The origin of biological nitrogen fixation in maize landraces around Totentepec

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Abstract

In species with social hierarchies, the death of dominant individuals typically upheaves the social hierarchy and provides an opportunity for subordinate individuals to become reproductives. Such a phenomenon occurs in the monogyne form of the fire ant, *Solenopsis invicta,* where colonies typically contain a single wingless reproductive queen, thousands of workers and hundreds of winged non-reproductive virgin queens. Upon the death of the mother queen, many virgin queens shed their wings and initiate reproductive development instead of departing on a mating flight. Workers progressively execute almost all of them over the following weeks.

To identify the molecular changes that occur in virgin queens as they perceive the loss of their mother queen and begin to compete for reproductive dominance, we collected virgin queens before the loss of their mother queen, six hours after orphaning and 24 hours after orphaning. Their RNA was extracted and hybridized against microarrays to examine the expression levels of approximately 10,000 genes. We identified 297 genes that were consistently differentially expressed after orphaning. These include genes that are putatively involved in the signaling and onset of reproductive development, as well as genes underlying major physiological changes in the young queens.

Introduction

Maize (Doyle & J.L. 1990), with a global output exceeding 844 million tons in 2010, is the most vital crop in the world [3]. Aside from traditional use for human consumption, maize has important use as livestock feed, cooking oil, and biofuel [4]. The projected global human population of 9 billion in the year 2050 is expected to require 70% more food than is presently produced, a major portion of which must come from maize [4]. With the advent of the Green Revolution, reported increases in yield have been due to intense application of synthetic nitrogen fertilizers. 60% of such fertilizers are used for cereal production [5], which entirely doubled between 1966 and 2000 [4, 6]. Yet fertilizer-use by cereals is inefficient, at a rate of less than 50% [7]. This inefficiency results in nitrate leaching into soil and groundwater supplies, thereby generating profound environmental concerns and health risks [8]. Furthermore, present rates of fertilizer over-application cannot be sustained [9], leaving an ever-growing need to develop alternative solutions for increased crop yield. This has led to recent efforts and accumulating evidence to indicate nitrogen fixation in cereal grains of agronomic importance [5, 8, 10, 11, 12]. As stated previously, our research collaborative has accrued evidence for biological nitrogen fixation in Totontepec maize (highlighted in Preliminary Results). With this foundation, I will work to genetically characterize nitrogen fixation and aerial root development in Zea mays.

In recent years, genetic mapping in maize has been greatly enhanced by development of the maize Nested Association Mapping panel [13]. With a joint-linkage association approach, this population has allowed for the genetic characterization of complex traits. Traits that were not intensely selected during maize domestication and improvement have trait architectures governed by numerous loci of small effect. Flowering time and leaf architecture, for instance, each are controlled by more than 30 QTL [14, 15]. This is in stark contrast to the genetic architectures for known domestication and improvement traits. Teosinte branched1 (tb1, governing apical dominance) and teosinte glume architecture1 (tga1, governing seed coat lignification) are two loci of extreme effect that govern radical differences between teosinte and modern maize domesticates [16, 17]. In addition, kernel carotenoid content, brought under intense selection within the past century due to its beneficial health effects for humans and livestock, is governed by only three loci of large effect [3, 18, 19, 20]. This forms the rationale for aim 1. Based upon the hypothesis that nitrogen fixation in maize is governed by aerial root development and mucilage production [to act as a chemo-attractant for diazotrophic microbes], this trait would have been present in ancestral teosinte populations – namely, Zea mays spp. Mexicana (hereafter Mexicana) which develops an abundance of aerial roots (Figure 4A). Following the model of trait architecture for pre-domestication traits, and including mild selection pressures by humans, I anticipate a moderate number of loci of small effect contributing to nitrogen fixation in maize.

