BIOLOGICAL REVIEWS
Cambridge Philosophical Society
Biol. Rev. (2014), **89**, pp. 255–269.
doi: 10.1111/brv.12052

Origin and function of the major royal jelly proteins of the honeybee (*Apis mellifera*) as members of the *yellow* gene family

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ABSTRACT

In the honeybee, *Apis mellifera*, the queen larvae are fed with a diet exclusively composed of royal jelly (RJ), a secretion of the hypopharyngeal gland of young worker bees that nurse the brood. Up to 15% of RJ is composed of proteins, the nine most abundant of which have been termed major royal jelly proteins (MRJPs). Although it is widely accepted that RJ somehow determines the fate of a female larva and in spite of considerable research efforts, there are surprisingly few studies that address the biochemical characterisation and functions of these MRJPs. Here we review the research on MRJPs not only in honeybees but in hymenopteran insects in general and provide metadata analyses on genome organisation of *mrjp* genes, corroborating previous reports that MRJPs have important functions for insect development and not just a nutritional value for developing honeybee larvae.

Key words: *Apis mellifera*, major royal jelly protein, MRJP, caste differentiation, social insect, Hymenoptera, royal jelly, genome organisation, apalbumin, royalactin.

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I. INTRODUCTION

During the first 3 days after hatching from the egg, the larvae of the western honeybee *Apis mellifera* are fed with royal jelly (RJ) - a secretion of the hypopharyngeal gland of young worker bees that nurse the brood (Snodgrass, 1925). After the third day, female larvae destined to develop into queens exclusively receive RJ, whereas larvae that develop into worker bees are fed with a mixture of RJ, honey and pollen. Therefore it is widely agreed that RJ somehow determines the developmental fate of a female larva. RJ is on average composed of 60–70% water, 12–15% crude protein, 10–16% sugar, 3–6% lipids and traces of vitamins, salts and free amino acids (von Planta, 1888; Rembold, 1983). Although it has been proposed that RJ harbours substances which contribute to the induction of queen differentiation, surprisingly little is known about the functions of individual components of RJ especially the protein moiety (Schmitzová *et al.*, 1998; Blank *et al.*, 2012).

Here we review research into major royal jelly proteins (MRJPs) not only in honeybees but also in other insects and evaluate the functions that have been suggested for these proteins. Analytical research on RJ proteins began in the 1960s (Patel, Haydak & Gochnauer, 1960) and the proteins were often named after their molecular weights or simply numbered according to their discovery order, resulting in a huge variety of names for the same proteins (Table 1). The first RJ protein to be identified was the most abundant and was termed major royal jelly protein 1 (MRJP1; Hanes & Šimúth, 1992). Following this terminology, today the nine most abundant proteins of RJ are also termed major royal jelly protein (MRJP1 to MRJP9) and the encoding genes (*mrjp1* to *mrjp9*) have been identified in the honeybee genome (Honeybee Genome Sequencing Consortium, 2006). Whereas the first five *mrjps* (*1–5*) were identified by cloning and sequencing of their cDNA (Klaudiny *et al.*, 1994*a*; Albert, Klaudiny & Šimúth, 1996; Ohashi, Natori & Kubo, 1997; Schmitzová *et al.*, 1998; Albert *et al.*, 1999*a*), *mrjp6–9* were detected by a homology search in a honeybee-brain-expressed sequence tag (EST) library or in the genome assembly of the honeybee version 2.0 (Albert & Klaudiny, 2004; Drapeau *et al.*, 2006; Honeybee Genome Sequencing Consortium, 2006).

MRJPs are synthesized with an N-terminal signal peptide consisting of 16–20 amino acids (Fig. 1) and are therefore destined to be secretory proteins. The mature MRJPs comprise 400–578 amino acids and have theoretical molecular weights between 45 and 68 kDa (Table 2). Most of the MRJPs are rich in essential amino acids for honeybees [Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val (de Groot, 1953)] and some have a very high content of nitrogen serving as storage proteins for biologically accessible nitrogen (Schmitzová *et al.*, 1998; Albert *et al.*, 1999*a*; Albert, Klaudiny & Šimúth, 1999*b*). Therefore, and due to the fact that MRJPs1–5 represent with up to 90% the most abundant proteins of RJ, it has been repeatedly suggested that at least MRJPs1–5 have mainly a nutritional function (Schmitzová *et al.*, 1998; Albert *et al.*, 1999*a*,*b*).

