single, dominant factor involved in regulation of colony queen identity and number in introduced *S. invicta* in the USA. Normal queenright colonies containing workers with the *b* allele tolerate multiple reproductive queens also bearing the allele, whereas colonies containing only homozygous *BB* workers tolerate only a single queen, which also must bear the *BB* genotype. The experimental results illustrating the nearly complete penetrance of *Gp-9* under diverse conditions are fully consistent with the allele and genotype distributions observed in the two forms in the wild.

#### WORKER *GP-9* GENOTYPE COMPOSITION PREDICTS COLONY SOCIAL ORGANIZATION

The preceding data led to the development of a specific phenomenological hypothesis of the relation between colony *Gp-9* genotypic

composition and social organization in *S. invicta*: the presence of workers bearing *b*-like alleles in a colony induces expression of the polygyne social phenotype, whereas the absence of such workers induces the monogyne phenotype. The resulting simple prediction that colony social organization can be altered by experimentally manipulating worker genotype frequencies has been tested in two studies. In the first, queens of each social form were cross-fostered into previously dequeened colonies of the alternate form (Ross and Keller 2002). Over a period of months, the adult worker genotype compositions gradually changed to those characteristic of the alternate form (i.e., monogyne colonies that adopted a polygyne queen acquired increasing frequencies of workers bearing allele *b*, whereas polygyne colonies that adopted a monogyne queen gradually lost workers with the *b* allele). As predicted, all experimental colonies switched their social phenotype over the course of the study (as assessed by introducing multiple *Bb* reproductive queens), and these changeovers consistently occurred when the proportion of workers bearing the *b* allele crossed a threshold of 5–15%. An alternative, nongenetic explanation for the results, that workers became increasingly habituated to their adopted queens, and thus more tolerant of introduced queens of the same *Gp-9* genotype (and intolerant of queens of the alternate genotype), is inconsistent with the results of Ross and Keller (1998), which suggest a lack of such queen influences. Moreover, it does not explain why the changeovers typically did not occur for several months, only when the frequencies of workers with allele *b* crossed the 5–15% threshold.

In order to entirely eliminate a role for any such queen effects in inducing social changeovers, a second study fostered *b* allele-bearing polygyne worker brood into monogyne test colonies that retained their original queens (Gotzek and Ross, forthcoming). As controls, monogyne test colonies received brood lacking allele *b* pooled from many other monogyne colonies. (This was done to match the genetic variation present in the fostered polygyne brood.) All treatment colonies switched their social phenotype (behaved as polygyne colonies when temporarily queenless) at sev-

eral weeks after brood introduction, when emergence from the added brood resulted in 12–42% of all adult workers bearing the *b* allele. No control colonies changed. As predicted, treatment colonies generally reverted back to the monogyne phenotype once their frequencies of adult workers with allele *b* again dropped below 15% due to replacement of the adult foreign workers by offspring of the resident queen.

Both of these studies support the phenom-enological hypothesis and, equally significantly, they suggest that only a relatively low proportion of workers (5–15%) need possess the *b* allele for polygyne behavior to be expressed by the entire colony. A major unresolved issue surrounding this latter finding is how a relatively small fraction of workers can bring about a major change in an emergent, group-level behavior (Ross and Keller 2002, see also below). Because the behavior of all colony members, including *BB* workers, apparently is altered by the presence of *b*-bearing workers, this seems to represent a case of indirect genetic effects, in which expression of an individual social behavior is conditional on the presence of certain genetic variants in other members of the social group (e.g., Linksvayer and Wade 2005).

A SINGLE MENDELIAN FACTOR MARKED BY *GP-9* REGULATES SOCIAL ORGANIZATION IN THE CLOSE RELATIVES OF *S. INVICTA*

The strong association of colony *Gp-9* genotype composition with social organization is not confined to introduced *S. invicta*. In the native range, polygyne colonies of this species invariably possess *b*-like alleles (variants similar in their amino acid sequence to the *b* allele from the introduced range), whereas monogyne colonies lack them, instead possessing only *B*-like alleles (variants similar to the *B* allele from the introduced range) (Krieger and Ross 2002, 2005; Mescher et al. 2003). Moreover, this same association has been found in the four closest relatives of *S. invicta* in their native South American ranges (*S. macdonaghi*, *S. megergates*, *S. quinquecupis*, and *S. richteri*), where polygyny is again associated with the presence of *b*-like alleles in a colony, and monogyne is associated with the presence

of only *B*-like alleles (Krieger and Ross 2002, 2005). The association of *b*-like alleles with polygyny in these other fire ant species has been particularly well documented in native *S. richteri* (Hallar et al. 2007). Significantly, *S. invicta* and these close relatives are the only members of a large group of fire ants centered in South America in which *b*-like alleles have been discovered. In addition, they are the only members of this group known to display both monogyne and polygyny. Thus, there is a phylogenetic association between the occurrence of social polymorphism and the presence of a major *Gp-9* allele polymorphism in South American fire ants.

Phylogenetic analyses of *Gp-9* nucleotide sequences from 21 New World *Solenopsis* species have revealed that the *b*-like alleles from the five socially polymorphic South American fire ants form an exclusive, relatively recently derived clade (Krieger and Ross 2002, 2005). Hence, the association of this class of alleles with polygyny apparently has evolved only once in the genus, and its retention in the different species represents a trans-species polymorphism. As has been inferred for other such polymorphisms (Klein et al. 1998), persistence of the *b*-like allele lineage across multiple speciation events may signal the historical action of balancing selection. Such selection presumably reflects the distinct demographic advantages of each form of social organization within each socially polymorphic species (see Ross and Keller 1995; Tschinkel 2006).

We note that *Gp-9* polymorphism is not linked to polygyny in all *Solenopsis* species. The monogyne and polygyne forms of *S. geminata*, a quite distant fire ant relative of *S. invicta* (Pitts et al. 2005), possess identical *Gp-9* alleles where they co-occur in northern Florida (Ross et al. 2003). There, the polygyne form displays greatly reduced genetic variation compared to the monogyne form, suggesting that it underwent a recent bottleneck and that loss of allelic variation at genes encoding recognition cues may have contributed to the emergence of polygyny by eroding worker discrimination capabilities. Importantly, such loss of genetic diversity can be ruled out as a factor promoting polygyny in *S. invicta* (Ross et al. 2003). Making matters



even more complicated in *S. geminata*, a polymorphism in the signal sequence distinguishes *Gp-9* alleles of polygyne ants from Chiapas, Mexico from all other sequences of this or any other *Solenopsis* species, suggesting yet another possible route to polygyny in fire ants (Krieger and Ross 2005). A common element in the various postulated genetic causes of polygyny in fire ants is changes in worker discrimination abilities that function in the regulation of colony queen number.

