**Supplementary Methods**

**RNA extraction and cDNA hybridization**

All samples were homogenized in 600 µl of RLT buffer (Qiagen RNeasy). RNA samples, with the exception of those from adult males, were precipitated in 50% ethanol before being processed according to the RNeasy protocol. Because some unknown components in the adult male cuticle inhibited binding of RNA to the Qiagen columns, we precipitated total RNA from the RLT buffer with isopropanol overnight before dissolving the RNA pellets in 1 ml of TRIzol® reagent, following the manufacturer’s instructions. For all samples, we used the Turbo DNA-free kit (Life Technologies) to remove residual DNA from the total isolated RNA. Finally, we amplified the mRNA ~10-100 fold and hybridized it to our cDNA microarrays as described in [1].

**Microarray analysis**

Our microarray slides contained 18 438 fire ant cDNAs representing 5957 genes, after excluding spots that failed to yield a single PCR product and spots that matched neither RNA sequence data nor any gene sequences in Swiss-Prot and TrEMBL based on BLAST searches (Supplementary information file 1 and Table S2). Of these 5957 genes (45.4% of the predicted fire ant genes), 3082 had multiple clones. All sample hybridizations were compared to a common reference [1] and hybridized on the same batch of microarray slides. Microarray slides were scanned using an Agilent Microarray Scanner, with the median foreground and background spot intensities extracted for the red and green label signals using GenePix® Pro software. We performed background subtraction (using the parameter normexp+offset=50 [2]), transformed the red/green intensity ratios to log2 expression data, and normalized the data with the limma package in R 2.11.1. We performed Print-tip Loess normalization – normalization of the red and green intensities within arrays – as well as between-array normalization across all 90 samples. However, we subsequently removed one slide of a haploid male pupa sample from our analysis because its gene expression profile had no correlation to other haploid male pupae (Pearson’s *r*=-0.04), while the correlation among other haploid males was high (Pearson’s *r* > 0.84). One potential cause of this outlier is that the pupa was close to death, something we have occasionally observed for samples in a separate timecourse experiment. For statistical analyses, we compared only individuals of the same age-class. We applied empirical Bayes statistics to estimate moderated *t*-statistics for specific pairwise comparisons with the limma package, adopting a false positive discovery rate (FDR) of 1% for all *F* tests and each pairwise *t* test. We used statistical cutoff rather than an absolute cutoff because small changes could well be significant and important

Because most clones of the same gene showed similar expression values (Table S3), a given gene was considered as differentially expressed if at least one clone corresponding to it was differentially expressed. Differences in expression for the remaining clones may be due to: 1) no DNA on the spotted microarrays; 2) annotation error and 3) alternative splicing (different clones correspond to different exons of single genes with variable expression patterns). We show only one of the significantly differentially expressed clones for each gene in the heat maps ─ the first clone listed alphabetically by name of the microarray spot ID. The data for all clones are presented in Table S2.

We defined ploidy-specific genes as genes that were significantly differentially expressed between diploid males and haploid males as well as between queens and haploid males (i.e., genes that were up-regulated in haploid males compared to both diploid males and queens or genes that were down-regulated in haploid males compared to both diploid males and queens). Likewise, sex-specific genes were defined as genes up-regulated in queens compared to both diploid and haploid males, or down-regulated in queens compared to both diploid and haploid males.

Because fire ant gene annotations using GO are incomplete, we manually searched for genes present on the microarray that were potentially involved in spermatogenesis and sperm maturation by looking at the descriptions of the 5957 genes for the keywords, “sperm”, “testis”, “accessory” and “dense fiber protein” (associated with sperm tail protein). A total of 12 genes were thus identified. Similarly, we identified 154 candidate genes involved in pheromone production or perception by searching for the keywords, “cuticle”, “cuticular”, “chito”, “chitin”, “chondroitin”, “peritrophin”, “desaturase”, “elongation of very long chain fatty acid”, “fatty”, “cytochrome p450”, “odorant binding protein”, “chemosensory protein”, and “allergen” because such genes are likely to encode proteins that are involved in hydrocarbon (semiochemical) biosynthesis, reside on the cuticle, or are associated with chemical communication in some other way.