Modern maize has a complex evolutionary history. Due to its tremendous phenotypic diversity [21, 22], maize originally was thought to be the product of multiple domestication events [23]. It now has been proven that all maize falls within a monophyletic clade derived from the wild teosinte Z. mays spp. Parviglumis (hereafter Parviglumis) [23], domesticated more than 8,700 years CBP in the Balsas River Valley [24]. Sequence analysis originally identified highland maize as genetically most similar to Parviglumis, which resides in lowland regions below 1,800 m elevation [23]. Recent work has elucidated this paradox. Relative kinship between highland maize and Parviglumis has been affected by significant admixture between highland maize and the related Mexicana (Figure 4B) [25, 26, 27], which resides in highlands above 1,700 m elevation [23] and diverged from Parviglumis 61,000 years ago [28]. This forms the rationale for aims 2 and 3. Due to the presence of abundant aerial roots in Mexicana, and lack thereof within Parviglumis (Figure 4A), I hypothesize that aerial root formation, and thus nitrogen fixation in maize, were the products of adaptive introgression from Mexicana. I anticipate greater presence of nitrogen fixation and aerial root abundance in highland maize varieties, with selective sweeps indicative of selection for prominent aerial root development. Furthermore, I expect nitrogen fixation to be largely absent in improved lines, due to drastically reduced need for this process and selection for reallocation of energy to other physiological processes.

Materials and methods

Ant collection and rearing

Eight monogyne *S. invicta* fire ant colonies, each containing at least 50 winged virgin queens and between 5,000 and 10,000 workers, were collected from a single population in Athens and Lexington, GA, USA in June 2006. There is genetic variability between colonies, however it is moderate because only few queens founded the North American *S. invicta* population when they were introduced from South America in the 1930s (**??**). All colonies were returned to the laboratory and reared for one month under standard conditions (**?**). Queen- and male-destined brood were identified by their large size and removed weekly to ensure that only field-reared queens be used in for our experiment. Indeed, laboratory-reared queens do not normally grow to the same sizes as field-reared queens and rarely shed wings or initiate reproductive development after orphaning (E. Vargo, NC State, Raleigh). We determined that each study colony was of the monogyne social form using several lines of evidence. Nest shape, nest density and worker size distribution were used to make initial identifications of social form in the field (**?**). Subsequently, monogyny was confirmed for each colony by the presence of a single, highly physogastric, wingless queen. Finally, the social form was further verified by electrophoretically

detecting only the *B* but not the *b* allele of *Gp-9* in pooled samples of 20 workers from each colony (lack of the *b* allele is diagnostic for monogyny in *S. invicta* in the USA (**????**)).

Orphaning simulation, RNA isolation and microarray hybridization

We removed the mother queen and collected virgin queens just before orphaning as well as 6 and 24 hours after orphaning (subsequently referred to as time points t_{0h}, t_{6h} and t_{24h}) to examine the onset of the molecular reaction to orphaning in virgin *S. invicta* queens. However, virgin queens emit pheromonal signals after orphaning that are similar to those of an functional queen and can thus influence each other (**??**). We attempted to minimize such effects and simplify interpretation of results by taking advantage of the fact that *S. invicta* is a very opportunistic species that changes nests often in its native habitat which includes large flood plains (**?**). We sampled queens according to the following setup: For t_{0h}, we haphazardly collected five virgin queens from the foraging area of each source colony and individually flash-froze them with liquid nitrogen in tubes containing 1g of 1.4mm Zirconium Silicate beads (QuackenBush). We placed ten additional virgin queens per source colony into individual small nests with 2g of mixed workers and brood. The density of workers and brood was comparable to that found in the source colonies. All virgin queens isolated in this manner are expected to shed their wings and initiate reproductive development (**??**). We thus simulated orphaning for a total of 80 virgin queens from a total of eight source colonies. We harvested half of the virgin queens thus treated after 6 hours (t_{6h}) and the remaining queens after 24 hours (t_{24h}). All collected queens were individually flash-frozen immediately after collection as described above. Samples were then stabilized until RNA isolation by the addition of 900 μl of cold Trizol reagent (Invitrogen) followed by homogenization with a FastPrep instrument (MP Biomedicals) and storage at -80°C. In summary, we had thus collected five queens at t_{0h}, five queens at t_{6h} and five queens at t_{24h} from each of eight source colonies, constituting eight biological replicates for our experiment (See also Supporting Figure 1). We chose to pool five individuals from each replicate to reduce the impact of between-individual differences (**?**), and conducted eight replicates to obtain sufficient statistical power with a feasible workload. In comparison, other studies that examined the effects of social context or mating used four replicates from a single *Drosophila* strain (**?**), six replicates using different bees from a single colony (**?**), six true biological replicates (**??**), and examined pools of individuals from sixteen independent pairs of ant colonies (**?**).

