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Letter

Evolution of the Yellow/Major Royal Jelly Protein family and the emergence of social behavior in honey bees

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The genomic architecture underlying the evolution of insect social behavior is largely a mystery. Eusociality, defined by overlapping generations, parental brood care, and reproductive division of labor, has most commonly evolved in the Hymenopteran insects, including the honey bee *Apis mellifera*. In this species, the Major Royal Jelly Protein (MRJP) family is required for all major aspects of eusocial behavior. Here, using data obtained from the *A. mellifera* genome sequencing project, we demonstrate that the MRJP family is encoded by nine genes arranged in an ~60-kb tandem array. Furthermore, the MRJP protein family appears to have evolved from a single progenitor gene that encodes a member of the ancient Yellow protein family. Five genes encoding Yellow-family proteins flank the genomic region containing the genes encoding MRJPs. We describe the molecular evolution of these protein families. We then characterize developmental-stage-specific, sex-specific, and caste-specific expression patterns of the *mrjp* and *yellow* genes in the honey bee. We review empirical evidence concerning the functions of Yellow proteins in fruit flies and social ants, in order to shed light on the roles of both Yellow and MRJP proteins in *A. mellifera*. In total, the available evidence suggests that Yellows and MRJPs are multifunctional proteins with diverse, context-dependent physiological and developmental roles. However, many members of the Yellow/MRJP family act as facilitators of reproductive maturation. Finally, it appears that MRJP protein subfamily evolution from the Yellow protein family may have coincided with the evolution of honey bee eusociality.

[Supplemental material is available online at www.genome.org.]

Explaining the evolution of group social behavior in terms of natural selection on individuals is one of the great triumphs of evolutionary biology (Hamilton 1964). Social behavior, the transition from individual survival by competition to integrated societies with cooperation, has multiple evolutionary origins (Wilson 1975). Eusociality, defined by overlapping generations, parental brood care, and reproductive division of labor, has most commonly evolved in the Hymenopteran insects, including bees, ants, and wasps (Wilson 1975).

The honey bee *Apis mellifera* is an excellent model for understanding the evolutionary genomics of eusociality. The complex social behavior of this species, which includes hive building, feeding of immature (larval) bees, and locating food resources, influences its important agricultural role as a producer of honey, beeswax, and propolis, and as a pollination facilitator (Williams 2000). The recent publication of the complete *A. mellifera* genome sequence (The Honey Bee Genome Sequencing Consortium 2006) will accelerate the identification and characterization of genes that modulate social behaviors.

A. mellifera caste determination occurs when young worker bees in the hive (nurse bees) produce, secrete, and feed a substance called Royal Jelly (RJ) to developing larvae. RJ is a natural source of essential amino acids, lipids, vitamins, acetylcholine, and other nutrients (Colhoun and Smith 1960; Schmitzova et al. 1998). In the initial 3 d of development, all larvae are fed RJ; but thereafter only larvae designated by workers to become queens receive RJ. In its place, a mixture of honey, pollen, and water is fed to larvae selected to become workers. Since individuals from the previous generation feed the young of the next generation, determining their developmental fate (fertile queen vs. sterile worker), all major aspects of *A. mellifera* eusociality are considerably influenced by RJ.

Major Royal Jelly Proteins (MRJPs) constitute ~90% of total RJ protein (Schmitzova et al. 1998; Sano et al. 2004; Santos et al. 2005; Scarselli et al. 2005). Eight *A. mellifera* loci encoding MRJPs (*mrjp1–mrjp8)* have been identified (Klaudiny et al. 1994a,b; Schmitzova et al. 1998; Albert and Klaudiny 2004), but little is known about the function of these genes or their protein products. We previously reported that the *mrjp1* gene is expressed in the mushroom bodies of the honey bee brain, implicating this gene in behavior (Kucharski et al. 1998). This result suggests that the MRJPs can be multifunctional, performing a nutritional role as a component of RJ and executing additional roles in various tissues including the brain. However, the expression patterns of the *mrjp* genes across development and in different sexes and castes have not been well-characterized.

MRJPs share a common evolutionary origin with the Yellow protein family, consisting of representatives from insects and some bacteria (Kucharski et al. 1998; Albert et al. 1999a,b; Maleszka and Kucharski 2000; Drapeau 2001). Two genes encod-

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ing Yellow-like proteins were recently discovered in *A. mellifera*, implying that the honey bee genome also contains a Yellow protein family (Albert and Klaudiny 2004). The characterized functions of Yellow-related proteins in flies suggest possible functions of honey bee Yellow-related and MRJP proteins.

The Yellow protein of *Drosophila melanogaster*, the patriarch of the Yellow protein family, is multifunctional. Since the early days of genetics, its dual requirement for normal pigmentation and behavior has been studied in considerable detail (e.g., Sturtevant 1913, 1915; Lindsley and Zimm 1992; Wittkopp et al. 2002a,b; Drapeau et al. 2003, 2005), and *yellow* is now known to be controlled, at least in part, by the fly's sex determination pathway (Radovic et al. 2002; Drapeau et al. 2003). These dual functions of Yellow appear to be conserved within *Drosophila* (e.g., Tan 1946; Wittkopp et al. 2002b; da Silva et al. 2005; Gompel et al. 2005; W.J. Etges, pers. comm.; J. Jaenike, pers. comm.; B. Prud'homme, pers. comm.). Other Yellow-related proteins appear to have evolved diverse functions in *D. melanogaster* (Fujii and Amrein 2002; Han et al. 2002; Artero et al. 2003; Giot et al. 2003; Claycomb et al. 2004).