We performed hierarchical clustering of the genes (microarray expression levels) and samples (individual ants) based on Pearson correlation of the expression values (expression values of all samples were compared to a common reference) by using the complete linkage clustering method to calculate the distances between groups in MeV v.4.6 [3].

**Gene annotation and enrichment test**

We used BLASTn [4] to compare the cDNA sequences in the microarrays against a gene model of *S. invicta* RNA sequence data derived from high-throughput RNA sequencing (unpublished), requiring for matches that (i) RNA sequence length be greater than 100bp and greater than 40% of cDNA sequence length and (ii) that sequence identity be above 95%. Annotation and GO terms obtained for the RNA transcripts by BLAST2GO [5] were assigned to the cDNA sequences that matched these transcripts. cDNA sequences that failed to match RNA transcripts were compared against the *S. invicta* proteome [6] using BLASTx [4], and we retained the annotation and GO terms of the best hits. For the remaining cDNAs that neither matched RNA transcripts nor the proteome of *S. invicta* (e.g., viral genes), we used the annotation and GO terms of the best hits from a direct BLAST search of the Swiss-Prot (reviewed) and TrEMBL (unreviewed) databases. Enrichment analyses were done using the TopGO software [7].

**Real-Time Quantitative Reverse Transcription PCR (qRT-PCR)**

We assayed the expression differences for Exons 4, 5, 6, and 7, as well as the exon splice junction between Exons 4 and 6, of the *doublesex* (*dsx*) gene in eight individuals of each ploidy/sex at each developmental timepoint (72 individuals) with qRT-PCR. Half of these samples (four individuals for each ploidy/sex and timepoint combination) were samples used in our microarray experiment and the other half were independent samples not used in the microarray experiment. We discarded eight individuals whose reverse transcription reactions were poor, so that the analyses were done on the following 64 samples: nine diploid male pupae, six haploid male pupae, eight queen pupae, four 1-day-old diploid male adults, six 1-day-old haploid male adults, eight 1-day-old adult queens, eight 11-day-old diploid male adults, seven 11-day-old haploid male adults, and eight 11-day-old adult queens.

We used TaqMan Reverse Transcription Reagents (Life Technologies) to synthesize cDNA from 1 µg of the total RNA of each individual with the following reagents: 5µl of 10× RT buffer, 2.5µl of 50 mM random hexamers, 1µl of 25 mM dNTPs, 1µl of 25 nM RNase inhibitor, 1µl of reverse transcriptase (200 U), and water to a final volume of 50µl. This mixture was incubated at 48°C for 30 min and then heat inactivated at 9 °C for 5 min. After the reverse transcription reaction, we added 30µl of 10 mM Tris to adjust the volume to 80µl. A 4-fold dilution of these cDNA samples was used for the quantitative PCR.

The qRT-PCR amplification mixtures were made by combining 2µl of diluted cDNA with 5µl of Power SYBR Green PCR Master Mix (Applied Biosystems), 300nM of each gene-specific forward and reverse primer (Table S4) and water to a final volume of 10µl. Reactions were run in triplicate on an ABI PRISM 7900HT Sequence Detector.

Results from Sequence Detection Systems software (Applied Biosystems; fluorescence intensity default threshold set at 0.2) were exported as tab-delimited files into qBasePLUS [8] for subsequent analysis. The 64 amplified RNA samples were analyzed for the *dsx* exons and 3 control genes (*GAPDH*, *RpL37* and *RpS9*). Outliers within each set of reaction triplicates were filtered (max dCt=0.5) and relative gene expression levels were calculated with PCR efficiency correction and reference gene normalization to the control genes. Statistical analyses (ANOVA) of relative gene expression levels and production of box and whisker plots were performed in R [9].

**References for Supplementary Methods**

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