Table 1. Different names for major royal jelly proteins 1–4 (MRJP1–4)

MRJP1	56 kDa protein	Kubo *et al.* (1996) and Ohashi *et al.* (1997, 2000)
	57-kDa protein	Kamakura *et al.* (2001*a*,*b*)
	Apalbumin 1	Bíliková *et al.* (2002, 2009), Bíliková & Šimúth (2010), Majtán *et al.* (2006), Scarselli *et al.* (2005), Šimúth (2001), Šimúth *et al.* (2004) and Tao *et al.* (2008)
	D III protein	Watanabe *et al.* (1996, 1998)
	p56kP-4	Kamakura & Sakaki (2006) and Ohashi *et al.* (1997)
	RJP-1	Kamakura *et al.* (2001*a*)
	RJP-3	Kucharski *et al.* (1998)
	RJPX	Klaudiny *et al.* (1994*b*)
	Royalactin	Kamakura (2011), Kamakura & Sakaki (2006) and Kamakura *et al.* (2001*b*)
MRJP2	50 kDa protein	Kubo *et al.* (1996) and Ohashi *et al.* (2000)
	55 kDa royal jelly glyco-protein (RJGP)	Kimura *et al.* (1996)
	Apalbumin 2	Scarselli *et al.* (2005) and Šimúth *et al.* (2004)
MRJP3	64 kDa protein	Kubo *et al.* (1996) and Ohashi *et al.* (1997, 2000)
	Apalbumin 3	Scarselli *et al.* (2005)
	RJP-1	Kucharski *et al.* (1998)
	RJP57-1	Beye *et al.* (1998), Klaudiny *et al.* (1994*a*,*b*) and Scarselli *et al.* (2005)
MRJP4	RJP-2	Kucharski *et al.* (1998)
	RJP57-2	Klaudiny *et al.* (1994*a*,*b*)

II. EXPRESSION AND SYNTHESIS OF MRJPs

All of the MRJPs have been found in RJ of *Apis mellifera* in several proteome analyses, except for MRJP8 (Schmitzová *et al.*, 1998; Sano *et al.*, 2004; Scarselli *et al.*, 2005; Li *et al.*, 2007, 2008; Schönleben *et al.*, 2007; Furusawa *et al.*, 2008; Yu, Mao & Jianke, 2010; Han *et al.*, 2011; Zhang *et al.*, 2012). Only one study on RJ of Africanized honeybees was able to identify MRJP8 within RJ (Santos *et al.*, 2005). It may not be surprising to find MRJPs not only in RJ but also in bee products that are handled by the workers. MRJP1–5 were also shown to occur in different honey types (Šimúth *et al.*, 2004; Bíliková & Šimúth, 2010; Di Girolamo, D'Amato & Righetti, 2012; Rossano *et al.*, 2012) and MRJP1 and 2 could be detected in the honeybee colony's pollen stores (Scarselli *et al.*, 2005; Bíliková & Šimúth, 2010). This 'bee