In this study, we describe the genomic organization and putative function of all genes encoding honey bee MRJP and Yellow proteins. We identify and describe two and eight novel *mrjp* and *yellow* genes, respectively. Then, we show that all 10 *mrjp* genes are tandemly arrayed in an ~60-kb cluster, and are flanked by five *yellow* genes. An analysis of the intron/exon structures of these genes and the sequences of their protein products suggests that the *mrjp* gene array evolved via multiple, rapid duplications of a specific *yellow* precursor, *yellow-e3*. Transcriptional profiles of *mrjp* and *yellow* genes in the honey bee suggest that despite some similarities, the proteins encoded by these genes have evolved diverse physiological and developmental functions since their origin. However, the data presented here, in combination with the available information from flies and ants, suggest a common theme: a requirement of a noteworthy subset of MRJP and Yellow proteins in sex-specific reproductive maturation. Overall, our findings reveal the recent origin of a novel protein subfamily (MRJP) from an ancient protein family (Yellow), and show how this has affected both sex-specific individual behavior and complex group social behavior. The Yellow/MRJP protein family significantly influences the nature of honey bee social organization.

Results

The *A. mellifera* genome contains 10 *mrjp* and 10 *yellow* genes

The availability of the *A. mellifera* genome assembly version 2.0 (The Honey Bee Genome Sequencing Consortium 2006) allowed us to perform BLAST and iterated PSI-BLAST searches to identify genes that encode proteins related to previously known MRJPs. This process recovered two groups of genes. The first group encodes nine highly related proteins with ~60% identity to each other (BLASTP: $E = 10^{-176}$ to 10^{-70}). Most of these have been previously characterized at some level, and they were named *mrjp1–mrjp9* (Table 1). One additional, novel member of this group is a pseudogene encoding an incomplete polypeptide, which would otherwise encode a tenth member of the MRJP protein group. We name this pseudogene *mrjp-Ψ*. Although we have not been able to detect a specific *mrjp-Ψ* transcript, it is conceivable that this pseudogene may be a segregating null allele in the population. The second group of genes with significant similarity to previously known *mrjp* genes consists of 10 more distantly related genes (BLASTP: $E = 10^{-40}$ to 10^{-10}) encoding the honey bee orthologs of *D. melanogaster* Yellow-related proteins (Table 1).

We characterized the intron/exon structure of the 19 protein-encoding *mrjp* and *yellow* genes from *A. mellifera* by sequencing cDNAs from *mrjp* and *yellow* genes, and comparing these sequences with the appropriate genomic DNA sequences. Combined with previously collected data on *mrjp* cDNA sequences (Klaudiny et al. 1994a,b; Kucharski et al. 1998; Albert and Klaudiny 2004), we were able to determine the similarities and differences in intron/exon structure among these 19 evolutionarily related genes (Fig. 1; Supplemental Table S1). There are two noteworthy features. First, the intron/exon structure of the *mrjp* genes is highly conserved, with each gene having five introns within their coding sequences, located in exactly the same positions. The intron sizes do vary somewhat among *mrjp* genes, but because they are very similar, and also have the same transla-

Table 1. The gene family encoding the Yellow/MRJP proteins of *A. mellifera*

Gene name	Database ref.	Location	cDNA length	Number of introns[a]	GenBank acc. no.
mrjp1	GB14888	chromosome 11	1430	5 + 1	AF000633
mrjp2	GB16246	chromosome 11	1544	5 + 1	AF000632
mrjp3	GB16459	chromosome 11	1830 (±)	5 + 1	Z26318
mrjp4	GB11768	chromosome 11	1612	5 + 1	Z26319
mrjp5	GB10622	chromosome 11	1966 (±)	5 + 1	AF004842
mrjp6	GB13789	chromosome 11	1529	5	AY313893
mrjp7	GB11022	chromosome 11	1427	5 + 1	BK001420
mrjp8	GB14639	chromosome 11	1329	5 + 1	AY398690
mrjp9	GB16324	chromosome 11	1793	5	DQ000307
yellow	GB19464	chromosome 10	1436	5 + 1	
yellow-b	GB16705	GroupUn		4 + 1	
yellow-e	GB17225	chromosome 11		4 + 1	
yellow-e3	GB1889	chromosome 11	1286 (+)	4 + 1	
yellow-f	GB17489	GroupUn	1447	4 + 1	AY661557
yellow-g	GB10842	chromosome 11		2	
yellow-g2	GB18218	chromosome 11		2	
yellow-h	GB18654	chromosome 11	2187	3 + 1	
yellow-x1	GB18300	GroupUn		0	
yellow-x2	GB19132	chromosome 12		2	

[a]+1 means the presence of an additional intron in the 5′-UTR.

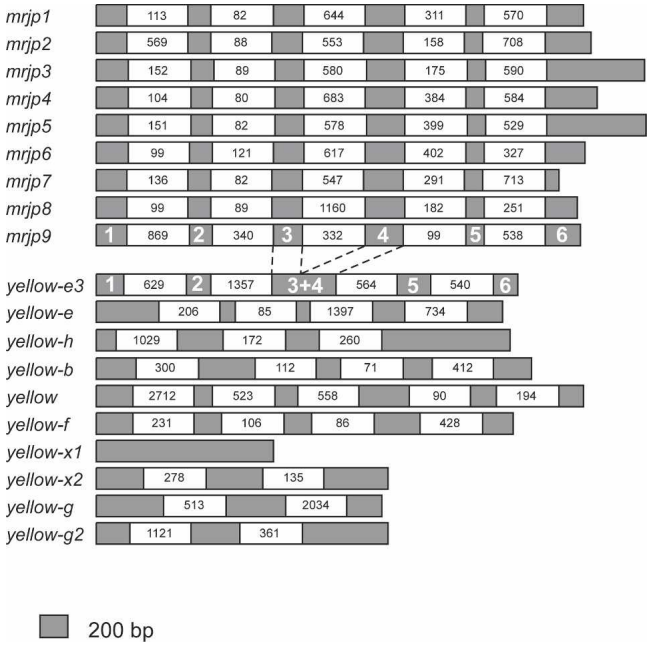

Figure 1. Intron/exon structure of honey bee *mrjp* and *yellow* genes. Exons are depicted as black boxes, to scale. To emphasize structural similarities among *mrjp* genes, introns are depicted with empty boxes of uniform size, displaying actual sizes in base pairs. The intron/exon structure of the *yellow-e3* gene is very similar to that of the *mrjp* genes.