Total RNA was isolated from all individuals using the Trizol protocol. RNA was pooled from 5 individuals per source colony for each time point and treated with DNA-free (Ambion). Subsequently, impurities were filtered away with MicroCon-30 spin columns (Millipore), and RNA quality was assessed on a 1% agarose gel prior to amplification using the MessageAmp II kit (Ambion). Amplified

mRNA samples from the eight colonies at three timepoints (t_{0h}, t_{6h} and t_{24h}) were labeled, hybridized to microarrays made from 22,560 independent fire ant cDNA spots (Microarray construction described in **?**), and scanned as previously described (**?**). This was done according to a dye-balanced loop design (Supporting Figure 1). For all procedures, precautions including randomization of sample order were taken to avoid introducing unwanted biases.

Microarray analysis

We followed a standard microarray analysis procedure, guided by the documentation of the Bioconductor limma package (**???**). In brief, median signal and background levels for each probe were extracted from scanned microarray images using Axon Genepix software. The limma 2.16 package (**?**) in R 2.8.1 (**?**) was used for normexp background correction, print-tip loess normalization within arrays, and aquantile normalization between arrays (**?**). The arrayQualityMetrics package (**?**) and custom R scripts were used for quality control. The 18,444 *Solenopsis invicta* cDNA spots yielding a single PCR band (**?**) and passing visual and automated inspection were used for analysis. We constructed a design matrix incorporating effects for sampling times (t_{0h}, t_{6h} and t_{24h}), biological replicate (eight colonies) and the two dyes. The model that is fit to each gene may thus be represented as "$expression = timepoint + replicate + dye$". The limma package was used for bayesian fitting of the model. Differential expression was determined for the contrasts "t_{24h} vs. t_{0h}", "t_{24h} vs. t_{6h}", and "t_{6h} vs. t_{0h}" according to the nested F method in limma. Briefly, a moderated F test determined that 521 microarray clones were differentially expressed for at least one of the contrasts with a 10% False Discovery Rate (FDR; **?**). Subsequently, significance of differential expression was assigned to one or several contrasts. In comparison, the effects of mating on honey bees queens were determined with 5% FDR (**?**), a comparison between fire ant workers from different social structures used 10% FDR (**?**), and the effect of the presence of brood on honey bee workers was determined with 30% FDR (**?**).

Sequence data, annotation and gene category analysis

The published sequences of all microarray clones (**?**) were assembled along with data from two runs of 454 sequencing of independently constructed cDNA libraries (Y. Wurm, D. Hahn and DD. Shoemaker; DH and DDS are at USDA-ARS, Gainesville). High quality sequence information was obtained for 16,227 out of the 18,444 *S. invicta* cDNA clones used for gene expression analysis. This was also the case for 475 out of the 521 significantly differentially expressed clones.

Annotation was obtained via several methods. First, we ran NCBI BLASTX 2.2.16 to compare assembled fire ant sequences with the non-redundant protein database (EMBL release 99). We retained

informative gene descriptions of hits with E-value < 10^{-5}. Second, Gene Ontology (GO) (**?**) annotations were inferred using BLASTX as previously described (**?**). Finally, each fire ant sequence was manually assigned a single descriptive category. The manually assigned gene category putatively encapsulates the general function of each sequence and is derived subjectively by examining the SwissProt or Ensembl database entries of the five best BLASTX hits (E-values < 10^{-5}), with an emphasis on GO, Interpro, and PANTHER annotations. The manual annotation comprises a total of 34 general gene categories (J. Wang, M. Nicolas and L. Ometto, University of Lausanne, Lausanne).