#### GP-9 ENCODES AN ODORANT-BINDING PROTEIN

Complete *Gp-9* sequences have been obtained for 21 *Solenopsis* species, with several sequences available for each of the more common fire ant species (Krieger and Ross 2002, 2005) and many dozen sequences available for native *S. invicta*. The exon/intron structure and respective lengths of the five exons of *Gp-9* are conserved across *Solenopsis*, and there is no evidence of significant historical or recent intragenic recombination; thus, variation in the coding region seems to have evolved solely or primarily by means of point substitutions (Krieger and Ross 2005). The 1700 bp gene of the "true" fire ants encodes a protein of 153 amino acids that, when cleaved of its 19-residue signal peptide, yields a mature protein with an estimated molecular mass of 14.7 kD (Krieger and Ross 2002).

BLAST searches using amino acid sequences initially revealed that *Gp-9* from *S. invicta* most closely resembles tortricid moth genes of the insect odorant-binding protein (OBP) family (Krieger and Ross 2002). With the advent of new sequences from this family,

*Gp-9* now is judged to be most similar to different OBP genes, *OBP56e* from the mosquito *Anopheles gambiae* and *OBP3* from the honey bee (as of November 2006). GP-9 shares several important structural features with other insect OBPs, including its size, presence of a signal sequence, and six cysteine residues arranged in a highly characteristic pattern (Vogt 2003, 2005). Most importantly with respect to its classification, GP-9 groups phylogenetically with other insect OBPs (Box 1). Because initial phylogenetic analyses failed to resolve the placement of GP-9 with respect to other OBPs (Vogt 2003, 2005), we added as outgroups to the original data set ten chemosensory proteins (CSPs), a family believed to be closely related to OBPs (Vogt et al. 1999; Nagnan-Le Meillour and Jacquin-Joly 2003). Although the overall topologies of the resulting trees for all 169 sequences vary considerably depending on the alignment and tree-building methods used, GP-9 sequences from *Solenopsis* always form a monophyletic group placed within the OBP family (Figure 2). Indeed, GP-9 falls within a major clade including many of the OBPs regarded as the "gold standard" proteins of this family (based on their localization to chemosensilla and ability to bind ligands; Vogt 2003). Phylogenetic analyses of a reduced data set also strongly support the conclusion that *Gp-9* is an OBP gene (Figure 2).

OBPs that have been characterized biochemically are small, water-soluble, extracellular carrier proteins that usually bind small hydrophobic ligands. Originally described from the antennae of Lepidoptera, proteins of this family subsequently were shown to be present in the lumen of the antennal olfac-

#### Box 1

The current consensus is that protein families should be recognized on the basis of the evolutionary relationships of the molecules (e.g., Vogt 2003, 2005; Higgs and Attwood 2005) rather than other features, such as their structures or presumed functions (e.g., Leal 2003, 2005). Thus, classifications of proteins serve as guides to the common evolutionary origins and divergence of the genes encoding them. Although similarity of function can be postulated from common membership in a family and, indeed, is important for gene annotation and candidate gene discovery (Bork et al. 1998; Higgs and Attwood 2005; Brown and Sjölander 2006), assignment of function necessarily remains provisional pending specific genetic, biochemical, and physiological experiments.

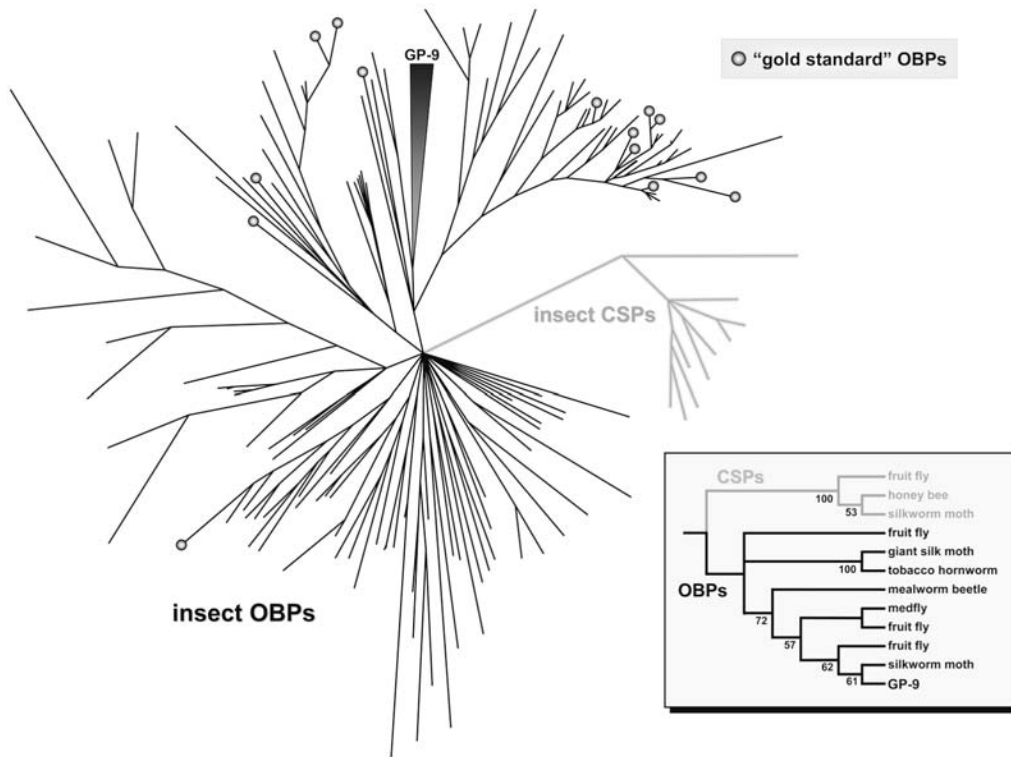


FIGURE 2. PHYLOGENETIC POSITION OF *SOLENOPSIS* GP-9 SEQUENCES RELATIVE TO ODORANT-BINDING PROTEIN (OBP) AND CHEMOSENSORY PROTEIN (CSP) SEQUENCES FROM OTHER INSECTS

The large phylogeny contains exemplar GP-9 sequences from ten *Solenopsis* species as well as 159 odorant-binding protein (OBP) and chemosensory protein (CSP) sequences. The small phylogeny contains a GP-9<sup>b</sup> sequence from *S. invicta* as well as eight exemplar OBP and three exemplar CSP sequences. Amino acid sequences were aligned using various algorithms (ClustalX and MUSCLE on the complete data, T-Coffee and DIALIGN on the data subset) then subjected to phylogenetic analysis using neighbor joining, maximum parsimony, and Bayesian inference. The depicted phylogenies resulted from Bayesian inference, with the larger one based on a MUSCLE alignment and the smaller one on a T-Coffee alignment. Posterior probability support values greater than 50% are shown as percentages for the smaller phylogeny. The “gold standard” OBPs have been implicated in chemoreception.

tory sensilla and to be capable of binding and transporting pheromones or food odorants (Picimbon 2003; Vogt 2003, 2005; Xu 2005); these gold standard OBPs thus appear to be crucial molecular components of insect chemoreception that function in transporting hydrophobic chemostimulants to receptors on the dendrites of sensory neurons. More recently, some OBP genes have been shown to be expressed in nonchemosensory organs (Vogt 2003, 2005; Forêt and Maleszka 2006), suggesting that the products in these cases function in transport roles other than those serving peripheral chemoreception. The sites

of expression of *Gp-9* and identity of the ligand(s) to which its product binds are unknown, although the protein is abundant in the hemolymph of adult females (Dietrich Gotzek, personal observation). Thus, *Gp-9* may fall in the ranks of those OBP genes encoding products that do not function in the traditional manner assumed for the gold standard OBPs.