tional phase, this suggests that this group of genes recently evolved from a common progenitor. Second, the intron/exon structure of the *yellow* genes is, in general, not conserved within the *yellow* group or with the *mrjp* genes. This observation is consistent with the dissimilarity between *mrjp* and *yellow* loci in genomic DNA and predicted/actual protein sequences.

yellow genes are common within arthropods; *mrjp* genes are rare

Because the nutritional MRJP component of RJ is essential for eusocial behaviors of the honey bee, we used all publicly available genomic DNA sequences and EST data from other animals to determine whether the distribution of *mrjp* genes was restricted to a subset of species, in particular, bees. We were able to draw two conclusions from the available data. Every arthropod for which sufficient sequence information exists possesses a "core" group of *yellow*-like genes (Table 2). We found *yellow*-like genes in a diverse group of arthropods, including flies, bees, mosquitoes, beetles, and moths, and this group ranges in size from eight to 14 members within a given species. Genes encoding Yellow-like proteins may be largely restricted to arthropods. We found no evidence that these genes exist in the complete genome sequences of the worm *Caenorhabditis elegans* and the yeast *Saccharomyces cerevisiae*.

Interestingly, however, we discovered that several bacterial species harbor either one or two *yellow*-like genes (see below). Such bacteria include the species *Pseudomonas syringae*, *Deinococcus radiodurans*, *Magnetospirillum magnetotacticum*, *Solibacter usitatus*, *Burkholderia fungorum*, and *Geobacter metallireducens*. This finding suggests that *yellow*-like genes are ancient, but have been lost from many lineages (see also Maleszka and Kucharski 2000). Horizontal gene transfer from insects to bacteria could also explain this distribution of *yellow*-like genes, but we believe such transfer is unlikely in practice.

In great contrast to the wide *yellow*-like gene distribution among various species, *mrjp*-like genes are thus far restricted to the honey bee genus *Apis* (Table 2). In addition to *A. mellifera*, the genomes of three related honey bee species—*Apis cerana*, *Apis dorsata*, and *Apis florea*—have genes encoding MRJPs (Albertova et al. 2005; Imjongjirak et al. 2005; Su et al. 2005). These data support the following two conclusions: The *mrjp* duplication events were recent, and the distribution of *mrjp* genes within animals is very restricted. It remains to be determined, however, precisely how limited this distribution is. A few recently initiated genome sequencing efforts, such as the *Nasonia vitripennis* project, will be pivotal for understanding the distribution of these genes in Hymenopteran species.

A tandem array of *mrjp* genes in the honey bee genome

The *A. mellifera* genome sequence contains spatial information that allowed us to determine the proximity of *mrjp* and *yellow* genes to one another. We observed that five *mrjp* genes and the *mrjp*-Ψ gene clustered together on one large linkage group (Group 11.23, one of the 16 linkage groups of the honey bee karyotype, representing chromosome 11), and that three others clustered on another (Group Un.927). A third linkage group (Group Un.1029) contained the remaining *mrjp* gene. Based on the similarities in intron/exon structure and protein sequence among the *mrjp* genes (see above), it is likely that the *mrjp* genes evolved via a series of recent duplications. This predicts that they should be in close genomic proximity to each other. This, and additional evidence from cDNA/EST sequences and computer predictions, suggested that the three aforementioned groups and an additional group, Un.51, should comprise one linkage group. This larger linkage group could not have been assembled in the overall genome project because of insufficient trace coverage to date.

We successfully reconstructed an ~833-kb interscaffold containing the large contig 11.23 and the three previously unoriented smaller contigs by sequencing overlapping DNA fragments obtained by PCR amplification. This "finishing" sequence resulted in a genomic landscape consisting of all 10 *mrjp* genes and

Table 2. The number of known *yellow* and *mrjp* genes in insects, worms, and yeast

Organism	*yellow*-like genes	*mrjp*-like genes
Complete genomes		
Apis mellifera	10	10
Drosophila melanogaster	14	0
Drosophila pseudoobscura	8	0
Aedes aegypti	13	0
Anopheles gambiae	14	0
Tribolium castaneum	10	0
Bombyx mori	14	0
Caenorhabditis elegans	0	0
Saccharomyces cerevisiae	0	0
Incomplete genomes		
Apis cerana	ND	7[a]
Apis dorsata	ND	5[a]
Apis florea	ND	1[a]

(ND) Not determined.
[a]Number obtained by cDNA sequencing or gene sequencing via degenerate PCR.

additionally five *yellow* genes (see Fig. 3, below). All 10 *mrjp* genes are positioned in a tandem array roughly 60 kb in size. The four most proximal *yellow* genes—*yellow-e*, *yellow-e3*, *yellow-g2*, and *yellow-h*—are all oriented in the same direction as the *mrjp* array. Interestingly, *yellow-g2* and the last *yellow* gene, *yellow-g*, are close, and in opposite orientation to each other, suggesting that their transcription is controlled via a bidirectional promoter. This finding—that the entire group of 10 *mrjp* genes is arranged in a relatively small tandem array—supports the theory that this important group of genes evolved from a common ancestral gene. The progenitor of the *mrjp* genes is most likely a *yellow*-like gene (see below).

