MOLECULAR BASIS OF LETHALITY OF AN ALLELE IMPLICATED IN SOCIAL EVOLUTION IN FIRE ANTS

by

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(Under the direction of Kenneth Ross)

ABSTRACT

Gp-9 has a major effect on social organization in fire ants. Queens and workers of polygyne *Solenopsis invicta* homozygous for the *b*-like allele *b* suffer reduced viability, and *bb* queens never survive. The *b* allele acts as a recessive lethal. This allele differs from other *b*-like alleles (designated *b′*), and all other *Gp-9* alleles, by encoding a lysine residue at position 151, leading to the hypothesis that this substitution is responsible for its deleterious effects. We compared *b′b′* and *bb* homozygotes, in reproductive queens of *S. richteri* and *S. invicta*, and in workers of *S. invicta* from native polymorphic populations. 20% of *S. richteri* queens were *b′b′* homozygotes, and 5% of *S. invicta* workers bore genotype *b′b′*, compared to the lack of *bb* homozygotes in the same populations. Thus, the lysine substitution at position 151 in the GP-9 protein confers the deleterious effects of the *b* allele in homozygous condition.

Keywords: fire ants; genetics of adaptation; *Gp-9*; lethal allele; odorant-binding proteins; polygyny; *Solenopsis invicta*.

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CHAPTER 1

INTRODUCTION

The gene *Gp-9* has a major effect on expression of colony social organization in fire ants, with the presence of *b*-like alleles in a colony invariably inducing multiple-queen (polygyne) organization. Queens and workers of polygyne *Solenopsis invicta* homozygous for the *b*-like allele designated *b* suffer reduced viability compared to other genotypes, and *bb* queens never survive to become egg-layers. Thus, the *b* allele effectively acts as a recessive lethal. This allele differs from the remaining *b*-like alleles (designated *b′*), as well as all other *Gp-9* alleles, by encoding a lysine residue at position 151 in the protein product, leading to the hypothesis that this substitution is responsible for its deleterious effects. We tested this hypothesis by comparing frequencies of *b′b′* and *bb* homozygotes, first in reproductive queens of *S. richteri* and *S. invicta*, then in workers of *S. invicta* from native populations polymorphic for the two *b*-like alleles. We found that almost 20% of *S. richteri* queens were *b′b′* homozygotes, compared to the virtual absence of *bb* homozygotes among *S. invicta* queens, and that at least 5% of *S. invicta* workers bore genotype *b′b′*, compared to the apparent lack of *bb* homozygotes in the same sampled populations. Our data thus implicate the lysine substitution at position 151 in the GP-9 protein as conferring the deleterious (lethal) effects of the *b* allele in homozygous condition, presumably by impairing the protein's function through interference with ligand binding/release or hindrance of proper dimer formation. Given the inferred evolutionary history of fire ant *Gp-9* sequences, we conclude that these deleterious effects are not pleiotropic consequences of variation that evolved under selection for an alternative social strategy.

CHAPTER 2

LITERATURE REVIEW

An important goal in evolutionary biology is to understand the genetic basis of major adaptations in wild populations (Orr & Coyne, 1992; Orr, 1998, 2005). Progress toward this goal requires data on the number and type (structural or regulatory) of genes influencing expression of alternate phenotypes, the spectra of mutational effects at these genes, and the degree of pleiotropy and epistasis that such genes exhibit (Baker *et al.*, 2001; Colosimo *et al*., 2005; Nachman, 2005; Storz, 2005). One remarkable case in which data of this sort have been generated concerns a single gene of major effect on the type of social organization diplayed by fire ant colonies. The gene *Gp-9* encodes a member of a family of proteins (odorant-binding proteins) thought to be involved in chemoreception in insects (Krieger & Ross, 2002). Association studies have demonstrated conclusively that the form of social organization in the fire ant *Solenopsis invicta* depends on the presence of specific allelic variants of *Gp-9* in a colony. In the U.S.A., where the species is introduced, two coding region variants occur (designated *B* and *b*). Monogyne colonies, which have only a single reproductive queen, contain only the *B* allele, whereas polygyne colonies, which have multiple reproductive queens, contain both the *B* and *b* alleles (Ross, 1997; Ross & Keller, 1998; Krieger, 2005). The complete association of *Gp-9* allele *b* with the expression of polygyny in *S. invicta* is significant because the two social forms differ in many significant reproductive and life history attributes other than colony queen number (Ross & Keller, 1995a); thus, varation at this single gene appears to underlie the expression of major alternative adaptive syndromes.

Recent studies have begun to catalog the selective forces acting on *Gp-9*, the nature of molecular variation at the gene, and the phenotypic effects of this variation (see Krieger, 2005). Selection has been implicated as playing an important role in this system based in part on observations of unusual genotype distributions in polygyne queens of introduced *S. invicta*. Whereas all three genotypes are represented among young, pre-reproductive queens, only the

heterozygous *Bb* genotype is found among older, reproductive queens (Ross, 1997). *BB* homozygotes are systematically executed by nestmate workers as these queens mature in the parent colony (Keller & Ross, 1993, 1998, 1999; Ross & Keller, 1998; DeHeer *et al.*, 1999); thus, aggression by polygyne workers toward queens lacking a *b* allele explains the absence of the *BB* genotype among reproductive queens in polygyne nests. No evidence exists for similar aggression against *bb* queens, yet this genotype also is effectively absent in reproductive queens and is rare in mature adult workers as well. Several lines of evidence suggest that its rarity may result from endogenous defects associated with the genotype. Young *bb* queens apparently do not undergo normal maturation after emergence from the pupa; indeed, they rarely gain weight during this period whereas queens with the other genotypes gain substantial weight due to accumulation of extensive energy reserves (Keller & Ross, 1993, 1999; DeHeer *et al.*, 1999). Adults workers with the *bb* genotype are significantly smaller than other workers (Goodisman *et al*., 1999), suggesting that possession of this genotype may detrimentally affect development in this caste also. Frequencies of the *bb* genotype decline in both castes during early adulthood (Ross, 1997; DeHeer *et al.*, 1999; Keller & Ross, 1999), leading to the conclusion that the *b* allele acts as an age-dependent deleterious recessive allele in *S. invicta* (recessive lethal in the case of queens). Given the currently available information, lethality of the *bb* genotype of *Gp-9* may reasonably be viewed as a pleiotropic effect of a genetic variant that has evolved primarily in the context of selection for alternative social and life history traits (see Anholt, 2004).