#### GP-9 IS FUNCTIONALLY IMPORTANT

Several lines of evidence suggest that *Gp-9* encodes a functionally important product. First, OBP homologs of *Gp-9* often play sig-

nificant roles in the sensory lives of insects, enabling them to locate mates or appropriate food sources (Vogt 2003). Further, the sex specificity and time course of expression suggest that *Gp-9* has an important function, which may relate to chemical communication involved in regulating social organization. In *S. invicta*, the gene is not expressed in the larval or pupal stages of either sex, and it is expressed in adult males at only relatively low levels (Ross 1997; Liu and Zhang 2004). In adult workers and queens, the protein is undetectable in newly emerged individuals, increases in abundance to high levels by approximately 7–10 days of age, then remains abundant throughout adult life (Ross 1997; Liu and Zhang 2004). Mature workers exhibit relative GP-9 mRNA levels four-fold higher than nonreproductive queens (Liu and Zhang 2004). These expression patterns parallel the patterns of performance of social behaviors involved in regulating queen number; males are not involved in such activities, and mature adult workers increasingly discriminate among young adult queens of different *Gp-9* genotype as these queens mature.

A further clue that *Gp-9* is functionally important comes from the fact that several individual traits are associated with *Gp-9* genotype in *S. invicta*. The best-studied traits involve physiological and behavioral phenotypes of nonreproductive and young reproductive queens. Among samples from the United States, *Gp-9* genotype is strongly associated with the weight of young queens, an effect caused by differential accumulation of fat during adult maturation (Keller and Ross 1993a,b, 1995, 1999; Ross and Keller 1998; DeHeer et al. 1999; DeHeer 2002). Mature *BB* queens are the heaviest individuals with the greatest fat reserves (regardless of social form of origin), *Bb* queens are of intermediate weight, and *bb* queens are the lightest. (Data from the native range suggest similar inhibitory effects on queen weight for all alleles of the *b*-like class (Mescher 2001).) Extensive reserves are needed to carry a young monogyne (*BB*) queen through independent, claustral colony founding (Markin et al. 1972; DeHeer et al. 1999; DeHeer 2002), but they are not needed by *b*-bearing queens, which typically seek adoption by an established colony before

initiating reproduction. No differences in thorax size between adult queens of different genotypes have been found (Keller and Ross 1993a), which suggests a lack of influence of *Gp-9* on overall size (determined in the larval stage) in this caste.

*Gp-9* genotype is also associated with the dispersal and oviposition behaviors of young adult queens. Homozygous *BB* queens of both forms disperse widely during mating flights, *Bb* queens disperse more locally (as expected if these queens seek out established polygyne nests to join), and *bb* queens rarely undertake mating flights at all (DeHeer et al. 1999). Under laboratory conditions that mimic those following dispersal, *BB* queens have earlier onset of oogenesis and higher fecundity than queens bearing the *b* allele (Keller and Ross 1993b; Ross and Keller 1998; DeHeer 2002). Rapid onset of reproduction is a trait suited to queens that found colonies independently (*BB* queens) because successfully founding a colony in this way depends on the rapid production of many workers (Markin et al. 1973). On the other hand, it is not imperative for young queens joining established colonies (most *Bb* queens) to begin rapid egg production soon after mating.

These phenotypic differences among queens early in their reproductive lives apparently carry over to egg-layers in mature colonies. Monogyne (*BB*) queens are capable of producing more eggs per unit body weight than polygyne (*Bb*) queens (Vander Meer et al. 1992), suggesting some inherent limitation on the metabolic efficiency of *Bb* queens related to egg production (Tschinkel 2006). Altogether, these results show that queen *Gp-9* genotype is associated with a suite of individual phenotypic characteristics that contribute to the different reproductive syndromes of the two social forms.

Somewhat surprisingly, adult body weight in workers and males has also been associated with *Gp-9* genotype. Similar to queens, individuals of these castes bearing the *b* allele tend to be lighter than individuals lacking it (Goodisman et al. 1999). Presence of the *b* allele in polygyne workers thus partly explains the relatively smaller size of workers of this form (Greenberg et al. 1985; Goodisman et al. 1999).

An unusual phenotypic effect of *Gp-9* genotype suggesting functional importance of the gene is the reduced viability of *S. invicta* workers and queens bearing genotype *bb*. Adult workers with the genotype are under-represented or even absent in population samples, and *bb* queens rarely survive to become egg-layers (Ross 1997; DeHeer et al. 1999; DeHeer 2002; Fritz et al. 2006; Hallar et al. 2007). The recessive deleterious effects of the *b* allele of *S. invicta* apparently are not characteristic of the other *b*-like alleles found in this species and in *S. richteri* (Hallar et al. 2007). The *b* allele features a radical, charge-altering amino acid substitution not found in any other *Gp-9* alleles, including other *b*-like alleles. While it is conceivable that this mutation arose in association with a mutant allele at another gene producing the deleterious effect, and that the two mutations have since remained in complete disequilibrium, an equally reasonable conclusion is that the radical substitution influences the normal function of GP-9. Supporting evidence for this view is the inference that the altered residue occurs at a position involved either in ligand binding/unloading or in the formation of a biologically active dimer (Krieger 2005; Hallar et al. 2007). In either case, a charge-changing substitution at this position might be expected to confer deleterious effects on the *b* allele by altering the normal functioning of its product.

Perhaps the most important evidence for the functional importance of *Gp-9* comes from tests of historical selection acting directly on this gene (Krieger and Ross 2002, 2005). These tests reveal a significant excess of nonsynonymous (amino acid replacing) substitutions relative to synonymous (silent) ones in the stem lineage of the *b*-like allele clade of the socially polymorphic South American fire ants. The implication is that positive selection has driven the molecular evolution of *Gp-9* (selection at linked genes cannot produce these patterns), and that such selection acted specifically in the context of the joint emergence of *b*-like alleles and polygyne social organization in these ants. Such selection can be hypothesized to have operated via the ligand-binding properties of GP-9, as two of the amino acids

uniquely shared by all *b*-like alleles are predicted to be binding-pocket residues (Krieger and Ross 2005).