Overrepresentation of manually assigned gene categories and GO categories was determined, respectively, using exact one-sided Fisher tests in R and the Elim test from the topGO Bioconductor package (**?**) limited to categories containing at least 10 fire ant genes. These included 514 Biological Processes, 131 Cellular Components and 171 Molecular Functions.

Comparison with data from other species

We wanted to determine the extent to which gene expression differences linked to changes in social context and reproductive status in this study are likely to play similar roles in other insects. To do this, we downloaded lists of significantly over- and under-expressed genes from studies that examined the transition to reproduction in flies, bees and mosquitos (**????**) as well as the fixed differences in reproductive status between honey bee queens and workers (**?**). Mapping between microarray probes and coding sequences was either provided by the study's authors (for **?**), obtained by BLASTN of probe sequences to coding sequences (for **?**) or downloaded (for **???**) from BioMart (**?**). Orthology information was required to compare lists of signficant genes between ants and the other species, however such information is practically nonexistant. This is in part because and only partial transcriptome and no proteome or genomic sequence data are published for ants. To obtain orthology information, we modified the Inparanoid ortholog identification pipeline (**?**) as follows: BLASTX was replaced with TBLASTX and stringency was reduced so that match areas must span at least 25% of the longer sequence with actual matching segments aligning with at least 10% of the longer sequence. We independently ran this modified Inparanoid pipeline on the assembled fire ant sequences and the complete set of coding sequences of each of the following species: *Drosophila melanogaster* (Flybase release 5.9), *Apis mellifera* (Honey Bee Official Gene Set pre-release 2) and *Anopheles gambiae* (AgamP3.4).

To determine the extent of overlap between two lists of significant genes, we constructed a 2-by-2 contingency table containing: the number of orthologous genes in both lists, the number of genes examined in the relevant studies but not part of the significant lists, and the numbers of genes that were examined in both studies but in only one of the two lists of significant genes. Subsequently, we

conducted an exact one-sided Fisher test to determine whether the number of genes in both lists was higher than would be expected by chance. Only significant results ($p<0.05$) are reported.

We determined the extents of overlap between two lists of significant genes from our study (the 146 genes upregulated at one point after orphaning and the 152 genes downregulated at one point after orphaning) and the lists of genes from each of the other studies. This was possible for a reduced set of genes that are both putatively orthologous between fire ants and the species from the other study and present on both the ant microarray and the microarray used in the other study. For the **?** study, we report significant overlap comparing the list of genes upregulated at one point after orphaning in our study with the list of 549 bee genes in the Honey Bee Official Gene Set that were more highly expressed in honey bee queens than in reproductive workers as well as the list of 619 bee genes that were more highly expressed in honey bee queens than in sterile workers. For the **?** study, we report significant overlap comparing the list of genes upregulated at one point after orphaning in our study with the list of 441 bee genes that were more highly expressed in mated than virgin honey bee queens, as well as with the list of 356 genes that were more highly expressed in honey bee queens that were mated but not yet laying eggs than in queens that were mated and egg-laying. For all remaining comparisons of pairs of lists of significant ant and honey bee genes, the overlaps were either non-significant, or were not examined because they concerned five genes or less.

For the **?** study, we obtained results comparing our two fire ant gene lists with a combined list of 1,663 *Anopheles* genes that were either more highly expressed in females 2h, 6h and 24h after mating than in virgins or more highly expressed 6h than 2h or 24h than 6h after mating, as well as with the complementary list of 1,586 genes that were less highly expressed in virgins than in mated female *Anopheles*. For the **?** and **?** studies, we compared our results with all individual lists of *Drosophila* genes that were differentially expressed in response to different aspects of mating, as well as with a combined list of all mating-response genes they had identified.