Molecular variation at *Gp-9* has been explored in native South American populations of *S. invicta* and closely related fire ant species, and some important general patterns have emerged. The *b* allele of introduced *S. invicta* is just one member of a class of "*b*-like" alleles that invariably are associated with polygyny in the South American species exhibiting polymorphism in colony social organization (Krieger & Ross, 2002, 2005). Alleles of this class, which appear to derive from a single *b*-like ancestor, differ from all "*B*-like" alleles of these species in the amino acids they encode at three positions; *b*-like alleles encode a glycine residue at position 42 and isoleucine residues at positions 95 and 139, whereas *B*-like alleles encode serine, methionine, and valine residues, respectively, at these positions. Remarkably, the *b* allele of *S. invicta* is unique among the *b*-like variants, as well as among all other *Gp-9* alleles sequenced to date in *Solenopsis*, because it encodes a lysine rather than a glutamic acid residue at position 151,

a substitution that causes a charge change in the protein product (Krieger & Ross, 2002, 2005). It is therefore unclear whether the deleterious effects of allele *b* in homozygous condition are attributable to the presence of amino acid residues at positions 42, 95, and 139 characteristic of all *b*-like alleles or, instead, are attributable to the presence of the unique charge-altering residue it encodes at position 151. Recent comparative analyses making use of the solved three-dimensional structures of several insect odorant-binding proteins suggest that the residue at position 151 may play a crucial role in the proper functioning of GP-9 protein as a carrier molecule, by affecting either its ability to bind and release ligands or its ability to form biologically active dimers (Krieger, 2005). Thus, a radical substitution at this position, such as occurred in the *b* allele lineage in *S. invicta*, might be expected to yield a defective protein that ultimately has lethal or otherwise deleterious effects in homozygotes.

The hypothesis that the unique substitution at position 151 in the *b*-allele product is responsible for its recessive deleterious effects is testable by virtue of the naturally occurring variation at *Gp-9* in South American populations of *S. invicta* and its close relatives. Specifically, the existence in these populations of *b*-like alleles that induce polygyny yet resemble *B*-like alleles in terms of the amino acid they encode at position 151 (designated collectively as *b′* alleles) leads to two simple predictions. First, polygyne reproductive queens from such populations frequently should be homozygous for these *b*-like alleles (that is, many should possess the *b′b′* genotype), in contrast to the effective absence of *bb* homozygotes in *S. invicta*. Second, adult workers of the polygyne form of native *S. invicta* should bear the homozygous *b′b′* genotype significantly more frequently than the *bb* genotype. In this study, we tested these predictions using queens of polygyne *S. richteri* (a close relative of *S. invicta* known to possess *b′* but not *b* alleles in the polygyne form) and workers of polygyne *S. invicta* from native populations segregating both *b′* and *b* alleles. The explicit hypothesis-testing framework we adopt is acknowledged to be an especially powerful empirical approach to illuminating genetic features of important adaptations in wild populations (Lipton, 2005; Phillips, 2005).

CHAPTER 3

MATERIAL AND METHODS

Samples

Twenty-seven polygyne colonies of *S. richteri* were sampled from the native range at a site near Rosario, Santa Fe Province, Argentina. Colony inhabitants were frozen in liquid nitrogen in the field for transport back to the laboratory, where they were held for long-term storage in a -80°C freezer. Two to four dealate (wingless) queens were selected haphazardly from each colony for study (total of 79 individuals). Polygyny of the colonies, initially inferred in the field using well established criteria (Greenberg *et al.*, 1985), subsequently was confirmed by dissecting the spermathacae (sperm storage organs) and ovaries to demonstrate that multiple queens per nest were mated and laying eggs. The head+thorax of each was separated from the gaster, with the former regions used for protein extraction and the latter used for DNA extraction. All implements used in dissection and separation were cleaned repeatedly with EtOH to avoid contamination of DNA across specimens.

Sixty-one polygyne *S. invicta* colonies were sampled from the native range at two sites, one near Corrientes, Corrientes Province, Argentina (32 colonies) and the other near Formosa, Formosa Province, Argentina (29 colonies). These two geographic populations of *S. invicta* are strongly differentiated at a number of neutral nuclear markers (Ross *et al.*, 1997), meaning that they likely represent evolutionarily separate lineages that constitute legitimate replicates for estimating *Gp-9* genotype frequencies. Sampled colony inhabitants were placed in liquid nitrogen in the field pending storage in a -80°C freezer. Polygyny has been confirmed previously for each colony by means of inspection of multilocus genotype arrays using numerous polymorphic allozyme loci (Ross *et al.*, 1997), by dissection of dealate queens (Ross *et al.*, 1996), and/or by determination of *Gp-9* allele composition (Ross, 1997; Mescher *et al.*, 2003). The type(s) of *b*-like alleles present in each *S. invicta* colony (*b* or *b')* has been established previously on the basis of joint protein electrophoretic and allele-specific PCR data (Ross, 1997; Mescher *et al.*, 2003). Two to four adult workers were selected haphazardly from each colony

for this study (total of 123 individuals), and the head+thorax of each was separated from the gaster for protein and DNA extraction, respectively. (Dealate queens from these populations were not available for this project.)