#### WHAT MUST BE LEARNED ABOUT *GP-9*

##### *GP-9*: CANDIDATE GENE OR MARKER?

Despite much being known about the association of *Gp-9* with social traits in *S. invicta*, a great deal more remains to be learned about the potential direct involvement of the gene in regulation of social organization. Given the overwhelming evidence that social organization acts like a trait with a simple genetic basis, the issue can be distilled to the question of whether *Gp-9* is a legitimate candidate gene, or simply a marker for other genes that produce the observed effects on social behavior and with which *Gp-9* is in complete gametic disequilibrium. Although decisive functional and manipulative genetic studies have yet to be undertaken, there is circumstantial support for each viewpoint.

##### Direct Involvement of *Gp-9* in the Regulation of Social Organization

GP-9 is an insect odorant-binding protein (OBP). This family includes several proteins that have been implicated as molecular components in signal transduction during detection of chemostimulants (reviewed in Vogt 2003, 2005). Consistent with a role in olfaction or gustation, many OBP genes are expressed solely in the antennae or other structures bearing chemosensilla (i.e., mouthparts, legs, wings). Given the evolutionary and structural affinities of GP-9 with the gold standard OBPs implicated in chemoreception, a possible role for the protein in fire ants is transport of pheromones employed in the regulation of colony queen number, an idea made plausible because the core feature of such regulation is discrimination among queens by workers based on specific chemical signals emanating from the queens (Keller and Ross 1998; Ross and Keller 1998). One hypothetical scenario is that GP-9 functions in the manner traditionally conceived for insect OBPs, as a transporter of odorants from cuticular pores to receptors on sensory neurons in chemosensilla, and that the *B*-like and *b*-like protein forms differ in their binding prop-

erties. Such biochemical differences have been postulated to confer different recognition capabilities on workers of different genotypes, with different colony phenotypes of collective worker tolerance toward queens emerging from the different worker genotype compositions in each form (Krieger and Ross 2002). Unfortunately, tests to confirm one basic premise of this scenario, that *Gp-9* is expressed in appropriate chemosensilla of workers, have not yet been conducted.

A related scenario acknowledges the likely role of GP-9 as a pheromone transporter protein, but in a manner different from that traditionally conceived for OBPs. Evidence has mounted for considerable heterogeneity in expression patterns and, by implication, specific functions of insect OBPs (Vogt 2005; Forêt and Maleszka 2006). Several are expressed not in chemosensory organs, but in such sites as the hemolymph (Graham et al. 2001; Paskewitz and Shi 2005), male accessory gland (Paesen and Happ 1995), and other tissues devoid of known chemosensory structures (Galindo and Smith 2001; Hekmat-Safe et al. 2002). In *S. invicta*, GP-9 is present in the hemolymph of adult females (Dietrich Gotzek, personal observation), while it is not expressed in the antennae of males (Guntur et al. 2004) (antennae of females have not yet been examined). OBPs are not present as major proteins in the antennae of other ant females (Ishida et al. 2002; Ozaki et al. 2005). These data suggest the possibility that GP-9, and perhaps other hemolymph OBPs, may be involved not in pheromone detection, but in some other molecular component of chemical communication (Calvello et al. 2003). Indeed, Pelosi et al. (2005) speculate that GP-9 might act to transport a pheromone or its precursor from the site of production to relevant organs of activation or release. (Anatomical separation of the sites of pheromone production and release has been described in fire ant queens (Vargo and Hulsey 2000).) Of course, involvement of GP-9 in hemolymph ligand transport does not necessarily rule out a role for it in signal transduction within chemosensilla (see Forêt and Maleszka 2006). Flexibility and interchangeability of roles in chemical communication analogous to that suspected for

OBPs has been proposed as well for the related CSP (chemosensory protein) family in insects (Calvello et al. 2003; Pelosi et al. 2005).

A more speculative scenario posits GP-9 as a hemolymph carrier protein serving some primary function other than chemical communication, perhaps as a transporter of small hydrophobic endocrine factors (Pelosi et al. 2005). Again, CSPs may provide analogous examples if they are confirmed to function not only as transporters of chemostimulants, but also as general lipid carriers involved in more wide-ranging functions, including regulation of development (e.g., Vogt et al. 1999; Nagnan-Le Meillour and Jacquin-Joly 2003; Wanner et al. 2005). One possibility is that GP-9 has some regulatory metabolic function, similar to lipocalin transport proteins (Åkerstrom et al. 2000), and may influence individual signal production or perception via indirect physiological routes. Regardless of the specific transport function of GP-9 in fire ants, its direct involvement in regulation of colony social structure is suggested by the fact that the protein is highly up-regulated in the individuals that participate in the process, mature queens and workers (Ross 1997; Liu and Zhang 2004).

Another line of evidence that suggests *Gp-9* may be directly involved is the invariant association of *b*-like alleles with polygyny in all members of the socially polymorphic clade of South American fire ants (Krieger and Ross 2002, 2005; Hallar et al. 2007). The persistence of this association is difficult to explain if *Gp-9* does not directly affect social organization, because the inexorable pressure of recombination over evolutionary time should lead to the decay of gametic disequilibrium between variation at *Gp-9* and the actual genes involved, even if they are tightly linked to *Gp-9* (Hedrick et al. 1978), yielding a concomitant decay in the association between *b*-like alleles and polygyny. Considering the 600 Mb genome size (Li and Heinz 2000), and assuming the presence of 10,000–20,000 genes, a gene occurs on average every 30–60 kb in *S. invicta*. Using conservative estimates of the extent of crossing over between linked human genes (Sabeti et al. 2006), and assuming a minimum age of 500,000 years for the *b*-like allele clade, anywhere from five to sev-

eral dozen crossing-over events are expected to have occurred between *Gp-9* and adjacent genes since the origin of the clade. This example is consistent with empirical findings from outbreeding species with large effective population sizes (like fire ants) that gametic disequilibrium often drops to insignificant levels over distances as small as 1 kb (Vasemägi and Primmer 2005). Thus, in the presence of typical recombination rates, the apparently ancient, obligate association between *b*-like alleles and polygyny is only likely to have been preserved if *Gp-9* directly influences social organization. Of course, some mechanism that permanently suppresses recombination between *Gp-9* and other linked genes of direct effect could exist, as discussed below.

Perhaps the most compelling evidence that *Gp-9* is directly involved in the expression of social organization is the inference that selection has acted both to drive the molecular divergence of *b*-like alleles from the ancestral *B*-like alleles and to maintain these derived alleles in all socially polymorphic South American fire ant species. The evident elevated rate of nonsynonymous substitutions in the stem lineage of the *b*-like allele clade implicates positive selection as having acted specifically on *Gp-9* to propel the adaptive molecular evolution of the *b*-like lineage in the context of the origin of polygyny (Krieger and Ross 2002, 2005). Moreover, the maintenance of the *b*-like clade as a trans-species polymorphism through multiple speciation events implicates balancing selection in protecting these alleles from loss through drift (Klein et al. 1998).