Results

Differential gene expression after orphaning

Four hundred seventy-five of the 16,227 sequenced cDNA clones, putatively representing 297 genes, were significantly differentially expressed between the samples of virgin queens collected 0 hours, 6 hours and 24 hours after orphaning (respectively t_{0h}, t_{6h} and t_{24h}). The remaining genes were either expressed similarly before and after orphaning, were highly variable between biological replicates, or yielded signals too weak for reliable assessment of differential expression. Among the 297 significantly

differentially expressed genes, four were upregulated within 6 hours of orphaning, while one was downregulated. One hundred forty-four genes were more highly expressed twenty-four hours after orphaning than at t_{0h} or at t_{6h} including one of the four genes that was already upregulated after 6 hours, while a total of 152 genes were significantly downregulated after 24 hours (Figure 2). One of the genes significantly upregulated after 6 hours was significantly downregulated between 6 and 24 hours. The significant genes are listed in Supporting Tables 1 and 2. These gene expression changes precede or are independent of wing shedding since none of 40 virgin queens collected 6 hours after orphaning and only three of 40 virgin queens collected 24 hours after orphaning had shed their wings.

Gene set enrichment analysis

We bioinformatically annotated the genes that were significantly upregulated or downregulated after orphaning and compared their annotations with the annotations of all genes examined on the microarray by using two different annotation methods. From our manually assigned annotation categories, two gene categories were overrepresented among upregulated genes. These were *proteasome* (11 observed, 1.2 expected, exact one-sided Fisher test $p = 1 * 10^{-7}$) and *protein transport* (10 observed, 1.5 expected, exact one-sided Fisher test $p = 4 * 10^{-6}$). No other manually assigned annotation categories were overrepresented among up or downregulated genes. From the BLAST-inferred Gene Ontology categories, several categories were overrepresented among up- and downregulated genes (complete list in Table 1). In particular, genes putatively part of the *proteasomal complex* were overrepresented among the upregulated genes (7 observed, 0.7 expected, $p = 0.0003$, topGO Elim test, adjusted for 10% False Discovery Rate (FDR)). Among downregulated genes, those putatively located in *microsomes* and involved in *oxidation reduction* were overrepresented (respectively 6 observed, 0.5 expected, FDR adjusted topGO Elim test $p = 0.0007$, and 14 observed, 3.3 expected, FDR adjusted topGO Elim test$p = 0.0005$). Additionally, genes that putatively have *aromatase activity* were overrepresented among the significantly downregulated genes (5 observed, 0.3 expected, FDR adjusted topGO Elim test $p = 0.0014$). In fact, all five of these genes are putative *Cytochrome P450*s.

Genes related to Juvenile Hormone metabolism

Among the 297 genes significantly differentially expressed in orphaned compared to non-orphaned queens, five have sequence similarity to genes from other species that are involved in Juvenile Hormone (JH) metabolism or response. In particular, three putative JH esterases were significantly downregulated after orphaning, while one was significantly upregulated. Additionally, a putative JH epoxide hydrolase was significantly downregulated after orphaning. Several putative JH inducible genes as well as a

putative JH esterase-binding gene showed non-significant increases in expression level after orphaning (Figure 3).

Comparison of fire ant results with data from honey bees

To determine whether the differentially expressed genes identified in our study are also differently expressed between reproductive and non-reproductive individuals in honey bees, we compared our results with the studies of **?** and **?**. The first of the two studies identified genes differentially expressed between brains of honey bee queens and workers. We identified a subset of 902 ant-bee orthologs examined in both that study and ours. Genes upregulated in orphaned fire ant queens were enriched for genes upregulated in brains of queen bees relative to brains of reproductive workers (12 observed, 7.5 expected, exact one-sided Fisher test $p = 0.005$). There was no significant overlap between other pairs of lists of genes from the two studies. Among the twelve genes that overlap between the groups of significantly upregulated ant and bee genes (Supporting Table 3), four are part of the manually assigned gene category *proteasome* (0.2 expected, exact one-sided Fisher test $p = 1 * 10^{-4}$).

The other study identified genes differentially expressed between virgin and mated honey bee queens (**?**). Among 2,286 ant-bee orthologs examined in our study as well as the bee study, 13 genes were more highly expressed in response to orphaning in fire ants and in response to mating in honey bee queens (7.7 expected, exact one-sided Fisher test $p = 0.038$, genes listed in Supporting Table 4). Among the thirteen genes that overlap between the two gene lists, four are part of the manually assigned gene category *proteasome* (0.2 expected, exact one-sided Fisher test $p = 1 * 10^{-4}$). There was no significant overlap between other pairs of lists of genes from the two studies.