Protein and DNA extraction

The head+thorax of each ant was homogenized in 30 µl of 50 mM Tris/HCl buffer to yield protein extracts for electrophoresis. The gaster was used as source tissue to extract total genomic DNA for PCR using the PureGene Kit (Gentra Systems). Sterile grinders and sterile aerosol-barrier pipette tips were used in this and all other DNA procedures to avoid contamination. Extracted DNA was diluted for PCR (1:75) using molecular biology grade H_2O.

Electrophoresis of GP-9 protein

Electrophoretic banding phenotypes were scored for each individual by running out the total protein extract in an 11% horizontal starch gel and staining with a nonspecific protein stain. Gels were run at 250 V for two hours then stained for one hour in a 0.05% nigrosine/napthol blue black solution (the dyes were dissolved in a 1:4:5 ratio of acetic acid, H_2O, and EtOH; DeHeer *et al.*, 1999). The background stain was removed through repeated rinses with destain solution (a 1:4:5 ratio of acetic acid, H_2O, and EtOH). High mobility bands were inferred to bear a glutamic acid residue at position 151 in the protein product (e.g., Krieger & Ross, 2002, 2005); that is, these bands represent the products of either *B* or *b′* alleles in *S. richteri* and *S. invicta*. Low mobility bands (those migrating 95% as far as the high mobility bands; Ross *et al.*, 1999) were inferred to bear a lysine at position 151; that is, these bands represent the products of *b* alleles (Krieger & Ross, 2002, 2005). Multiple standards of known *Gp-9* genotype were run on each gel to aid in assessing sample band mobilities.

PCR/RFLP assay of *Gp-9* genotype

A low-stringency 1st-stage PCR was performed using the diluted template DNA of individual ants. The cycling profile for this PCR was initial denaturation at 94.0°C (2 min), followed by 30 cycles of 94.0°C (30 s), 58.0°C (40 s), and 72.0°C (60 s), with a final step at 72.0°C (5 min). Reaction mixtures included the following: 1x PCR buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl), 1x Q-solution (Qiagen), 0.1 mM dNTPs, 0.5 µM forward primer (*Gp-9_169.for*; 5′ GGCCAGCAAAACCAATC 3′), 0.5 µM reverse primer (*Gp-9_490.rev*; 5′ GTATGCCAGCTGTTTTTAATTGC 3′), 1.0 U *Taq* DNA polymerase, and 2.0 µl of diluted

template DNA. This 1st-stage PCR amplified a 828 bp fragment of the *Gp-9* gene including the codon for amino acid 95. The PCR products were run out in a 1% agarose gel containing EtBr and visualized on a UV transilluminator. The 828 bp bands comprising the *Gp-9* amplicons were excised from the gel and used to seed a high-stringency 2nd-stage PCR that amplified the same *Gp-9* fragment. Each excised gel plug was diluted 1:75 in H_2O for this subsequent PCR.

The 2nd-stage PCR was performed using the following touchdown cycling profile: 94.0°C (2 min); two cycles of 94.0°C (30 s), 64.0°C (40 s), and 72.0°C (60 s); two cycles of 94.0°C (30 s), 63.0°C (40 s), and 72.0°C (60 s); five cycles of 94.0°C (30 s), 62.0°C (40 s), and 72.0°C (60 s); 25 cycles of 94.0°C (30 s), 60.0°C (40 s), and 72.0°C (60 s); and a final step at 72.0°C (5 min). Reaction mixtures for this PCR included the following: 1x PCR buffer, 0.1 mM dNTPs, 0.15 µM each of primers *Gp-9_169.for* and *Gp-9_490.rev*, 1.0 U *Taq* DNA polymerase, and 2.0 µl of the diluted amplicon from the 1st-stage PCR as template. The 2nd-stage PCR re-amplified with very high specificity and yield the 828 bp target sequence initially amplified in the 1st-stage PCR (e.g., Ross *et al.*, 2003), making the product especially suitable for digestion with a restriction enzyme.

Four µl of this 2nd-stage product from each sample was mixed with the restriction enzyme *Bsa*A I and appropriate buffer (Buffer 3, New England BioLabs) in an RFLP assay designed to detect the nucleotide substitution at *Gp-9* codon 95 that distinguishes *b*-like from *B* alleles in fire ants (Krieger & Ross, 2002, 2005). The amount of enzyme in each 25 µl reaction mixture varied from 2 to 4 µl, and the mixture was incubated for 12 or 24 h at 37° C. Higher amounts of enzyme and longer digestion periods were used to ensure complete digestion of difficult products. The digestion products were run out in a 1.5% agarose gel stained with EtBr. *Bsa*A I cuts the *B* allele amplicon at one site, yielding two fragments (545 bp and 283 bp). The enzyme cuts the amplicons of *b*-like alleles at two sites, yielding three fragments (428 bp, 283 bp, and 117 bp). Thus, homozygotes for the *B* allele yield two bands of predictable size, homozygotes for *b*-like alleles yield three bands, and heterozygotes for the two types of alleles yield four bands.