In addition to the 15 tightly linked genes on chromosome 11, we localized the remaining five *yellow*-like genes in the honey bee genome. These genes, in contrast to the five that flank the *mrjp* array, are distributed in separate scaffolds, and are therefore located throughout the genome.

The *mrjp* array arose via initial duplication of *yellow-e3*

The most likely scenario by which the *mrjp* tandem array evolved is by the duplication of a *yellow*-like gene located near the extant *mrjp* cluster. Two specific genes, *yellow-e3* and *yellow-h*, which directly flank the *mrjp* array, are the best candidates to be the progenitor of all *mrjp* genes. Because novel genes resulting from recent duplication events will retain the intron/exon structure of their template gene, we used information about the position of introns within the *mrjp* and *yellow* genes to determine whether any *yellow*-like gene could be classified as a strong candidate for the *mrjp* template. We found that the *yellow-e3* intron/exon structure bears a remarkable similarity to that of the *mrjp* genes: Specifically, they share four out of five introns in the same relative positions, and in the same phase (Fig. 1; Supplemental Table S1). The *yellow-h* introns are not similar to those in *mrjp* genes (Fig. 1).

MRJPs are a monophyletic group within the Yellow/MRJP family

Using the protein sequences of *mrjp* and *yellow* genes from insect and microbial species, we constructed an unrooted phylogenetic tree using neighbor-joining (Fig. 2). We observed that MRJPs form a distinct monophyletic branch that appears to have evolved from an insect Yellow-like most recent common ancestor. Our phylogenetic tree suggests that this most recent common ancestor to the MRJPs is Yellow-e3 (Fig. 2). These protein data provide additional support for the hypothesis that the *mrjp* group of genes evolved recently via rapid and multiple duplication of *yellow-e3*.

The precise branching pattern of the MRJPs reflects the recent gene duplication in this region of the genome. Specifically, MRJP pairs forming terminal clades in the MRJP branch of the tree, MRJP5 + 6, MRJP2 + 7, and MRJP8 + 9 (Fig. 2), are encoded by neighbor *mrjp* genes in the tandem array (Fig. 3).

More generally, this phylogenetic analysis demonstrates that for many of *D. melanogaster* Yellow proteins, close honey bee relatives exist. Because these are likely to represent honey bee orthologs, they were given the same names as their fruit fly counterparts (see Drapeau 2001). Additionally, we discovered two "orphan" Yellow-like proteins that have no obvious *D. melanogaster* counterparts (Fig. 2). For this reason, we have named the genes that encode them *yellow-x1* and *yellow-x2*. We note in passing that *yellow-x1* is the only intronless member of this gene family.

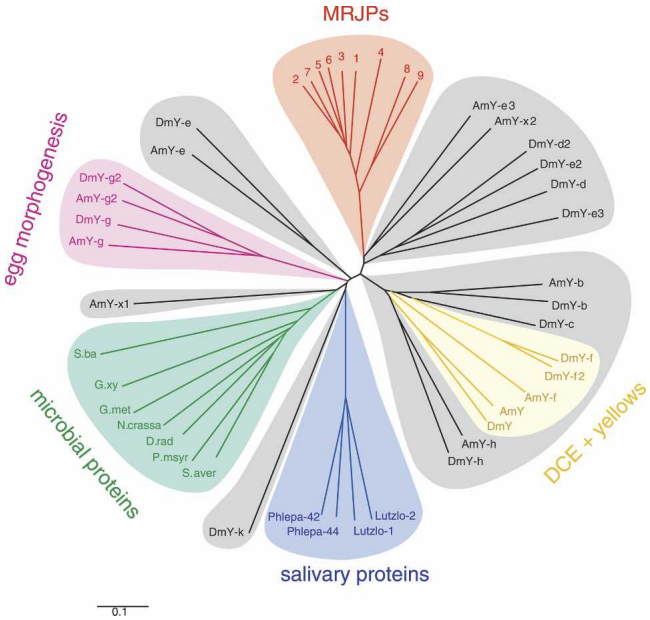

Figure 2. Phylogeny of the Yellow/MRJP protein family. An unrooted tree was constructed with aligned protein sequences from bees, flies, and bacteria using neighbor-joining (see Methods). The Yellow/MRJP protein family is composed of numerous color-coded subfamilies, each characterized by a group of closely homologous proteins. One of these subfamilies, the MRJPs, has thus far only been found in honey bees. Other subfamilies characterized by similar protein sequences are also known to have similar functions, and are so labeled. (AmY) *Apis mellifera* yellow; (DmY) *Drosophila melanogaster* yellow; (Phlepa) *Phlebotomus papatasi*; (Lutzlo) *Lutzomyia longipalpis*; (S.ba) *Shewanella baltica*; (S.aver) *Streptomyces avermitilis*; (G.met) *Geobacter metallireducens*; (G.xy) *Gluconobacter oxydans*; (N.crassa) *Neurospora crassa*; and (D.rad) *Deinococcus radiodurans*.

As noted before (Kucharski et al. 1998; Albert et al. 1999a), and as clearly demonstrated by our phylogenetic analysis, the Yellow-like and MRJP protein groups are evolutionarily related. Therefore, we name the total set of proteins the Yellow/MRJP protein family. We also name the proteins belonging only to the MRJP group the MRJP subfamily, reflecting the fact that these proteins are a distinct group within the larger, more ancient Yellow group.

The *mrjp* and *yellow* genes have diverse expression patterns

Some of the *mrjp* genes have a well-characterized nutritive role, and accordingly are highly expressed in worker bee hypopharyngeal glands (HPG) that secrete RJ. Because all nine MRJPs have been found in the RJ proteome (Schmitzova et al. 1998; Sano et al. 2004; Santos et al. 2005; Scarselli et al. 2005), the MRJP subfamily most likely evolved in association with this function. We tested the hypothesis that MRJPs evolved additional, diverse functions following gene duplication.