Comparison of fire ant results with data from dipterans

To determine whether the differentially expressed genes identified in our study are also involved in the transition towards reproduction in other insects, we compared our results with those from studies conducted in *Anopheles* and *Drosophila*. The comparison of our results with those of a study on the effects of mating in female *Anopheles gambiae* mosquitoes for 1,682 orthologs ant-*Anopheles* orthologs (**?**) revealed that genes whose level of expression increased after orphaning in *S. invicta* queens are enriched for genes that are upregulated after mating in *Anopheles* (36 observed, 20.6 expected, exact one-sided Fisher test $p = 8 * 10^{-5}$, genes listed in Supporting Table 5). There was no significant overlap between other pairs of lists of genes from the two studies. Six of the thirty-six genes identified in both studies are part of the manually assigned gene category *proteasome* (0.5 expected, exact one-sided Fisher test $p = 3 * 10^{-5}$).

Similar gene expression studies were also performed in the fruitfly *Drosophila melanogaster*. We found no significant overlap between expression changes due to orphaning in fire ant queens and changes due to mating in female *Drosophila* (**??**), nor between orphaned fire ant queens and specific aspects of *Drosophila* mating: the mating process itself (without receiving sperm), receiving sperm, or receiving particular accessory proteins normally part of sperm (**?**).

Discussion

We used microarrays to conduct a genome-wide survey of gene expression in virgin *Solenopsis invicta* fire ant queens over the 24 hours that follow orphaning from their mother queen. We identified five genes that are consistently differentially expressed within six hours of orphaning. These early response genes may be responsible for some of the additional 292 gene expression changes that take place within 24 hours of orphaning. The annotations of the differentially expressed genes indicate that they potentially are involved in many different functions, including signaling reproductive status, reproductive development, proteasomal activity, protein transport, and regulation of chromatin structure and transcription. We discuss each in turn.

Genes potentially involved in signaling of reproductive status

The pheromones that the mother queen uses to signal her presence and fertility are currently unknown. Our study revealed that *Glutathione S-transferase* (GST) is the only gene downregulated in virgin queens 6 hours after orphaning. Furthermore, an additional GST as well as five *Cytochrome P450*s are significantly downregulated in virgin queens within 24 hours of orphaning. Both GSTs and *Cytochrome P450*s are known to be involved in degrading foreign and endogenous compounds (**?**). We speculate that the virgin queens may use these genes to degrade fertility signals produced by the mother queen. This could be important if maternal fertility signals also triggered reproductive development in the virgins. Alternatively, virgin queens may produce their own fertility signals, and simultaneously degrade them using the GSTs and *Cytochrome P450s*, hence permitting them to avoid aggression from the workers yet be able to rapidly increase levels of fertility signals when orphaned.

We also identified three upregulated genes putatively related to olfactory signals, two *chemo-sensory proteins* (CSPs) and one *odorant binding protein* (OBP). The CSPs and OBP may play the roles of carrier proteins (**??**) possibly involved in the production of reproductive status signals. Interestingly, the gene with the highest sequence similarity to the OBP is *Gp-9*, a gene that is linked to odor differences between queens (**??**) and to the selective execution of queens which lack the small *b* allele at this locus in multiple-queen colonies of *S. invicta* (**?????**). The upregulated OBP could similarly be involved in the production

of a qualitative signal by virgin queens.