Joint interpretation of protein electrophoresis and PCR/RFLP

Electrophoresis of GP-9 protein indicates whether an individual has two, one, or no copies of alleles encoding a glutamic acid residue at position 151 (*B* or *b′* alleles) and whether it has two, one, or no copies of the *b* allele encoding a lysine at this position. The PCR/RFLP assay

indicates whether an individual has two, one, or no copies of *B* alleles and whether it has two, one, or no copies of *b*-like alleles. In conjunction, the two techniques fully specify the *Gp-9* genotype of an individual queen of *S. richteri* or worker of *S. invicta*.

Estimation of *Gp-9* genotype frequencies

Polygyne colonies of native fire ants comprise more or less extended families (e.g., Ross *et al.*, 1996, 1997), so genotypes from a single nest cannot be considered to be independent. In order to obtain unbiased genotype frequency estimates while making use of all the data, we employed a resampling procedure in which a single genotype was drawn at random from each nest to yield a distribution of independent genotypes. Genotype frequencies for each species and population were estimated as the arithmetic mean frequencies in 1000 such resampled genotype distributions. The 95% confidence intervals (CIs) about these genotype frequency estimates were obtained by two methods, one overly liberal and one overly conservative. For the liberal method, 1000 bootstrap samples were drawn from the original data set (which contains non-independent genotypes), and the 25 highest and 25 lowest frequency estimates were dropped. For the conservative method, the resampling and bootstrap approaches were combined; each of the 1000 bootstrap samples was drawn from a separate distribution of resampled genotypes (one per colony), and the 25 highest and 25 lowest frequency estimates were dropped.

Simulation model of selection and gene flow affecting *Gp-9* genotype frequencies in polygyne *S. richteri*

Queens of polygyne *S. invicta* bearing the *bb* genotype have low viability as adults, apparently because of endogenous physiological defects associated with the genotype (DeHeer *et al.*, 1999; Keller & Ross, 1999). We wished to learn if such defects associated with the *b′b′* genotype in dealate queens of polygyne *S. richteri* need be invoked to explain the observed *Gp-9* genotype frequencies in this species. (The finding of no queens bearing the *b′b′* genotype would constitute de facto evidence of such defects.) Therefore, computer simulations were carried out to predict the effects of selection against *b′b′* queens on *Gp-9* genotype frequencies in *S. richteri*, given varying levels of gene flow from the monogyne social form of the species (with its presumably different *Gp-9* genotype frequencies). Mating between polygyne queens and monogyne males of *S. richteri* is assumed to occur by analogy with *S. invicta*, where such inter-form crosses apparently are common (e.g., Ross & Keller, 1995b; Shoemaker & Ross, 1996). By comparing

simulation results under varying scenarios to the observed *Gp-9* genotype frequencies in polygyne dealate queens of *S. richteri*, we were able to infer levels of selection and inter-form gene flow compatible with these empirical data. We did not conduct similar simulations for *S. invicta* because the large workers sampled for this study are unlikely to display unbiased genotype frequencies necessary for proper model parameterization and evaluation (Goodisman *et al.*, 1999).

The first class of simulations started with *Gp-9* genotype frequencies in the polygyne queens (mothers) in generation 1 set to the empirical point estimates. Other classes of simulations assumed that genotype distributions in the first generation mothers corresponded to the lower or higher confidence bounds about the point estimates. Polygyne mother queens in each generation were obliged to mate with pre-determined proportions of males of each social form, with the proportions held constant throughout each simulation. In generation 1, polygyne fathers were assumed to produce gametes with *Gp-9* haplotype frequencies indentical to the allele frequencies of the polygyne mother queens; in each successive generation, polygyne fathers were assumed to produce gametes at frequencies identical to the allele frequencies of the polygyne mothers of the previous generation (male fire ants are the impaternate haploid offspring of queens). Immigrant fathers of the monogyne form were assumed to produce only *B* gametes in every generation (the monogyne form of *S. richteri* appears to be fixed for such alleles [Krieger & Ross, 2002, 2005], as is the monogyne form of *S. invicta* [Ross, 1997; Ross & Keller, 1998, Krieger & Ross, 2002]). Complete mortality of pre-reproductive queens bearing the *BB* genotype, presumably due to intolerance displayed toward them by polygyne workers, was assumed in each generation (see below; also Ross & Keller, 1998 for *S. invicta*). Thus, only *Bb′* and *b′b′* queens served as mothers throughout each simulation. All simulations assumed random mating with respect to *Gp-9* genotype, no differential survival of gametes according to *Gp-9* haplotype, and Hardy-Weinberg genotype proportions in zygotes.

Values for the two parameters of interest, the relative magnitude of pre-reproductive mortality (selection) against *b′b′* queens and the proportions of queens mating with males of each form, were varied systematically in models of each class to determine which combinations of values yielded model output compatible with the observed data. In the models started with genotype frequencies set at the lower or higher bounds around the point estimates, the observed

frequencies also were assumed to equal these lower or higher bounds. Each simulation was run for 20 generations, at which point genotype frequencies output by the simulations always stabilized.

CHAPTER 4

RESULTS

Solenopsis richteri dealate queens

Polygyny was confirmed in all 27 *S. richteri* study colonies by means of dissecting the spermathacae and ovaries of the 79 sampled dealate (wingless) queens. All of these queens possessed well developed (functional) ovaries, and all but four of them (from two different colonies) were mated (indicated by the presence of an opaque, whitish spermatheca; e.g., Goodisman & Ross, 1999). Thus, each of these *S. richteri* colonies contained multiple reproductive queens, the defining feature of polygyny.