We compiled expression data for the genes encoding the honey bee Yellow/MRJP protein family (Table 3). To determine developmental-stage-, sex-, and caste-specific expression patterns, and to quantify expression levels, we used data from three different EST libraries, PCR amplifications, Northern blots, and microarrays, including expression data from the literature. As expected, we found no evidence for expression of the *mrjp-Ψ* pseudogene. Expression of a given *mrjp* gene manifests itself as a single Northern blot band, suggesting that no alternative splicing

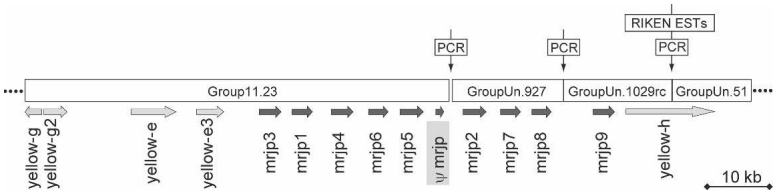

Figure 3. Genomic landscape of genes encoding MRJPs. Four scaffolds from the *A. mellifera* genome project were joined by PCR amplification and sequencing, forming an 883-kb interscaffold (see Results). All 10 *mrjp* genes are aligned in a tandem array ~60 kb in size. This, in combination with sequence similarity, suggests a recent series of duplication events in the formation of the MRJP protein subfamily. The *mrjp* array is flanked by five *yellow* genes, all but one of which read in the same direction as the *mrjp* loci. The orientation of the *yellow-g* and *yellow-g2* genes suggests the presence of a shared, bidirectional promoter.

takes place in these genes. As previously noted, *mrjp* genes are highly expressed in the HPG, and therefore all show head expression. But, perhaps unexpectedly, expression of *mrjp1* and *mrjp3* was found not only in workers, but also in queens and drones (males) (Fig. 4; Table 3). The *mrjp1* gene is additionally expressed in adult worker brains, suggesting a role in behavior. The *mrjp9* gene is expressed in the adult venom gland, where its product performs a currently unknown function.

We also found expression of *mrjp* genes throughout honey bee development. Various *mrjp* genes are expressed in embryos, larvae, and pupae, albeit at lower levels than seen in adults (Fig. 4; Table 3). We also observed several temporal changes in expression, exemplified by *mrjp5*. This gene has high expression in worker heads until Day 5 after emergence, low expression until Day 26, and undetectable expression thereafter (Fig. 4). Interestingly, the *mrjp5* profile is distinct from that of *mrjp1* (Kucharski et al. 1998), suggesting that *mrjp* genes are not coregulated despite being closely arranged in the genome. In total, these data suggest that the nine protein-coding *mrjp* genes not only have strong expression in the worker HPG for a nutritive function, but have also recently evolved distinct temporal, spatial, sexual, and caste-specific gene expression patterns, and presumably protein functions.

Like their *mrjp* cousins, the *yellow*-related honey bee genes also have diverse expression patterns (Table 3). Most are expressed in worker heads at various developmental stages, but more distinct expression patterns are evident. For example, *yellow-g* shows queen-specific ovary expression, and is also expressed in very young embryos, consistent with a maternal expression pattern (Fig. 4; Table 3). The *yellow-h* gene is predominantly expressed in developing queens at the larval stage, suggesting a specialized role for this gene's product (Fig. 4; Table 3). In contrast, *yellow-f* is activated at the late embryonic stage, where its expression continues throughout larval and pupal developmental stages (Fig. 4). The *yellow-f* gene is also highly expressed in the adult brain. The gland expression of *yellow-e3* supports the notion that this gene is a progenitor of the Major Royal Jelly protein family.

A general conclusion that emerges from these analyses is that MRJPs perform context-dependent functions; the nutritional role of MRJP will have different phenotypic implications in the gland as compared to brain functions or developmental processes. The biological significance of a given MRJP product will depend on when and where its message is expressed. Furthermore, we conclude from these data that while a common underlying theme may exist, the honey bee Yellow-related proteins also are largely multifunctional, performing different functions in different tissues at various stages of development. The MRJP subfamily evolved from a Yellow progenitor for the purpose of a nutritive role in RJ. However, the MRJPs have recently evolved diverse roles throughout development and in adults.

The Yellow/MRJP family and sex-specific reproductive maturation

Because most of our knowledge about the functions of proteins in the Yellow/MRJP family comes from genetic mutant and microarray studies in *D. melanogaster*, we can use this valuable information as a backdrop for predicting, and understanding, the roles that Yellow-like and MRJP factors perform in honey bees. A review of the literature inevitably leads to the conclusion that Yellow-like proteins have dissimilar expression patterns, mutant effects, protein binding partners, and by implication, functions (Supplemental Table S2; Martin and Ollo 1996; De Gregorio et al. 2001; Diagana et al. 2002; Stanyon et al. 2004). Despite this disparate data, however, a common theme emerges: The Yellow protein family of *D. melanogaster* mainly appears to be required for sex-specific reproductive maturation and/or development (Supplemental Table S4; Wayne and McIntyre 2002; Gilliland et al. 2005). The classic example of Yellow, required for pigmentation (often sex-specific, particularly in the abdomen and sex-combs) and male sexual behavior characteristics (obviously sexually dimorphic), is representative of this admittedly diverse group of proteins. These data are consistent with what we know about the functions of Yellow/MRJP family members in *Apis*. Specifically, the MRJPs have a nutritional function in RJ that modulates caste determination, and we have also shown sex-specific expression of numerous *mrjp* and *yellow*-like genes, sometimes in reproductive tissues (Fig. 4; Table 3). Thus, we propose a universal role of Yellow-like proteins in sex-specific reproductive maturity.