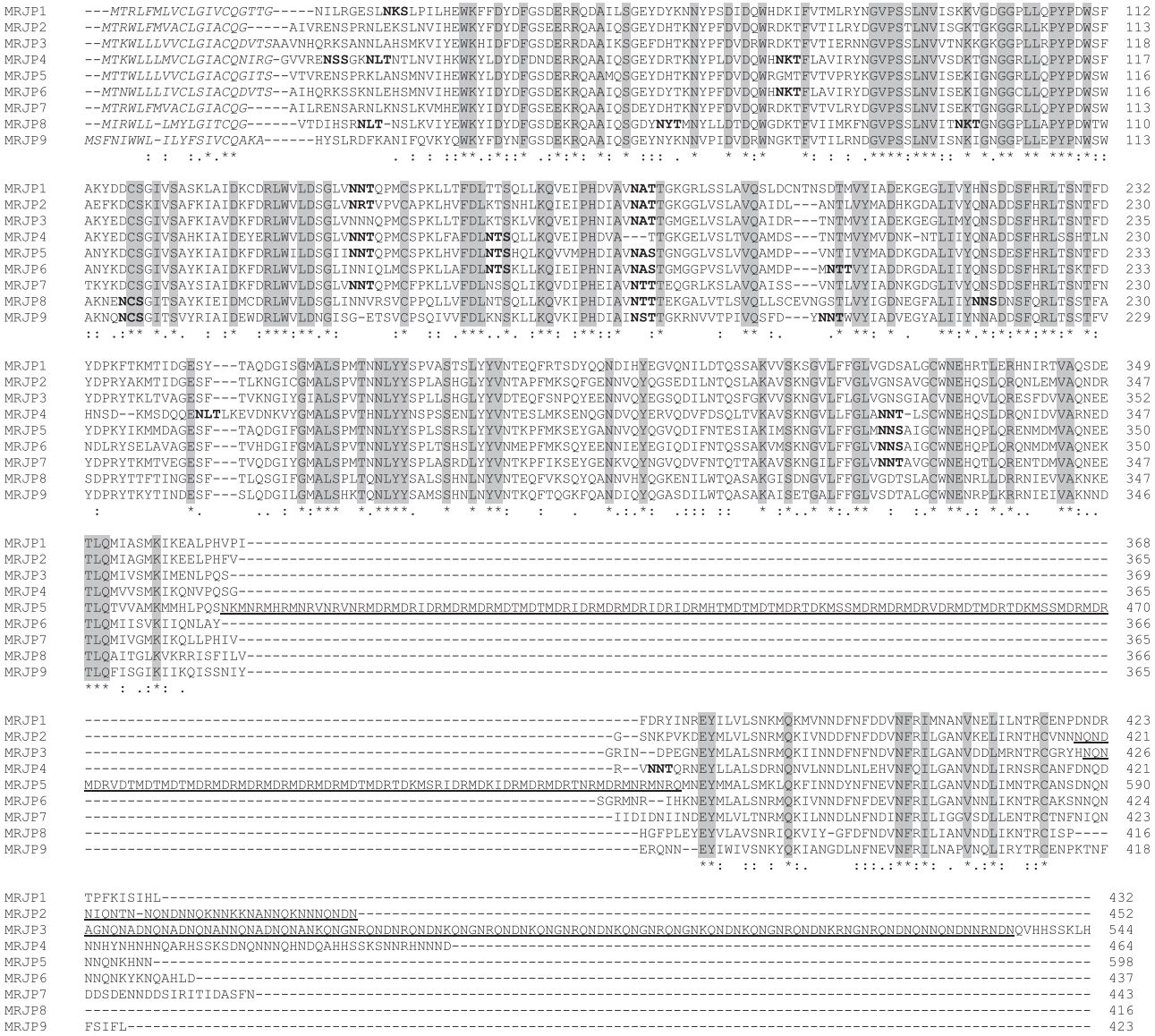

Fig. 1. Sequence alignment of major royal jelly protein 1–9 (MRJP1–9) of *Apis mellifera*. Sequences were aligned using Clustal Omega of the European Molecular Biology Laboratory - European Bioinformatics Institute (EMBL EBI) (http://www.ebi.ac.uk/Tools/msa/clustalo/). The signal peptides for MRJP1-5 were adopted from Schmitzová *et al.* (1998) and Sano *et al.* (2004). The putative signal peptides for MRJP6-9 were determined using the SignalP 4.0 server (Petersen *et al.*, 2011). Grey shading and asterisks, conserved amino acids; colon, groups of strongly similar properties; period, groups of weakly similar properties; underlining, repetitive regions; italic, signal peptides; bold, predicted glycosylation sites (NetNGlyc 1.0 server - http://www.cbs.dtu.dk/services/NetNGlyc/).

bread' is enzymatically and microbially processed pollen mixed with honey, nectar and salivary gland secretions, and is the main source of protein for worker honeybees (Casteel, 1912; Ševčik, 1950). More surprisingly might be the presence of MRJP8 and 9; these are both rare in RJ but could be detected in honeybee venom (Peiren *et al.*, 2005, 2008; Blank *et al.*, 2012).