The *Gp-9* genotype of each *S. richteri* queen was determined by joint application of protein electrophoresis and a PCR/RFLP assay. Each of the 79 queens was found to possess at least one copy of a *b*-like allele at *Gp-9*; that is, no *BB* homozygotes were discovered (see Table 1). The *b*-like alleles carried by these queens were in every case inferred to be *b′* alleles, based on the observed high mobility of the protein product in an electrophoretic gel consistent with a glutamic acid residue at position 151. This confirms previous reports that only *b′* alleles are represented among the *b*-like alleles of this species (Krieger & Ross, 2002, 2005). The fact that each polygyne reproductive queen of *S. richteri* possessed a *b*-like allele parallels earlier findings that polygyne reproductive queens of *S. invicta* always bear alleles of this class (Ross, 1997; Ross & Keller, 1998; Krieger & Ross, 2002), presumably because queens lacking such alleles are executed as they mature and become reproductively active (Keller & Ross, 1998; Ross & Keller, 1998).

Frequencies of the *Bb′* and *b′b′* genotypes in the sampled polygyne *S. richteri* queens, as estimated by a resampling procedure, are shown in Table 1. Approximately one-fifth of *S. richteri* queens in our sample possessed the homozygous *b′b′* genotype. The 95% confidence intervals (CIs) about the genotype frequency estimates obtained by an overly liberal and an

overly conservative method suggest that between 5% and 30% of all egg-laying queens in the polygyne *S. richteri* population we sampled actually possessed the *b′b′* genotype. This is in contrast to the effective absence among dealate *S. invicta* queens of homozygotes for the *b*-like allele designated *b* (Ross, 1997; Goodisman & Ross, 1999; Shoemaker *et al.*, 2005), the only *Gp-9* allele known to encode a lysine residue at position 151. These results thus provide support for the hypothesis that the replacement of glutamic acid by lysine at position 151 in GP-9 has led to the lethal effects of the *b* allele in homozygous condition in queens.

We used computer simulations to determine whether the observed *Gp-9* genotype frequencies for dealate *S. richteri* queens are compatible with equal survival of the *Bb′* and *b′b′* genotypes. In these simulations, we took into account the possibility that polygyne queens of *S. richteri* mate with conspecific males of the alternate, monogyne, social form, which is assumed to be monomorphic for the *B* allele. We found that simulations assuming no differential survival of the two genotypes predicted the observed 18.8% frequency of genotype *b′b′* when 36% of polygyne queens mated with males of the polygyne form and the remaining 64% mated with males of the monogyne form. Moreover, substituting reasonable confidence bounds (5%, 30%) for our point estimate of the *b′b′* frequency in the simulations also led to prediction of the observed frequencies without the need to incorporate selective mortality (by assuming 90% and 45% inter-form matings, respectively). Thus, elevated mortality of *b′b′* queens relative to *Bb′* queens need not be invoked to explain the observed genotype distributions, assuming that some moderate to high level of mating occurs between polygyne queens and monogyne males. On the other hand, in the complete absence of such matings a selection coefficient of 0.685 (proportion of *b′b′* queens not surviving to reproduction) is required to explain the *Gp-9* genotypic data for *S. richteri*.

***Solenopsis invicta* adult workers**

The *Gp-9* genotype of each adult worker was determined by combined protein electrophoresis and PCR/RFLP, and the frequencies of the six genotypes possible with three alleles (*B*, *b*, *b′*) were estimated separately for each geographic population using a resampling procedure (Table 2). Four of the six possible genotypes were observed. Most notably, workers bearing the *b′b′* genotype were discovered, although the genotype is not common at either geographic locality (the estimated CIs suggest that between 1% and 15% of all polygyne workers at each site

possessed this genotype). The presence of such workers at both sites again contrasts with the complete absence in our samples of workers bearing genotype *bb*. Moreover, we note that *bb* workers also were not found in an earlier study of *S. invicta* from these same populations in which 12 workers from each of 79 polygyne colonies (44 from Corrientes and 35 from Formosa) were assayed electrophoretically (Ross, 1997). Thus, there appears to be a real, biologically meaningful, difference in the frequencies of homozygotes for the two *b*-like *Gp-9* alleles in polygyne *S. invicta* workers, with the observed difference supporting the hypothesis that the lysine residue at position 151 in the *b* allele product is responsible for the deleterious effects in homozygotes.

Another genotype, *bb′*, also was not detected in the workers that we examined. The absence of this genotype presumably can be explained by the fact that the two *b*-like alleles rarely co-exist within single polygyne *S. invicta* colonies. For instance, earlier electrophoretic analyses of the two study populations revealed that only one of 26 polygyne colonies in Corrientes and none of 20 polygyne colonies in Formosa could be inferred to have both *b* and *b′* alleles represented among the colony's reproductive queens (K. G. Ross, unpublished data). Our analyses are consistent with these previous results in that only two of our 61 study colonies (both from Corrientes) were found to contain both *b*-like alleles (these occurred in different workers in each nest). A general conclusion that can be drawn from these results is that polygyny in native *S. invicta* is associated with the presence in a colony of either *b*-like allele of *Gp-9*, but that the two *b*-like alleles rarely co-exist in such colonies.

Given the existence of two apparently distinct classes of polygyne colony in native *S. invicta*, it may be appropriate to re-estimate our worker *Gp-9* genotype frequencies with respect to the relevant class of polygyne nest. When done for focal genotype *b′b′*, our resampling approach yields estimates of 0.149 (liberal CI 0.026-0.237) and 0.178 (liberal CI 0.037-0.259) for its frequency in the subsets of Corrientes and Formosa polygyne nests bearing the *b′* allele. The zero frequency estimate for genotype *bb* necessarily remains unchanged when the reference population is restricted to just the subset of nests bearing the *b* allele. Thus, it appears that about one-sixth or more of adult workers are *b*-like homozygotes in the subset of native polygyne nests bearing the *b′* allele, whereas no workers are *b*-like homozygotes in the subset of nests bearing the *b* allele.