Discussion

Yellow protein family evolution through metazoans and bacteria

The observation that members of the Yellow/MRJP family are found not only in insects but also scattered through diverse bacterial species suggests that Yellow-like proteins are evolutionarily ancient. Hence, *yellow*-like genes have been lost from numerous lineages (e.g., enterogenic bacteria), and retained in those where they perform an important function. An alternative, and we believe less probable, hypothesis is that *yellow*-like genes in bacteria arrived via horizontal transfer from insects (Makarova et al. 2001). The fact that all bacterial Yellow-like proteins, along with the only non-insect eukaryotic *N. crassa* Yellow, are monophyletic (Fig. 2) militates against horizontal transfer as a viable explanation for the data. The hypothesis proposing multiple, independent transfer events predicts that the transferred bacterial Yellow protein would have a most recent common ancestor among the insects, and across seven proteins this was never the case (see also True et al. 2005 for another example).

Another common aspect of the Yellow/MRJP protein family is that most members have hydrophobic leader peptides at their N termini, suggesting that they are secreted factors. This may make sense in light of the fact that MRJPs have a nutritive func-

Table 3. Developmental-stage-, sex-, and caste-specific expression patterns of the *yellow* and *mrjp* genes

Gene	Tissue	Stage	Caste	Expression level	Evidence	Putative function	Relevant reference
mrjp1	Head, brain	L, P, NB, N, F	Worker (HPG, B) Drone(L, P, G) Queen (O,L)	●●●●	Northern blot, ESTs, PCR, microarray, proteomics, in situ	Bee milk, other	Schmitzova et al. 1998 Kucharski et al. 1998 Whitfield et al. 2002 Sano et al. 2004 Santos et al. 2005 Scarselli et al. 2005
mrjp2	Head		Worker (HPG)	●●●	Northern blot, ESTs, proteomics	Bee milk	Schmitzova et al. 1998 Kucharski and Maleszka 2002 Sano et al. 2004 Santos et al. 2005 Scarselli et al. 2005
mrjp3	Head	E, NB, N, F	Worker (HPG) Drone (G) Queen (O, L)	●●●●	Northern blot, ESTs, PCR, microarray, proteomics	Bee milk, other	Schmitzova et al. 1998 Klaudiny et al. 1994a,b Whitfield et al. 2002 Sano et al. 2004 Santos et al. 2005 Scarselli et al. 2005
mrjp4	Head	P, NB, N, F	Worker (HPG)	●●●●	Northern blot, ESTs, PCR, microarray, proteomics	Bee milk	Klaudiny et al. 1994a,b Whitfield et al. 2002 Santos et al. 2005 Sano et al. 2004
mrjp5	Head	N, F	Worker (HPG)	●●●	Northern blot, ESTs, PCR, microarray, proteomics	Bee milk	Whitfield et al. 2002 Sano et al. 2004 Santos et al. 2005 This study
mrjp6	Head		Worker (HPG)	●●	ESTs, microarray, proteomics	Bee milk	Whitfield et al. 2002 Santos et al. 2005
mrjp7	Head	N, F	Worker (HPG)	●●●	ESTs, microarray, proteomics	Bee milk	Whitfield et al. 2002 Santos et al. 2005
mrjp8	Head	N, F	Worker	●	EST, microarray		Whitfield et al. 2002
mrjp9	Head, venom sac	N, F	Worker (HPG, VGS)	●●	ESTs, proteomics	Bee milk, bee venom component	Santos et al. 2005 Peiren et al. 2005
mrjp-Ψ				Not detectable	Northern blot		Pseudogene, this study
yellow	Head	P, N, F	Worker	●	ESTs, microarray		Whitfield et al. 2002 This study
yellow-b	Head	N, F	Worker	●	EST		This study
yellow-e	Head	N, F	Worker	●	ESTs		This study
yellow-e3	Head	P, N, F	Worker (HPG)	●	ESTs, microarray		Whitfield et al. 2002 This study
yellow-f	Embryo >24 h, brain	E, P	Worker (B) Queen (L) Drone (L, P)	●●	ESTs, PCR		This study
yellow-g	Embryo <24 h	E	Worker (E) Queen (O)	●●	PCR		Maternal transcript, this study
yellow-g2							Genomic organization suggests a bidirectional promoter with AmY-g.
yellow-h	Head, larva	L	Queen (L)	●	ESTs, PCR		This study
yellow-x1	Head/brain	P, N, F	Worker	●●●	ESTs		This study
yellow-x2	Head	N, F	Worker	●	ESTs		This study

(NB) Newly born; (N) nurse (<21 d old); (F) forager; (L) larva; (P) pupa; (E) embryo; (HPG) hypopharyngeal gland; (G) genitals; (O) ovary; (B) brain; (VGS) venom gland(s)/sac.
Note: the relative expressions refer to the highest observed level.

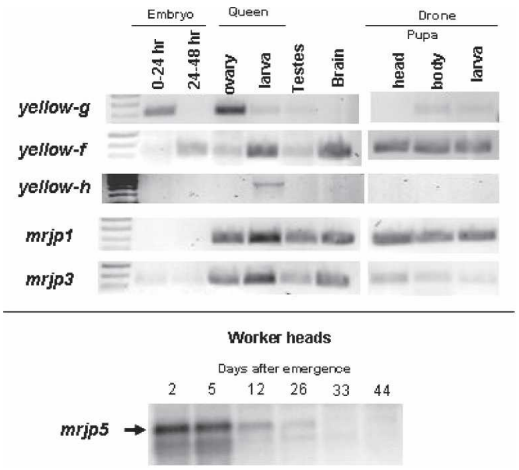

Figure 4. Developmental-stage-, sex-, and caste-specific expression of a subset of *yellow* and *mrjp* genes. Shown are data from *yellow-f*, *yellow-g*, and *yellow-h*, and also *mrjp-1*, *mrjp-3*, and *mrjp5*. RT-PCR products were run on gels for visualization of gene expression. The primers are listed in Supplemental Table S3. The expression of mrjp5 was examined by Northern blotting using a PCR-amplified insert of a cDNA clone as a probe (nucleotides 15–1942 of NM_001011599). See Methods for more details.

tion in the RJ that is secreted from the HPG. The *D. melanogaster* Yellow protein, the best-studied of all the members of the Yellow/MRJP protein family, is a secreted factor that may have hormonal function (Drapeau 2003; see below).