In general it is believed that the expression of *mrjps* is a function of age in worker honeybees. During the first days of life freshly emerged workers function mostly as cell cleaners before they take care of the brood as nurses (Seeley, 1982). Various studies showed increased expression levels of *mrjps* or increased amounts of the RJ proteins in hypopharyngeal glands of these nurses but not in foragers, i.e. workers older than 20 days gathering nectar, pollen, water and resin but not responsible for the brood (Klaudiny *et al.*, 1994*b*; Kubo *et al.*, 1996; Ohashi *et al.*, 1997; Kucharski *et al.*, 1998; Feng, Fang & Li, 2009; Ueno *et al.*, 2009). This emphasises the nutritional function of MRJPs as the genes are expressed primarily in the hypopharyngeal glands of brood-raising nurses and not of foragers. However, it is known that foragers can be reset to function as nurses dependent on

Table 2. Molecular characteristics of honeybee major royal jelly proteins (MRJPs) predicted from genome sequence data. Signal peptide sequences and post-translational modifications are not included

MRJP	Amino acids	Predicted phosphorylation sites	Predicted glycosylation sites	M_W (kDa)	pI
1	413	S: 13/T: 2/Y: 09	3	46.86	5.03
2	435	S: 05/T: 4/Y: 06	2	49.15	6.65
3	524	S: 09/T: 2/Y: 09	1	59.49	6.50
4	444	S: 14/T: 4/Y: 08	8	50.67	5.74
5	578	S: 16/T: 8/Y: 11	4	68.13	5.95
6	417	S: 09/T: 2/Y: 10	5	47.58	6.01
7	426	S: 11/T: 9/Y: 09	3	48.66	4.85
8	400	S: 04/T: 2/Y: 05	6	45.06	5.81
9	403	S: 06/T: 2/Y: 09	3	46.27	8.62

For determination of theoretical molecular weight (M_W) and isoelectric point (pI) the program ProtParam of the Swiss Institute of Bioinformatics ExPASy Bioinformatics Resources Portal was used (Artimo *et al.*, 2012). The phosphorylation sites were predicted with the help of the NetPhos 2.0 server (S-serine, T-threonine, Y-tyrosine) (Blom *et al.*, 1999). The protein sequences used for the analyses were adopted from the UniProt protein knowledgebase (UniProtKB) (UniProt IDs: MRJP1-O18330, MRJP2-O77061, MRJP3-Q17060, MRJP4-Q17061, MRJP5-O97432, MRJP6-Q6W3E3, MRJP7-Q6IMJ9, MRJP8-Q6TGR0, MRJP9-Q4ZJX1).

colony conditions (Rösch, 1930). Such older workers acting as nurses can again synthesise MRJPs (Ohashi *et al.*, 2000). Thus, the hypopharyngeal gland cells can respond flexibly according to gland function; the cells produce MRJPs when they are needed for brood feeding. In addition, workers in colonies where the queen is lost activate their ovaries to produce eggs and synthesize MRJP1 and 2 in their glands like normal nurse bees (Nakaoka, Takeuchi & Kubo, 2008). All of these results indicate that some MRJPs have an age-dependent function in the hypopharyngeal glands and are produced in brood-raising nurse bees but not in foragers.

Kucharski *et al.* (1998) reported *mrjp1* expression in the Kenyon cells of the mushroom bodies in the honeybee brain, a new, then unknown function for MRJPs. Since then, the expression of *mrjp1–8* in the brain has been shown repeatedly (Whitfield *et al.*, 2002; Thompson *et al.*, 2006; Garcia *et al.*, 2009; Peixoto *et al.*, 2009; Hojo *et al.*, 2010). MRJP1 was found to be up-regulated in nurses compared to queens, foragers or drones and to be located in the antennal lobe, optical lobe and mushroom bodies (Garcia *et al.*, 2009; Peixoto *et al.*, 2009). The expression of *mrjp1* in the mushroom bodies was higher in worker bees sampled from colonies compared to isolated ones (Hojo *et al.*, 2010), that are known to have a decreased learning ability (Ichikawa & Sasaki, 2003). MRJP2 and 7 were only detected in brains of nurses but not in foragers (Garcia *et al.*, 2009) and they have transcripts in the worker mushroom bodies (Hojo *et al.*, 2010). Furthermore, the expression of *mrjp2* and *7* was up-regulated in queenright workers (sterile workers from colonies headed by a queen) compared to ovary-activated workers from a mutant honeybee strain in which queenright workers are reproductively active (Thompson *et al.*, 2006). All of these expression data lead to the conclusion that MRJPs have important functions for honeybee physiology in general and not just the nutritional value for developing larvae.