CHAPTER 5

DISCUSSION

Molecular basis of lethality of the *b* allele in *S. invicta*

The objective of this study was to compare the frequencies of homozygotes for the two different types of *b*-like alleles of *Gp-9* in fire ants in order to test the hypothesis that a substitution unique to the *b* allele of *S. invicta* (lysine at position 151) is responsible for the deleterious (lethal) effects of the allele in the homozygous state. A first comparison was between reproductive queens of *S. richteri*, a species in which only the *b′* version (which lacks the substitution) occurs, and reproductive queens of *S. invicta*, a species in which the *b* variant is common. A second comparison was between adult workers of *S. invicta* from colonies containing the *b* allele and adult workers of this species from colonies containing the *b′* allele, a comparison that was made in two distinctive native populations. These comparisons test two simple predictions of the hypothesis: (i) polygyne queens of *S. richteri* commonly should be homozygous for *b*-like alleles (should possess the *b′b′* genotype), in contrast to the virtual absence of *bb* homozygotes in *S. invicta*, and (ii) workers of polygyne *S. invicta* should bear the homozygous *b′b′* genotype significantly more frequently than the *bb* genotype.

We found that almost one-fifth of the *S. richteri* queens in our study were *b′b′* homozygotes, with the estimated confidence intervals suggesting that somewhere between 5% and 30% of queens in the source population actually possessed the genotype. These figures are to be contrasted with the near complete absence of *bb* homozygotes among reproductive *S. invicta* queens from *b*-containing polygyne nests (a handful of such queens have been detected in samples of many thousands from South America and the U.S.A. [Ross, 1997; Goodisman & Ross, 1999; Shoemaker *et al.*, 2005]). We also discovered adult workers of *S. invicta* bearing the *b′b′* genotype at frequencies of 5-6% (the estimated confidence intervals suggest that between 1% and 15% of all polygyne workers in each study locality possessed this genotype). Recalculation of worker genotype frequencies with respect to the specific type of source colony yielded revised estimates of 15-18% for the *b′b′* genotype. Importantly, we detected no *bb*

workers in samples from the same localities, and a more extensive prior survey of these populations similarly found no workers bearing this homozygous genotype (913 polygyne workers surveyed; Ross, 1997). Thus, our results confirm the predictions of the hypothesis that the charge changing glutamic acid→lysine substitution at position 151 in the protein encoded by the *b* allele of *S. invicta* is responsible for the recessive deleterious effects of this allele. Moreover, our results apparently rule out the alternative explanation that these effects are attributable to the presence of one or more of the amino acid residues at positions 42, 95, and 139 that are unique to all *b*-like allele products.

We have no reason to believe that estimates of *Gp-9* genotype frequencies obtained for reproductive polygyne queens of *S. richteri* and *S. invicta* in the present and earlier studies are biased. On the other hand, at least one important source of bias potentially plagues estimates of these frequencies in adult workers. Goodisman *et al.* (1999) reported a significant association of *Gp-9* genotype with worker mass in samples of *S. invicta* from Georgia, U.S.A., with *bb* workers smaller than *Bb* workers, which, in turn, are smaller than *BB* workers (fire ant workers exhibit a continuous size distribution; Porter & Tschinkel, 1985; Tschinkel *et al.*, 2003). Workers collected for the present study generally were among the larger workers in each colony, so it is possible that our estimates of frequencies of *bb* workers in the native range (zero in both populations) are biased downwards. Some evidence for this comes from estimates of the frequency of this genotype in polygyne workers in the U.S.A., which range from 0.2% to 3.1% (Ross, 1997; Goodisman *et al.*, 1999). It is not known if possession of a *b′* allele is similarly associated with reduced worker size in native *S. invicta*, but if so this may have caused a downward bias in our estimates of frequencies of *b′b′* workers as well.

The structure of GP-9 protein has been predicted with structure prediction software by using the solved three-dimensional structures of several insect odorant-binding proteins (OBPs) as templates (Krieger, 2005). Position 151 is a location on the GP-9 protein that may well be crucial to the proper biological functioning of the molecule. One reason to suspect so is that it maps to a residue in the C-terminal tail of a silkworm moth OBP, a part of the protein thought to play a role in unloading the ligand at its receptor by undergoing a pH-dependent conformational change (Horst *et al*., 2001; Lee *et al*., 2002). Moreover, in three other insect OBPs (Kruse *et al.,* 2003; Lartigue *et al.,* 2003; Lartigue *et al*., 2004), GP-9 position 151 maps to a residue in the

irregular C-terminal structure that is part of the binding-pocket wall. The C-terminus of GP-9 is slightly shorter than the C-terminal tail of the silkworm moth protein but longer than the C-terminus of the other insect OBPs, so it is difficult to say which type of solved structure is a better model for GP-9 protein. In either case, a radical, charge-changing substitution in the C-terminus may be expected to affect the protein's ability to bind and/or release its ligand (assignment of GP-9 to the OBP family implies that it acts as a carrier of small, hydrophobic molecules, but the identity of such ligands presently is unknown).

Parts of the C-terminal tail of the silkworm moth OBP are of additional functional importance because they are involved in formation of the biologically active dimeric protein (Sandler *et al*., 2000). Therefore, inhibition of proper dimer formation is a plausible alternative explanation of how GP-9 function is affected by a charge-changing substitution at position 151 (Krieger, 2005). Under either general explanation, the substitution of lysine for glutamic acid encoded at this position apparently was the pivotal event responsible for conferring recessive deleterious effects on the *b* allele by impairing the normal functioning of its protein product.