Genes known to be involved in reproductive development in social insects

The level of Juvenile Hormone (JH) increases with the onset of reproduction in many female insects. In particular, high JH titers have been linked to reproductive dominance in bumble bees as well as in *Polistes* wasps, but not in honey bees where JH has been shown to regulate the labor tasks between workers (reviewed in **?**). After orphaning young *S. invicta* queens, JH synthesis rate increases and JH body content peaks prior to wing shedding (**?**; see also Figure 1). The ectopic application of synthetic JH to virgin queens leads to wing shedding even if the mother queen is present (**?**), whereas applying an inhibitor of JH synthesis represses wing shedding in orphaned virgin queens (**?**). The fact that JH level increases after orphaning is consistent with our findings that four genes putatively involved in JH degradation are downregulated after orphaning. Indeed, downregulation of these genes should lead to reduced JH degradation and thus to increased JH levels. Our data also imply that JH degradation genes are highly expressed before orphaning, and thus that JH is already being produced and simultaneously degraded before orphaning. Thus, maintenance of low JH levels in virgin queens prior to orphaning may be due to the simultaneous production and degradation of JH. This has also been suggested to occur in bumble bee workers by **?** who found that the rate of *in vitro* JH synthesis does not reliably indicate hemolymph JH titers. Such dual control of JH titer by simultaneous production and degradation of JH is known to exist from studies in solitary insects (**??**).

Beyond the role of JH, two small-scale studies identified genes associated with reproductive differences in ants. In *S. invicta* queens, participation in a mating flight triggers wing shedding and reproductive development (**?**) and leads to the upregulation of at least seven genes (**?**). One of these genes, *Striated Muscle Activator of Rho Signaling* (STARS), was also significantly upregulated in our study 6 and 24 hours after orphaning. Five of the remaining genes, *Vitellogenin-1*, *Vitellogenin-2*, *Yellow-1*, *Yellow-2* and *Abaecin* were more highly expressed after orphaning, although not significantly so. A study in the black garden ant *Lasius niger* identified seven genes more highly expressed in mature queens than in workers (**?**). While none of these genes showed significant expression differences in our study, the mean expression level for four of them was non-significantly higher after orphaning in *S. invicta*. The remaining three genes were respectively absent from the *S. invicta* microarray, similarly expressed, or had non-significantly lower mean expression levels after orphaning.

Genes that are putatively proteasomal

Genes with similarity to proteasomal genes were highly overrepresented among the genes upregulated after orphaning. Proteasomes are responsible for degrading unneeded proteins. The proteasomal genes could be involved in degrading wing muscle tissue or storage proteins such as hexamerins and vitellogenins that would liberate amino-acids that can be used for reproductive development. Alternatively, the increased proteasomal activity after orphaning may trigger changes in gene expression or cellular proliferation via the respective degradation of transcriptional repressors or specific cyclins. Both possibilities are coherent with the overrepresentation of proteasomal genes among the genes that we identified as being upregulated after orphaning in ant queens and also after mating in bees and mosquitoes. This indicates that the role of proteasomal genes during the onset of reproductive development may be evolutionarily conserved. Furthermore, we detected significant downregulation of a gene with similarity to *Cellular Repressor of E1A-stimulated Genes 1* (*CREG1*) after orphaning. *CREG1* has been shown to inhibit growth in human cancer cells and to inhibit apoptosis of human muscle cells (**?**). The downregulation and degradation of this gene in virgin fire ant queens may similarly induce proliferation of ovarian tissue or the apoptosis of wing muscle cells.

Genes putatively involved in protein transport

Genes sharing sequence identity with those involved in protein transport were highly overrepresented among the genes upregulated after orphaning. Proteins need to be shuttled between intracellular compartments for post-translational modifications as well as signal transduction. Protein transportation is also essential for communication between cells via the secretion and uptake of proteins (**?**). The upregulation of putative protein transport genes in orphaned fire ant queens could be involved in changes in neuronal activity (**?**) as a response to orphaning. Alternatively, they may be involved in ovarian development.

Genes putatively involved in transcriptional changes and chromatin remodeling

Three lines of evidence indicate that major transcriptomic and epigenetic changes are taking place after orphaning in virgin fire ant queens. First, the upregulated genes include two putative *RNA polymerase* subunits as well as a putative *Mediator complex subunit* involved in protein-coding gene transcription (**?**). Second, a Zinc finger transcription factor domain containing gene is downregulated, while *STARS* and a RING finger transcription factor domain containing gene are upregulated. *STARS* may induce wing muscle degradation as previously suggested (**?**). Finally, genes similar to *Chromobox Homolog protein 1* and *Nucleoplasmin-like protein* are upregulated after orphaning. Both are important for chromatin

remodeling (**??**). Some or all of these gene expression changes could be related to the post-orphaning increases in ovarian development and egg production (**??**).