#### Involvement of Other Genes in the Regulation of Social Organization

Several arguments can also be made to support the view that *Gp-9* plays no direct part in control of social organization or, at best, is but one of many genes involved. First, the mere idea that a single gene could have a large effect on a multifaceted, highly complex social phenotype is anathema to many biologists, as appears to be reflected in Wilson's view that "[s]ocial organization is the class of phenotypes furthest removed from the genes" (Wilson 1980:9). Even so, an apparent precedent of such a major effect of variation at a

single gene on complex social behavior has been reported in voles (e.g., Lim et al. 2004, 2005; but see Fink et al. 2006).

Much of the complexity in fire ant social organization involves the association of colony queen number with alternative suites of traits, including form-specific differences in individual size, dispersal and reproductive strategies, and the structure of colonies and populations (Ross and Keller 1995; Tschinkel 2006). Remarkably, *Gp-9* genotype has been associated with many of these diagnostic differences, the sheer diversity of which would seem to argue against variation at any single gene being entirely responsible. On the other hand, individual phenotypes of highly social organisms depend on interactions with colony mates, so that what appear to be independent traits may often be linked by virtue of common communication mechanisms. In the case of *Gp-9*, the product is an OBP that may function in chemical communication. If the variant proteins function upstream in behavioral interactions that require reciprocal production and perception of chemical signals, then downstream consequences of altered behaviors could be manifested in diverse physiological and behavioral traits. As a hypothetical example, if queens with altered chemosensory abilities as a result of their *Gp-9* genotype do not appropriately recognize and respond to workers attempting to feed them, this could lead to their loss of nourishment, failure to sequester fat during maturation, and inability to sustain flight sufficient for long-range dispersal.

One observation that seems to implicate involvement of other genes is the association between *Gp-9* genotype and weight in adult workers and males (Goodisman et al. 1999). This association very likely results from genotype-specific differences in larval development programs rather than differential accumulation of fat or other materials during adult maturation (Porter and Tschinkel 1985; Tschinkel 1993), yet *Gp-9* does not appear to be expressed until the adult stage in any caste (Ross 1997; Liu and Zhang 2004). It is difficult to imagine any scenario for the involvement of *Gp-9* in the expression of these traits consistent with a complete absence of its product in larvae. This fact alone suggests that there are other,

as yet unknown, genes in complete gametic disequilibrium with *Gp-9* that influence expression of at least some traits comprising the contrasting social syndromes. Indeed, theoretical expectations are for these genes to be recruited into the genomic segment containing both the gene(s) determining social form (marked by *Gp-9*) and factors suppressing recombination (Hey 1998; Mescher 2001), so that inheritance of this entire region becomes Mendelian. In this view, *Gp-9* is embedded in an epistatic network of genes that are resistant to being broken up and that regulate various aspects of the social syndrome (i.e., a coadapted gene complex or supergene; Palopoli and Wu 1996). Again, this resistance to recombination presumably is achieved by some chromosomal feature such as an inversion or other mechanism of crossing-over suppression (Mathiopoulos and Lanzaro 1995; Schimenti 2000). The existence of a nonrecombining unit of this type is at least minimally supported by the absence of evidence for historical recombination within a 2200 bp region including *Gp-9* (Krieger and Ross 2005).

#### FUTURE RESEARCH ON *GP-9* AS A CANDIDATE GENE

Given the at least minimally compelling evidence for a direct role of *Gp-9* in regulating fire ant social organization, we advocate studies directed at this gene and the physiological and behavioral pathways in which it functions as the most promising avenues of future investigation. Such an approach is, of course, subject to change pending concrete data disproving direct involvement of *Gp-9* and must, in any case, be augmented by sustained exploration for other candidate genes.

The prime obstacle to understanding the involvement of *Gp-9* in social regulation is the lack of knowledge of its functional role in individual ants and the colony as a whole. Thus, an important research priority is to describe the normal workings of the protein product at the biochemical, physiological, and behavioral levels. This can be done by discovering details of the protein structure, identifying its ligand(s), determining the effects of sequence variation on binding properties, and determining phenotypically

downstream effects on individual physiology and social interactions. Extrapolation from other OBPs seems risky because, in many respects, GP-9 appears not to be typical of the gold standard OBPs about which most is known (Vogt 2005).

Although nucleotide and amino acid sequences have been determined for *Gp-9* alleles from many *Solenopsis* species (Krieger and Ross 2002, 2005), the secondary, tertiary, and quaternary protein structures have not been solved. Inferential information has come from structure prediction modeling based on other insect OBPs with solved structures (Krieger 2005; Krieger and Ross 2005), an approach that may be robust at a general level given the conservation of basic structural features among even highly divergent OBPs (Picimbon 2003; Vogt 2003, 2005). As an example, the charge-changing amino acid replacement characterizing the *b* allele of *S. invicta* (Lys151Glu) maps to a position in the C-terminus of the predicted GP-9 structure that may play a role in ligand binding and/or unloading or may participate in dimer formation (Krieger 2005). Thus, plausible mechanistic explanations of how this radical substitution disrupts GP-9 function and causes the recessive deleterious effects of the *b* allele were formulated, and specific predictions were tested following structure modeling (Hallar et al. 2007). As useful as this approach may be, analysis of the solved structures of GP-9 variants could be additionally informative, for instance, by directly linking structural to binding properties of the *b*-like and *B*-like protein forms. Such analyses may also reveal whether GP-9 forms biologically active dimers (a matter of controversy with regard to other insect OBPs; Leal 2003) and, if so, whether all types of homodimers and heterodimers are equally likely to form and are equally active.

Of equal importance for understanding GP-9 function will be information on its natural ligand(s) and binding properties. Based on information from other insect OBPs, it is likely that GP-9 binds small, hydrophobic molecules (but see Rivière et al. 2003). High specificity for a single ligand apparently is not characteristic of OBPs implicated in chemoreception, so discrimination

among chemostimulants generally may be facilitated at the molecular level by differential binding of related ligands (Picimbon 2003; Rivière et al. 2003; Vogt 2005). By analogy, it is possible that GP-9 transports an array of related compounds in varying contexts and so may serve multiple functions, perhaps explaining the diverse phenotypic effects. A most relevant issue here is whether GP-9 normally binds compounds that act as pheromones or pheromone precursors in fire ants.

Expression studies revealing detailed caste-, tissue-, and age-specific expression patterns also will be required to help unravel GP-9 function. Liu and Zhang (2004) described general patterns of expression in the different life stages and castes, which seem to reflect relative protein abundances (Ross 1997), but complete information is needed on the adult tissues in which expression occurs and the changes in expression as individuals age or change reproductive status. GP-9 protein routinely is extracted from whole-thorax or head homogenates of adult females, and it is abundant in thoracic hemolymph (Dietrich Gotzek, personal observation), which suggests that it may circulate throughout the hemocoel. This simple information alone is of value in suggesting that *Gp-9* does not encode an OBP that functions exclusively in signal transduction in chemosensilla.