Complete lethality of the *bb* genotype in polygyne queens of *S. invicta* is an extreme phenotypic effect presumably associated with the failure of young queens to accumulate energy reserves and otherwise undergo normal reproductive maturation (Keller & Ross, 1999). An important issue is whether lesser, sublethal effects may be associated with possession of the *b′b′* genotype, effects that presumably would be attributable to one or more of the amino acid residues encoded at positions 42, 95, and 139 that distinguish all *b*-like from other *Gp-9* alleles. Our estimate of less than 20% for the frequency of genotype *b′b′* among reproductive *S. richteri* queens is less than half the frequency expected under Hardy-Weinberg equilibrium after accounting for complete loss of the *BB* class, a finding that would seem to support the existence of such sublethal effects. However, our simulations reveal that this frequency is expected in the absence of any reduced viability of *b′b′* queens when there is a moderate level of inter-form mating (polygyne queens mating with monogyne males, all of which are assumed to bear a *B* allele). Mating between polygyne queens and monogyne males apparently is common in *S. invicta* (e.g., Ross & Keller, 1995b; Shoemaker & Ross, 1996; Goodisman *et al.*, 2000) and so may occur commonly in *S. richteri* as well under appropriate circumstances. Such appropriate circumstances include the presence of large numbers of conspecific monogyne colonies in the

immediate vicinity of polygyne colonies, which appears to be the case at our study site (K.G. Ross, unpublished data). Although our data provide no compelling evidence to the contrary, valid confirmation of the hypothesis that *b′b′* queens do not suffer higher mortality than *Bb′* queens in polygyne *S. richteri* will require independent estimation of the frequency of inter-form matings or tracking of *Gp-9* genotype frequencies in known-age cohorts of polygyne queens. If confirmed, this would constitute strong ancillary evidence that the negative viability effects associated with genotype *bb* in *S. invicta* are attributable solely to the lysine residue encoded at position 151 by the *b* allele, with no contributory effects from the characteristic *b*-like residues at positions 42, 95, and 139.

Phylogenetic analyses of *Gp-9* sequences obtained from 21 diverse *Solenopsis* species have revealed that the *b* allele arose relatively recently within the clade of *b*-like alleles, and that it appears to be confined to *S. invicta* (where it occurs in polymorphic condition with the *b′* allele) and the closely related *S. megergates* (Krieger & Ross, 2005). The present study, which represents the first broad survey of intraspecific *Gp-9* variation outside of *S. invicta*, confirms that polygyne *S. richteri* possesses only the *b′* variant, as expected if the taxonomic distribution of the recently derived *b* allele is very limited. Our conclusion that the recessive deleterious effects of allele *b* stem from the lysine it encodes at position 151, rather than any of the three amino acids uniquely characterizing *b*-like alleles, means that lethality in homozygotes is not appropriately viewed as a pleiotropic consequence of *Gp-9* variation that evolved under selection for an alternative social strategy. Rather, the deleterious mutation was superimposed on existing variation responsible for inducing polygyny, with this pre-existing variation apparently having been subject historically to strong positive selection for this role (Krieger & Ross, 2002, 2005). This example illustrates the utility of coupling phylogenetic with molecular analyses of the genetic architecture of major adaptive traits in order to unravel the evolutionary history of variants and gain insights into the forces that have shaped the variation (see also Hammock & Young, 2004; Colosimo *et al.*, 2005).

Lethality of the *b* allele in homozygous condition is just one of the phenotypic effects associated with possession of this allele by young queens of *S. invicta*. Others include diminished weight gain during maturation due to lower accumulation of energy reserves (Ross & Keller, 1998; DeHeer *et al.*, 1999), a reduction in rates of wing shedding (a behavior linked to

the onset of reproduction) and of oogenesis once maturity is attained (Keller & Ross, 1993, 1999), and a decreased tendency to disperse long distances on mating flights (DeHeer *et al*., 1999). These effects on the physiology and behavior of young queens correlate with the alternative reproductive strategies characteristic of the polygyne social form of fire ants and other ants (Ross and Keller, 1995a). Whereas monogyne queens typically disperse widely during their mating flights and subsequently found new colonies independently (without the help of workers), polygyne queens generally disperse more locally and attempt to initiate reproduction within established polygyne colonies, which multiply by fissioning or budding. Thus, the phenotypic effects of the *b* allele in *S. invicta* contribute to a unique reproductive syndrome that is integral to polygyne social organization. Our results pinpoint the molecular change underlying the recessive lethal effects of the *b* allele, but the question of whether the same change is responsible for the other effects of this allele remains open because relevant data on the reproductive physiology and behavior of fire ant queens bearing the *b′* allele are not available. A reasonable working hypothesis is that these other effects that are core elements of the polygyny syndrome stem not from the amino acid substitution at position 151 unique to the *b* allele but rather from one or more of the three substitutions characterizing the entire clade of *b*-like alleles (cf. Krieger, 2005; see also Glazier *et al.*, 2002; Nachman, 2005). Recent discoveries that *Gp-9* sequences from monogyne fire ants can contain *b*-like residues at positions 42 or 95 (Ross *et al.*, 2003; Krieger & Ross, 2005), coupled with the inference that residue 139 is at a ligand-binding position (Krieger & Ross, 2005), suggest that the isoleucine in this latter position in the *b*-like allele product may be crucial for expression of the core polygyne traits. Of course, these physiological and behavioral traits may instead be controlled by variation in other genes that are tightly linked to and in strong linkage disequilibrium with *Gp-9*, a possibility that can be all but ruled out for the lethal effects in light of the predictive hypothesis-testing framework we employed.