Origin of the MRJP subfamily from an ancient progenitor

Owing to their high level of similarity in intron/exon structure and protein sequence, it has been hypothesized that the *mrjp* genes arose via multiple nearly simultaneous duplications (Albert et al. 1999a). Gene duplications often give rise to such local clusters of genes (Wagner et al. 2003). An alternative model of evolution is suggested by a local genomic alignment (via the UCSC Genome server; http://genome.ucsc.edu/cgi-bin/hqGateway) between a small region of *D. melanogaster* chromosome 4, encoding a calcium-binding protein (CG31999), unrelated to Yellows, and MRJP6. In this scenario, some of the *mjrp* genes may have evolved via mutation accumulation in an ancient pre-existing genomic locus that encodes distinct proteins in modern insects. Indeed, a phylogenetic tree based on DNA alignments confirms that *mrjp6* is weakly related to that region of chromosome 4 (Supplemental Fig. S1). However, the tree also shows that all *mrjp* genes are more closely related to *yellow-e3*, which is our candidate for the progenitor of the whole family. Furthermore, as shown in Supplemental Figure S2, the genomic alignment is not only interrupted by gaps but is largely confined to stretches of As and Ts with virtually no homologies in the more complex regions. It is therefore difficult to evaluate to what extent it reflects a meaningful evolutionary history.

Our data suggest that the entire *mrjp* cluster evolved from the *yellow-e3* gene. First, *yellow-e3* is one of two *yellow*-related genes directly flanking the *mrjp* gene cluster (Fig. 3). Second, of all the honey bee *yellow*-related genes, the intron/exon structure and protein sequence of *yellow-e3* are the most similar to those of *mrjp* genes (Figs. 1, 2). Third, and perhaps most importantly, microarray expression data suggest that the functions of Yellow-e3 and the MRJPs have more in common than Yellow-e3 does to the rest of the Yellow proteins. The *yellow-e3* transcript is highly expressed in the head and HPG, and generally follows a developmental pattern typical of *mrjp* genes. Overall, our findings reveal the recent origin of a novel protein subfamily (MRJP) from an ancient protein family (Yellow). This evolutionary event, combined with the use of MRJP-infused RJ as a nutritional supplement, greatly influenced honey bee behavior and hive social structure.

The function of MRJPs in honey bee Royal Jelly

The hypopharyngeal gland determines honey bee royalty within a structured society. Young worker bees (nurse bees) use the HPG to produce and secrete RJ, critical for the nutritional transformation of an immature female larva into a fertile queen bee. The absence of RJ leads immature females to a sterile worker bee fate. Hence, RJ not only creates colony-level social structure, but also modulates the sex-specific fertility of individuals. At the transition from young to old workers (i.e., nurses to foragers), bees cease the production of RJ as they take on their new role in the hive. Therefore, if MRJPs play an important role in RJ, we would expect that levels of gene expression and protein levels would decrease during and after this transition. This is, indeed, the case (Klaudiny et al. 1994a,b; Ohashi et al. 1997; Kucharski et al. 1998), and the effect has been shown to be reversible (Ohashi et al. 2000).

What is the molecular function of MRJPs in RJ? Recent evidence suggests that repetitive pentapeptide regions concentrated with nitrogen-rich amino acids may function as deposits of biologically accessible nitrogen (Albertova et al. 2005). Because nitrogen is an essential component of biogenic polymers such as nucleic acids and proteins, yet animals must obtain it from exogenous sources, it is often considered a limiting component of eukaryotic diets. Hence, high levels of nitrogen stored in MRJPs may be critical for rapidly growing young larvae and for the development of fertile queens. Other MRJP repeat regions may have additional functions. For example, the methionine-rich tripeptide repeat in the MRJP5 protein is ideal for the storage of sulfur, another limiting nutrient (S. Albert, unpubl.). Regarding other molecular functions of these proteins in the honey bee, a starting hypothesis consists of the assumption that MRJPs retain some of the ancestral roles that are associated with the Yellow proteins.

On the function of *D. melanogaster* Yellow

The *yellow* gene and the role(s) of its protein product have been characterized more extensively than any other in the Yellow family. As regards pigmentation (melanization), Yellow is required in cuticle cells for the presence of DOPA-melanin, and ectopically expressed Yellow is sufficient for the formation and deposition of melanin (Wittkopp et al. 2002a,b). Changes in the *yellow* locus underlie some changes in pigmentation patterns across evolutionary time (Wittkopp et al. 2002a,b; Gompel et al. 2005). Sex-specific abdominal pigmentation in *D. melanogaster* is Yellow-dependent and dependent on the Bric-a-brac protein, which integrates inputs from the homeotic and sex-determination pathways (Kopp et al. 2000). Yellow accumulation in some cells of the *D. melanogaster* male central nervous system (CNS) is dependent on sex-limited action of the Fruitless (FRU) protein (Radovic et al. 2002; Drapeau et al. 2003). The *yellow* gene may also be regulated by the Doublesex (DSX) protein, another transcription factor in the sex-determination cascade (Drapeau et al.

2005). These Yellow$^+$ CNS cells appear to be relevant to male courtship behaviors (Drapeau et al. 2003).