Ultimately, experiments involving genetic transformation by means of techniques such as precise allele substitution may be needed to definitively establish a causal connection between *Gp-9* variation and expression of social organization in fire ants (Glazier et al. 2002; Lim et al. 2004; Greenberg and Wu 2006). Such experiments that allow replacement of one natural allele by another while holding the genetic background constant may pose no special difficulties beyond those associated with any nonmodel organism for analyses of form-diagnostic individual traits. On the other hand, they will be especially challenging when applied to colony queen number, because this group-level social trait represents the collective outcome of decisions made by many individual workers. An obvious experiment would be the transgenic equivalent of the studies of Ross and Keller (2002) and Gotzek and Ross (forthcoming),

in which workers of a monogyne colony have a *Gp-9<sup>b</sup>* transgene substituted for one of their *B* homologs. Such genetic transformation will need to be achieved for a sufficiently large number of workers to make up 10% or more of the population of each test colony, however, and colonies with fewer than several thousand workers may not behave “normally” in the context of such experiments.

Even if transformation experiments were successful in implicating a role for *Gp-9* in control of social organization, a much broader integrative approach is required to fully establish the mechanistic, causal connections between molecular allelic variation and social organization (see Feder and Watt 1992; Watt and Dean 2000; Glazier et al. 2002; Vasemägi and Primmer 2005). Moreover, such a broad approach may yield a wealth of complementary results that, by themselves, can compellingly establish causality in nonmodel organisms (Glazier et al. 2002; Vasemägi and Primmer 2005). The broad objective of this approach as applied to GP-9 will be to discern the position and role of the protein in specific metabolic or signaling pathways, and to learn how variation in the protein affects the behavior of the pathway(s) and, therefore, individual and colony-level phenotypes. By necessity, this approach focuses on determination of pathway architecture, including identification of other genes contributing products to the pathway. A promising point of entry to characterizing this architecture is through microarray gene expression analyses (Wang et al. 2007), because clusters of genes that are coordinately expressed with *Gp-9* may participate in the same pathway (Qu and Xu 2006; Whitehead and Crawford 2006). In fact, some coordinately expressed genes serving related pathway functions may reside in close genomic proximity to *Gp-9* (Hurst et al. 2004; Ranz and Machado 2006), a possibility that raises the important issue of what genes are tightly linked to *Gp-9*.

Because social organization in fire ants comprises expression of alternate syndromes of traits involving worker behavior, queen reproductive physiology, and colony breeding strategies (Ross and Keller 1995), and because the *b* allele behaves in some respects as a selfish genetic element with detrimental



individual-level effects (Keller and Ross 1998), there are theoretical reasons to expect that *Gp-9* may be one component in a complex of tightly linked, coadapted genes that do not undergo recombination (Lyttle 1991; Palopoli 2000; Mescher 2001). Thus, an important goal of future research should be to identify genes in the genomic region surrounding *Gp-9*, and to learn whether they constitute a large non-recombining complex (supergene). A top-down approach of constructing a high resolution linkage map to delimit the boundaries of the chromosomal region in tight linkage with *Gp-9* should be combined with a bottom-up approach of obtaining sequence data for the region surrounding *Gp-9*. These sequence data will permit identification and annotation of additional candidate genes tightly linked to *Gp-9* that may be part of a supergene, and may also contribute toward resolution of the architecture of pathways in which GP-9 functions. An important subsequent step will be to determine the extent of gametic disequilibrium among allelic variants of these linked genes in native *S. invicta*; if a supergene exists, then recombination is not only expected to be low or absent among its members (including *Gp-9*), but particular combinations of alleles are expected to have been preserved over evolutionary time by selection (Mallet 1989; Mescher 2001; Zivanovic and Marinovic 2003). Long haplotypes that include the *b*-like alleles of *Gp-9* and specific variants of neighboring genes are expected invariably to be associated with polygyny.

A final important research objective will be to identify and characterize other OBP genes in *S. invicta*. These genes often occur as members of multigene families organized into genomic clusters (Vogt 2005; Forêt and Maleszka 2006), so it is possible that OBP paralogs linked to *Gp-9* occur in the fire ant genome. Presumably, some fire ant OBPs function in the manner traditionally conceived for these proteins, as molecular components of signal transduction in chemosensilla, while others may play supplementary roles in the expression of social behavior, perhaps even forming part of a supergene including *Gp-9*.

With extensive information available on the function of *Gp-9* and the genes with which

it interacts, it should be possible to develop explicit models of the causal links between molecular variation, individual phenotype, and colony social organization. Although conceptually and technically daunting, this enterprise fulfills the core aim of the field of sociogenomics, which is to understand the molecular genetic bases of alternative traits that provide the raw material for social evolution (Robinson 1999; Amdam et al. 2004; Robinson et al. 2005; Nedelcu and Michod 2006). In the spirit of advancing progress on this front, we present a new behavioral model for the regulation of colony queen number in fire ants that is consistent with currently available evidence. Although we assume that variation at *Gp-9* is the major causative factor in the expression of colony social organization, our model relies only on the fact that a single Mendelian factor playing this role is marked with complete fidelity by *Gp-9*.

#### A PROXIMATE MODEL FOR REGULATION OF COLONY QUEEN NUMBER IN FIRE ANTS

Several models have been proposed to explain features of the behavioral regulation of colony queen number in *S. invicta* (Fletcher and Blum 1983a; Keller and Ross 1999; Crozier 2002; Keller and Parker 2002; Ross and Keller 2002; Krieger 2005). A common element of many of these is the assumption of an optimal level of "queen reproductive pheromone" that must be maintained in a colony. Fletcher and Blum (1983a) hypothesized that the ability to produce such a pheromone varies among egg-laying queens in association with their fecundity (degree of physogastry), and that single monogyne queens, which are highly physogastric, produce enough pheromone to reach the optimal colony level and prevent workers from adopting supernumerary queens. Single polygyne queens, being far less physogastric, individually produce insufficient amounts of pheromone to reach the colony optimum, leading Fletcher and Blum (1983a) to speculate that polygyne workers accept additional queens until their combined pheromone production reaches this optimum. Models developed since the discovery of *Gp-9* assume that production of this hypothetical pheromone is linked more closely to a queen's *Gp-9* genotype than her fecund-

ity, with *Bb* queens producing less pheromone than *BB* queens (Crozier 2002; Keller and Parker 2002; Krieger 2005). Polygyne workers thus require multiple *b*-bearing reproductive queens to reach a colony's optimal pheromone level, while monogyne workers require only a single *BB* queen.