Association of *b*-like alleles with polygyny in South American fire ants

The fact that each of the 79 reproductive queens of polygyne *S. richteri* that we surveyed possessed a *b*-like allele (*b′* in this case) is an important ancillary finding of this study. It parallels the pattern reported in the extensively studied *S. invicta* in both the native and introduced ranges, where polygyne reproductive queens always bear either a *b* or *b′* allele (Ross,

1997; Ross & Keller, 1998; Krieger & Ross, 2002). Indeed, the requirement that egg-laying queens in polygyne nests possess a *b*-like *Gp-9* allele may be universal in the South American fire ant species that are closely related to *S. richteri* and *S. invicta* and display similar polymorphisms in colony social organization (Krieger & Ross, 2002, 2005). The presumed proximate explanation for this pattern, which is well established in *S. invicta*, is that workers in polygyne colonies recognize and become increasingly intolerant of queens lacking a *b*-like allele as these queens mature and attempt to become reproductively active (Ross and Keller, 1998; DeHeer *et al.*, 1999; Keller and Ross, 1999). The result of escalating aggression is that such queens are executed or forced to disperse from the colony before they attain reproductive status. More generally, our extension from *S. invicta* to *S. richteri* of the finding that only queens with a *b*-like allele become polygyne reproductives supports the emerging consensus that *b*-like alleles must be represented among colony inhabitants in order that polygyny be expressed in each of the South American relatives of *S. invicta* exhibiting this alternative form of social behavior (Krieger & Ross, 2002, 2005).

Polymorphism of *b*-like alleles in *S. invicta*

The polygyne form of *S. invicta* is polymorphic for the two major types of *b*-like alleles in its native South American range, with the *b* and *b′* alleles sometimes occurring within single geographic populations (Mescher *et al.*, 2003). Yet reproductive queens from any single polygyne colony in such populations nearly always bear just one of these alleles (K. G. Ross, unpublished data). Our data on polygyne *S. invicta* from two geographic populations in Argentina extend this finding to the worker caste; only 3% of our study colonies were found to contain workers with both *b*-like alleles. Two proximate explanations for the existence of apparently distinct classes of polygyne colonies in native *S. invicta*, one containing *b* alleles and the other containing *b′* alleles, can be considered. The first invokes the mechanism of positive assortative mating according to *Gp-9* genotype. That is, queens mate only (or predominantly) with males bearing the same *b*-like allele. Little is known about fire ant mating biology, especially the possibility of mate choice by queens, but volatile pheromones presumably are involved in the process of mate location in the aerial mating swarms formed by these insects (Markin *et al.*, 1971; DeHeer *et al.*, 1999), so mate choice based on chemical cues associated with *Gp-9* genotype is not implausible. A second mechanism, which may function alone or in

concert with the first, involves differential worker aggression toward maturing queens based on the *Gp-9* genotypes of both castes. In this scenario, workers in a polygyne colony prevent resident or immigrant queens that do not bear the prevalent type of *b*-like allele in the colony from becoming reproductives. Both explanations entail signalling and recognition systems tied to *Gp-9* genotype, a reasonable possibility given the substantial evidence that (i) *S. invicta* workers distinguish queens on the basis of complementarity of *Gp-9* genotypes using chemical cues (Ross & Keller, 1998; Keller & Ross, 1999), and (ii) GP-9 belongs to a class of proteins implicated in chemoreception in insects (Leal, 2003; Vogt, 2003).

The apparent balanced polymorphism of *b*-like alleles in *S. invicta* raises the issue of what evolutionary forces act to maintain the two alleles associated with polygyny in this species. The issue is especially intriguing in light of the apparently high segregational genetic load associated with possession of *b* rather than *b′* in polygyne colonies, which might be expected to lead to the eventual replacement of the former allele by the latter in the absence of any countervailing selective advantage to possession of allele *b*. Research into the genetic, physiological, and behavioral mechanisms responsible for the co-existence of two apparently discrete polygyne social systems controlled by different *b*-like alleles in *S. invicta* may be expected to shed light on the broader issue of how *Gp-9* regulates the expression of monogyny and polygyny in South American fire ants.

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Table 1 Frequencies of *Gp-9* genotypes in dealate queens of the polygyne form of *S. richteri* from Argentina.

Genotype		
BB	*Bb′*	*b′b′*
0	0.812	0.188
–	(0.763-0.925)	(0.075-0.225)
–	(0.630-0.963)	(0.037-0.370)

Frequencies were estimated as the means from 1000 random draws of single genotypes per nest. The 95% confidence intervals (CIs) around the genotype frequencies (in parentheses) were estimated using a liberal (upper row) or conservative (lower row) bootstrap approach (see text).

Table 2 Frequencies of *Gp-9* genotypes in adult workers of the polygyne form of *S. invicta* from Argentina.

	Genotype					
Site	*BB*	*Bb*	*bb*	*Bb′*	*bb′*	*b′b′*
Corrientes	0.486	0.211	0	0.253	0	0.050
	(0.333-0.576)	(0.121-0.318)	–	(0.152-0.364)	–	(0.015-0.136)
	(0.273-0.0.667)	(0.061-0.394)	–	(0.091-0.424)	–	(0-0.152)
Formosa	0.357	0.321	0	0.262	0	0.060
	(0.241-0.500)	(0.207-0.448)	–	(0.155-0.379)	–	(0-0.121)
	(0.179-0.571)	(0.143-0.536)	–	(0.107-0.464)	–	(0-0.179)

Frequencies were estimated as the means from 1000 random draws of single genotypes per nest. The 95% CIs around the genotype frequencies (in parentheses) were estimated using a liberal (upper row) or conservative (lower row) bootstrap approach.