Conclusion

This study represents the first genome-wide survey of gene expression changes in subordinate animals immediately following the sudden loss of the dominant individual. We identified 297 genes differentially expressed within 24 hours of orphaning in virgin *S. invicta* queens. Many of the observed gene expression changes are consistent with previous knowledge about the physiological changes in virgin queens after orphaning, and some genes related to the onset of reproductive development appear to be conserved across species from ants to bees and even mosquitoes. Additionally, we detected several genes possibly required for the perception or production of olfactory signals. These genes may play roles in triggering the onset of reproductive development in virgin queens or in signaling reproductive status to nestmates. Finally, we found evidence for activation of genes putatively involved in muscle degradation and ovarian development. However, much work remains to truly understand the molecular-genetic cascades of events involved in the competition for reproductive dominance between virgin queens. It will be particularly fascinating to understand the evolutionary pressures acting upon different genes involved in this process. A further challenge will be identifying the basis by which workers make decisions regarding which competing queens to execute and which to keep.

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Figure Legends

Figure 1 Timeline of post-orphaning events in the fire ant (based on the following studies: **??????**).

Figure 2: Numbers of genes significantly differentially expressed in young fire ant queens within six hours (left) and 24 hours of orphaning (right).

Figure 3: Expression levels of genes related to Juvenile Hormone (JH) metabolism and response in virgin fire ant queens that are either still in presence of their mother queen or have been orphaned for 6 or 24 hours. Only genes with multiple clones on the microarray are shown. Error bars represent the standard error of the mean expression levels as obtained by independent clones. Genes for which at least one representative clone is significantly differentially expressed after orphaning are indicated by triangles.

Table Legend

Table 1: Gene Ontology annotations that are significantly enriched among genes that are significantly upregulated or downregulated after orphaning.

Tables and Figures

Figure 1:

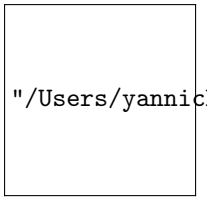

Figure 2:

Figure 3:

Table 1:

Author Information Box

This work is part of Y. Wurm's PhD thesis under the supervision of L. Keller. Y. Wurm and J. Wang use genetic tools to study the social lives of ants. L. Keller works on various aspects of evolutionary ecology such as reproductive skew, sex allocation, caste determination as well as the molecular basis of aging and behavior in ants.

Supporting Information

Supporting Figure 1: Graphical representation of microarray hybridizations. The unit of biological replication is the colony; each pool of five queens was hybridized to two different microarrays. Each vertice represents an amplified RNA sample and each edge represents a microarray hybridization (a total of $3 * 8 = 24$ hybridizations were conducted). Cy3-labeled samples are at the tails and Cy5-labeled samples are at the heads of arrows.

Supporting Table 1: List and annotations of all fire ant genes significantly upregulated for at least one of the following comparisons: 6h vs 0h, 24h vs 6h, 24h vs 0h. Some genes are significant according to multiple microarray clones.

Supporting Table 2: List and annotations of all fire ant genes significantly downregulated for at least one of the following comparisons: 6h vs 0h, 24h vs 6h, 24h vs 0h. Some genes are significant according to multiple microarray clones.

Supporting Table 3: List and annotations of all fire ant genes significantly upregulated for at least one of the following comparisons: 6h vs 0h, 24h vs 6h, 24h vs 0h and also significantly higher in brains of honey bee queens than reproductive workers

Supporting Table 4: List and annotations of all fire ant genes significantly upregulated for at least one of the following comparisons: 6h vs 0h, 24h vs 6h, 24h vs 0h and also significantly upregulated after mating in honey bee queens

Supporting Table 5: List and annotations of all fire ant genes significantly upregulated for at least one of the following comparisons: 6h vs 0h, 24h vs 6h, 24h vs 0h and also significantly upregulated in *Anopheles gambiae* females in response to mating according to Vectorbase gene expression data

Microarray Data: Will be uploaded to the Gene Expression Omnibus database (access information will be put here).