Several of these models also allow for an entirely different mechanism of regulation of queen number that features differences in worker response to or perception of the queen pheromone rather than differences in queen pheromone production. Thus, Keller and Parker (2002) suggested that *Bb* (polygyne) workers may have a higher optimum for colony queen pheromone than *BB* (monogyne) workers (also proposed by Fletcher and Blum (1983a) without regard to *Gp-9* genotype), while Crozier (2002) and Krieger (2005) suggested that individual *Bb* workers may suffer some impairment in their perception of the queen pheromone. However, these proposals fail to explain why the *BB* workers in a polygyne colony fail to function as normal "monogyne" workers and attack supernumerary *Bb* queens, even when they constitute as much as 95% of the colony's worker force (Ross and Keller 2002; Gotzek and Ross, forthcoming). A general model of queen regulation must explain why polygyne workers of all genotypes are complicit in tolerating multiple *Bb* reproductive queens.

The model presented below expands on these previous behavioral models by positing two different sets of individual traits in workers and queens that are influenced by their *Gp-9* genotypes and elicit worker behaviors that, in the aggregate, determine colony queen number (see also Keller and Ross 1998). The two trait sets constitute distinct components of the model that are presented as separate hypotheses.

#### WHICH QUEENS ARE ACCEPTED: THE SMELLY WORKER HYPOTHESIS

We postulate that workers and queens carrying a *b* allele produce a distinct chemical label (odor) qualitatively or quantitatively different from that produced by individuals lacking the allele (see also Keller and Ross 1999; Ross and Keller 2002). Workers encountering *b*-bearing individuals form a specific exclu-

sionary template (memory) that they use as a reference when making decisions about whether to tolerate or attack incipient or actual reproductive queens attempting to gain acceptance as egg-layers. Workers in colonies with *b*-bearing individuals accept only *b*-bearing reproductive queens because the queens' labels are compatible with the workers' *b*<sup>+</sup> templates; conversely, workers in colonies with only *BB* individuals accept only *BB* queens, whose labels are compatible with these workers' *b*<sup>-</sup> templates. Thus, this hypothesis explains how *b*-bearing queens can be tolerated when as few as 5% of workers in a colony bear the allele (presumably, worker contacts are sufficiently frequent that all nestmates contact even rare *b*-bearing workers within a brief period). We note that the hypothesis does not address the issue of whether any specific queen is actually added to the resident queen pool, only whether a queen is compatible with a colony's worker force and, thus, eligible for acceptance (the second hypothesis deals with queen addition). We further note that the hypothesized discrimination system likely differs from, but may interact with, another system ordinarily used in queen (nestmate) discrimination in monogyne colonies (see below; also Vander Meer and Morel 1998; Tschinkel 2006).

There are a number of plausible mechanisms by which an individual's *Gp-9* genotype could affect production of the hypothesized odor label. For instance, if GP-9 functions to transport one component of a "pheromone blend" from its site of production to relevant organs of activation or release, and the *b* allele product is nonfunctional in this role (i.e., *b* is a null allele; Krieger 2005), then *BB* individuals would produce a label dominated by the component carried by GP-9, and *b*-bearing individuals would produce a label with far less of it. In this view, the label differs qualitatively in its composition among the genotypes. Alternatively, if GP-9 has some general regulatory metabolic function, it may influence individual label production indirectly by influencing flux through relevant biosynthetic pathways. The compositional blend of the *Gp-9* label is similar among all individuals, but the label differs quantitatively among the genotypes.

There is reason to believe that this hypothesized label is cuticular in origin and that behavioral interactions are not required for its perception. Adding freshly killed adult *Bb* workers to monogyne colonies can induce conversion to polygyny (Dietrich Gotzek, personal observation). Also, significant nestmate aggression can be elicited against polygyne workers rubbed against the cuticle of mature *BB* queens, whereas a similar reaction is not observed if *Bb* queens are used (Keller and Ross 1998). Evidently, the label is not ordinarily shared among colony members because virgin polygyne *BB* queens would not be expected to meet escalating aggression as they mature in their natal colony if it were.

#### HOW MANY QUEENS ARE ACCEPTED: THE WIMPY QUEEN HYPOTHESIS

We further postulate that *S. invicta* females produce different amounts or blends of a pheromone that signal their actual or potential reproductive status to workers (see also Fletcher and Blum 1981, 1983a,b; Willer and Fletcher 1986; Keller and Ross 1999; Vargo 1999). Production of this hypothetical "queen-caste signal" is predicted to vary quantitatively or qualitatively with caste, age of prereproductive queens, and reproductive potential of mature queens, with the latter feature associated with *Gp-9* genotype (Figure 3). Young prereproductive queens yet to attain sexual maturity, as well as workers of any age, have low or no potential fertility and are hypothesized to create pheromone profiles that signal this to workers. Mature prereproductive queens ready to initiate oogenesis, as well as egg-laying queens, vary in their fertility potential depending on their *Gp-9* genotype, as signified by differences in accumulation of fat reserves during maturation, time to onset of oogenesis, initial fecundity, and metabolic efficiency of egg production (see above). This genotype-associated variation in reproductive potential is hypothesized to be communicated as well to workers via the queen-caste signal.

Specifically, we propose that possession of a *b*-like allele pleiotropically "down-regulates" expression of these fertility components as well as the queen-caste signal. Whereas prereproductive *BB* queens in polygyne colonies

begin producing a strong queen-caste signal once they approach sexual maturity, queens with allele *b* never produce this type or level of pheromone, even after having mated and commenced laying eggs (Figure 3). Once polygyne *BB* queens begin producing a signal by which workers recognize them as potential reproductive females, they are attacked because, in accordance with our first hypothesis, they lack the appropriate *Gp-9* odor label. Queens bearing *b*-like alleles are not perceived as being fully fertile by workers (they are pheromonal wimps), even after they become egg-layers, so workers always tolerate them as if they were immature prereproductives, which leads to polygyny. In essence, polygyne colonies can be viewed as being pheromonally permanently queenless. The exact number of supernumerary egg-laying queens tolerated by a polygyne colony probably depends on factors such as worker/queen ratios or frequencies of attempted infiltrations by newly mated queens.

#### ADDITIONAL CONSIDERATIONS FOR THE MODEL

It is likely that the *Gp-9* label proposed in the first hypothesis is superimposed on another system that also functions in queen (nestmate) discrimination in the constant *Gp-9* background of the monogyne form of *S. invicta*. Workers in monogyne colonies clearly imprint on their mother queen (presumably via chemical labels) and will kill any substitute reproductive queen presented to them, regardless of her *Gp-9* genotype. This discrimination can be extinguished, however, if the mother queen is absent from her colony for a period of two or more days, at which point a single substitute queen bearing genotype *BB* becomes acceptable (Fletcher 1986; DeHeer and Tschinkel 1998; Ross and Keller 1998).