Besides their expression in worker heads, *mrjp1* and *3* are also expressed in drones (head, body, larvae) and queens (ovary, larvae) (Drapeau *et al.*, 2006). In addition, some MRJPs are also influenced by bacterial challenge as MRJP3 is up-regulated and MRJP4 down-regulated in honeybee heads 8 h after infection with *Escherichia coli* (Scharlaken *et al.*, 2007). MRJP1–3 and 7 can also be found in the haemolymph of larvae (Randolt *et al.*, 2008); after infection with *Paenibacillus larvae* MRJP1–3 are down-regulated or depleted in the haemolymph (Chan *et al.*, 2009).

In light of these findings, it becomes clear that we are far from understanding all the functions of MRJPs in the honeybee. It appears we are only beginning to understand that RJ proteins are much more than just a nutritional feature of the larval diet. Indeed it seems that there are a wide variety of MRJP functions that are still largely to be detected.

III. PROTEIN CHARACTERISATION

All MRJPs are highly homologous to each other, share 111 conserved amino acids and the individual sequence identities range between 47 and 74% (MRJP4–MRJP9 and MRJP5–MRJP6, respectively) (Fig. 1). Furthermore, the proteins share four conserved cysteine residues, which typically cause the formation of disulfide bridges, and several highly conserved blocks of amino acids. This high conservation suggests that these regions are functionally essential either for the tertiary structure of the proteins or for binding of a certain substrate (Schmitzová *et al.*, 1998).

(1) Post-translational modifications

Klaudiny *et al.* (1994*a*) were first to propose that MRJPs might possess post-translational modifications (PTMs) as the sequence of MRJP3 and 4 showed potential asparagine (N)-linked glycosylation and phosphorylation sites. We searched for serine, threonine and tyrosine phosphorylation and N-glycosylation sites using the NetPhos 2.0 (Blom, Gammeltoft & Brunak, 1999) and NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) and detected 11–35 phosphorylation and 1–8 glycosylation sites for all nine MRJPs (Fig. 1, bold amino acids; Table 2).

A comprehensive study based on the combination of two-dimensional polyacrylamide (PA)-sugar chain mapping, mass spectrometry analyses and ^{1}H nuclear magnetic resonance (^{1}H-NMR) spectroscopy revealed N-linked oligosaccharides of high-mannose-type (72%), biantennary-type (8%) and hybrid-type structure (3%) in total RJ glycoproteins (Kimura *et al.*, 2000). Some of these glycosylations could be assigned to certain MRJPs. Hence, MRJP1 is N-linked glycosylated

(Ohashi *et al.*, 1997; Kamakura *et al.*, 2001*a*) at three different glycosylation sites: ESL**N**KSL (unique complex-type structure harbouring the T-antigen), VAV**N**ATT and GLV**N**NTQ (both high mannose-type structures) (Kimura *et al.*, 2010). Furthermore, it was shown that different MRJP1 molecules can be glycosylated differentially (Kimura *et al.*, 2010). MRJP2 has an N-linked sugar chain of typical high mannose-type structure (Kimura *et al.*, 1996) and a minor homolog of MRJP2 (MRJP2a), missing the first four and the last 24 amino acids, was shown two have two glycosylation sites: GLV**N**RTV (N-linked biantennary- or triantennary-type structure) and IAV**N**ATT (high mannose-type structure) (Bíliková *et al.*, 2009). For MRJP3 it was just shown that the protein is N-linked glycosylated (Okamoto *et al.*, 2003).

MRJP2, 5, and 7 were shown to be phosphorylated (Furusawa *et al.*, 2008; Zhang *et al.*, 2012), and for MRJP1–5 as well as 7, methylations at aspartatic or glutamic acid and deamidations were present (Zhang *et al.*, 2012).