At present, it is not known what the precise biochemical function of the Yellow protein is, but one theory can account for the dual roles of Yellow in pigmentation and behavior, and allows a relationship to be drawn between Yellow and the MRJPs. Yellow protein is secreted from cells because of a signal peptide that commands this action (Hannah 1953; Geyer et al. 1986; Kornezos and Chia 1992; Radovic et al. 2002; Wittkopp et al. 2002a; Drapeau et al. 2003). Yellow may then influence the properties of nearby cells via dopamine-like receptors and a hormone-like mechanism (Drapeau 2003). Activation of different signal transduction pathways in different cell types (cuticle vs. neural) would allow the activation of different downstream genes relevant to particular phenotypes (pigmentation vs. behavior) (Drapeau 2003). Like Yellow, the MRJPs have N-terminal signal peptides directing their secretion from cells, and post-secretion these signals are cleaved (Sano et al. 2004). Additionally, like Yellow (Geyer et al. 1986), MRJPs are glycoproteins with *N*-linked sugar chains (Kimura et al. 1996), and it is now known that glycosylation can regulate factors involved in extracellular signaling (e.g., Haines and Irvine 2003). Further study of the Yellow/MRJP protein family will lead to a greater understanding of these proteins as extracellular molecules. Because the requirement for Yellow in pigmentation and behavior is conserved within *Drosophila* (e.g., *Drosophila virilis* [Wittkopp et al. 2002b]; *Drosophila willistoni* [da Silva et al. 2005]), and because Yellow-g appears to have a similar function in flies and ants (Claycomb et al. 2004; Tian et al. 2004), we expect that *A. mellifera* Yellow-like proteins will share many similarities with those of *D. melanogaster*.

Genetic regulation of complex social behavior

The genes necessary and sufficient for complex social behaviors are largely a mystery. A few noteworthy exceptions include the *Gp-9* gene, which encodes an odorant-binding protein in the fire ant *Solenopsis invicta* (Kreiger and Ross 2002); a locus named *caste* with unknown function in the red harvester ant *Pogonomyrmex barbatus* (Volny and Gordon 2002); and the gene encoding the V1a receptor for the neuropeptide vasopressin in voles (Lim et al. 2004).

Although *A. mellifera* has traditionally been the most intensively studied of all the eusocial insects (e.g., Wilson 1971), little progress has been made toward identifying genes underlying its elaborate social behavior. Honey bee "behavior genes" that encode members of conserved signaling pathways are unlikely to be unique to eusocial insects (see Robinson et al. 2005). In contrast, *mrjp* genes are not only crucial for *A. mellifera* colony-level behavior, but are thus far unique to the genus *Apis*. A comparison of *mrjp* loci to one of the honey bee behavior genes, the protein kinase *foraging*, which has been implicated in the transition of workers from hive work to outside-the-hive foraging (Ben-Shahar et al. 2002), reveals a key difference—*foraging* has a well-characterized homolog in *D. melanogaster* that controls larval foraging behavior (e.g., Osborne et al. 1997). While this fact makes *foraging* no less interesting, it follows that the *foraging* locus predates the speciation of flies and bees, and that the function of *foraging* in the control of bee social behavior came after the initial evolution of such behavior. Because *mrjp* genes have thus far only been found in *Apis*, we speculate that they may have specifically evolved to modulate eusociality via nutrition. Here, nature and nurture converge during the determination of complex behavior.

Methods

Sample collection

Foraging honey bee workers were captured near hive entrances and snap-frozen in liquid nitrogen (LN). To ensure that fully mature workers were harvested, only those that carried pollen or nectar were selected. They are estimated to be 20–35 d old. To obtain newly emerged honey bees, a single brood frame was removed from the hive and incubated at 32°C with 80% humidity. Then, individual insects were collected within 5 min after emergence and snap-frozen in LN. Pupae and larvae were harvested directly from a brood frame and snap-frozen in LN. Eggs and embryos were collected from a standard Jenter device.

Molecular biology

Tissue dissection, RNA extraction, hybridization, and data analyses were carried out as described previously (Kucharski and Maleszka 2002, 2003). For RT-PCR amplification, RNA samples were reverse-transcribed using Superscript II enzyme (Invitrogen) and an anchored $d(T)_{20}VN$ primer following the manufacturer's protocol (Kucharski and Maleszka 2005). Cloning and sequencing of cDNAs were done as in Albert and Klaudiny (2004). Isolated DNA was cloned into PCR.2.1 (Invitrogen) and sequenced by fluorescent (BigDye) sequencing.

Bioinformatics

Sequenced cDNAs were assembled and analyzed by University Wisconsin GCG 10.3 (Accelrys) and EMBOSS program packages. BLAST and iterated PSI-BLAST searches were run either locally or using the NCBI BLAST server (http://www.ncbi.nlm.nih.gov/BLAST). Sequence alignments were done using CLUSTALW with the Gonnet protein weight matrix. Aligned sequences were subjected to phylogenic analyses using the neighbor-joining method; gaps in the sequences were not considered. Branch stability was tested by bootstrap analysis (1000 repetitions). Dendrograms were drawn using the Phylodendron server (http://iubio.bio.indiana.edu/treeapp/treeprint-form.html).

Details of *D. melanogaster* gene sequences, structures, and functions were obtained through FlyBase (http://www.flybase.org/) and associated external databases (Fly GRID, http://biodata.mshri.on.ca/fly_grid/servlet/SearchPage; Berkeley *Drosophila* Genome Project, http://www.fruitfly.org/cgi-bin/ex/insitu.pl; Yale *Drosophila* Developmental Gene Expression Timecourse, http://genome.med.yale.edu/Lifecycle/; Gene Ontology, http://www.geneontology.org/), and additionally from the primary literature. The honey bee genomic resources are available via BeeBase at http://racerx00.tamu.edu/bee_resources.html.

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