Our model does not directly explain why numerous *BB* alates are tolerated in queen-right monogyne colonies even after they attain sexual maturity. One possible reason is that workers with alternate *Gp-9* genotypes differ in the signal threshold at which they perceive a maturing queen as a future reproductive, with *Bb* workers having a lower threshold than *BB* workers. In this view, the

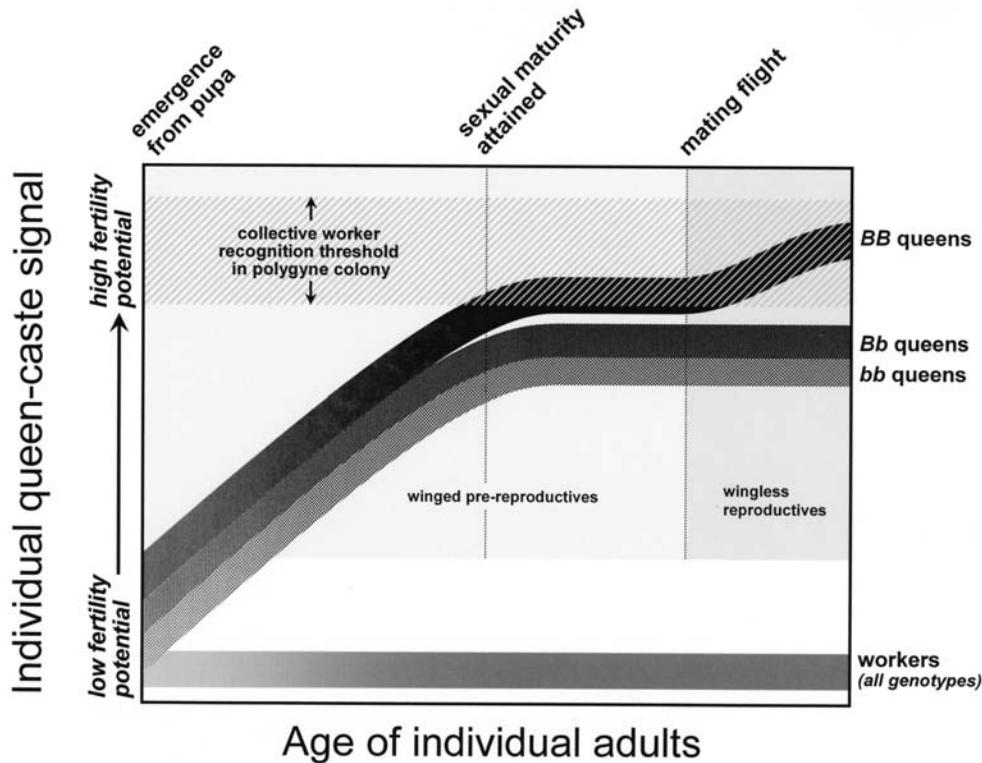


FIGURE 3. REGULATION OF QUEEN NUMBER IN A POLYGYNE *S. INVICTA* COLONY BY MEANS OF QUEEN-CASTE SIGNALS PRODUCED BY INDIVIDUAL ADULT FEMALES

Ribbons symbolize levels of queen-caste signal produced by females of different classes (variation among individuals within classes is emphasized by the use of ribbons rather than lines). Diagonal hatching indicates individual workers' threshold levels of signal at which a female is recognized as a potential or actual reproductive (variation among workers is represented by the width of the hatching). Increasing intensity of ribbon patterns with age signifies the increased expression of *Gp-9* as adult females mature.

hatched band in Figure 3 that depicts the collective worker threshold in a polygyne colony is a composite of a lower band for *Bb* workers and a higher band for *BB* workers (the band in a monogyne colony would be narrowed and elevated). Queen-caste signals of *b*-bearing queens remain below the thresholds of both *BB* and *Bb* workers in polygyne colonies, while mature *BB* queens' signals cross the individual thresholds of some *Bb* workers, inducing their execution by such workers (Keller and Ross 1998). Monogyne *BB* workers, with their higher thresholds, fail to perceive nestmate *BB* alates as potential reproductives, which explains the absence of aggression toward them. An alternative possibility is that the *BB* repro-

ductive queens in monogyne colonies suppress the production of queen-caste signal in maturing alates, while the *Bb* queens in polygyne colonies are incapable of doing so. In this view, the solid black ribbon in Figure 3 is depressed in a monogyne colony, so that few *BB* queens cross the collective worker recognition threshold.

Finally, our model can (with little accommodation) explain why a small minority of mature *BB* queens in polygyne colonies actually survive to take part in mating flights (DeHeer et al. 1999; Goodisman et al. 2000a; DeHeer 2002). These individuals are hypothesized to be relatively delayed in developing the typical *BB* queen-caste signal; thus, they

attain sexual maturity without crossing the collective worker threshold of recognition of reproductives, and so escape aggression before departing the nest on mating flights (see Figure 3).

Several questions must be answered to test and extend our model. Can odor cues extracted from *b*-bearing workers cause social conversion when presented to monogyne colonies? How do workers form exclusionary templates on the basis of brief and infrequent contacts with *b*-bearing nestmates? Do definable predictors of "potential reproductive status," such as the numbers of ovarioles comprising the ovaries, differ between polygyne queens of different *Gp-9* genotypes? Are compounds from the queen poison sac previously identified as "queen recognition pheromones" (Rocca et al. 1983a, b) natural ligands of GP-9?

#### CONCLUSIONS

Despite recent progress in our understanding of the genetic regulation of colony social organization in fire ants, much work remains. It is clear that expression of social organization in *S. invicta* is regulated by a single Mendelian factor, and this genetic architecture has been conserved over evolutionary time in the South American fire ants. Selection appears to have been involved both in driving the molecular evolution and in preserving the variation of one candidate gene comprising this factor, *Gp-9*. This gene is likely to be functionally important and, given the identity of its product as an odorant-binding protein, plausible scenarios for its direct involvement in the regulation of social organization can be envisaged. However, our current lack of knowledge of the functional role of the protein in individual ants, combined with our ignorance of the precise behavioral interactions by which colony queen number is regulated, has hin-

dered progress toward a fuller understanding of the importance of *Gp-9*. Future studies aimed at identifying the biochemical pathways in which the gene product functions, discerning the involvement of other pathway genes or genes in linkage disequilibrium with *Gp-9*, and revealing details of the chemical communication involved in queen acceptance or rejection by workers will go far toward painting a complete picture of the genetic architecture underlying regulation of colony queen number in fire ants. In doing so, such work will illuminate the major molecular, individual, and colony-level changes that transpired during the evolution of a key social adaptation.

As this research program unfolds, it can serve as a useful model for investigating the genetic basis of social evolution in other social animals that display naturally occurring polymorphisms in social organization, especially if such variation appears to be under the control of relatively few genes of major effect. The candidate factors must be dissected to learn if each locus represents a single gene or group of linked interacting genes, and each candidate gene emerging from this analysis must then be subjected to functional and manipulative studies. At the phenomenological level, the genetic, physiological, and behavioral data derived from such studies can perhaps best be assimilated by adopting the perspective that not only the direct effects of the genetic variants in group members, but also the indirect effects of variants expressed in their social interactions, can play a large role in the manifestation of social behavior (Wolf et al. 1998; Linksvayer and Wade 2005). This is clearly the case for *Gp-9* in fire ants.

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