This huge variety of well-documented PTMs leads to many isoforms of MRJPs which differ in both molecular weights and isoelectric points (pIs). As a consequence MRJP size separation *via* one-dimensional and two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) varies in almost every publication after the first description for MRJP1 in Hanes & Šimúth (1992).

(2) Repetitive regions

Repetitive motifs of repeating tri- or pentapeptides were found in MRJP2, 3 and 5 (Fig. 1, underlined sequences) (Klaudiny *et al.*, 1994*a*; Albert *et al.*, 1999*b*).

In MRJP3, 22 repeats of the pentapeptide (N/K/R) QN(A/G/D)(G/D/N) are located C-terminally between amino acid 424 and 535 of the sequence deposited in the UniProt database (ID: Q17060) (Albert *et al.*, 1999*b*; Originally, Albert *et al.*, 1996 described this region as extending to amino acid 523 but this was corrected by Albert *et al.*, 1999*b*). The repetitive region of MRJP3 shows length variability due to a different number of repeats at the DNA level among individual honeybees in a colony. To date, eight different alleles have been found in MRJP3 encoding 14, 22 or 25–30 pentapeptide repeats, respectively (Beye *et al.*, 1998; Albert *et al.*, 1999*b*). The repetitive pentapeptide motif was shown to exist also in *Apis cerana*, *Apis dorsata*, *Apis florea* and Africanized honeybees but the degree of polymorphism varied (Albert & Schmitz, 2002; Albertová *et al.*, 2005; Su *et al.*, 2005; Baitala *et al.*, 2010). The allele size of *A. dorsata* and *A. cerana* is, at 600–700 bp, much bigger than for *A. mellifera* leading to 34–38 repeats of the pentapeptide (Albert & Schmitz, 2002; Albertová *et al.*, 2005). Furthermore, the polymorphism seems to be less pronounced in *A. florea* with only two alleles found to refer to 24 and 26 repeats (Albertová *et al.*, 2005).

The repetitive motif of MRJP5 is repeated 58 times with dominance of the tripeptide DRM and interrupts the consensus sequence between amino acid 367 and 540 (UniProt ID: O97432) (Albert *et al.*, 1999*a*). Polymorphism connected with a genetically determined length variability was also reported for MRJP5 (in Schmitzová *et al.*, 1998 referring to Albert & Klaudiny, unpublished observations) but to date only two different alleles have been published for *A. mellifera* (Su *et al.*, 2005). The repetitive region of the tripeptide DRM was also found in *A. cerana* (five different alleles) and Africanized honeybees (six different alleles) with approximately the same size as in *A. mellifera* (Su *et al.*, 2005; Baitala *et al.*, 2010). In *A. dorsata* the region had only 23 repeats (Albert & Schmitz, 2002).

MRJP2 contains a repetitive motif comprising seven imperfect repeats of the pentapeptide NQ(N/K)XN directly at the *C*-terminus which has never been shown to differ in length for *A. mellifera*. By contrast, Su *et al.* (2005) reported on length polymorphisms of the same pentapeptide in *A. cerana* with eight or nine repeats.

In addition to the polymorphic repetitive region, there are several cases where single nucleotide polymorphisms (SNPs) cause amino acid changes for *mrjp3*, *4* and *5* (Schmitzová *et al.*, 1998; Albert *et al.*, 1999*b*; Albert & Klaudiny, 2004).

Since MRJP3 and 5 do not contain sequentially related penta- or tripeptide units, the repetitive region seems to have evolved independently twice (Albert *et al.*, 1999*b*). Nevertheless, the regions share the appearance of positively charged Arg/Lys residues and negatively charged Asp residues (Albert *et al.*, 1999*a*). Furthermore, the amino acids Arg, Lys, Glu and Asn, numerous in these regions, contain one to three nitrogen atoms in addition to the amine group nitrogen suggesting that the domains are storing nitrogen in a biologically accessible form (Albert *et al.*, 1999*a*,*b*).

The polymorphic regions combined with different PTMs (see Section III.1) can lead to various amounts of MRJP isoforms especially in the case of MRJP3 where 30 different isoforms have been identified in *A. mellifera* (Li *et al.*, 2008).

(3) Protein stability and oligomer formation

In spite of two decades of MRJP research, almost nothing is known about the structure, function and stability of the proteins. Recently Cruz *et al.* (2011) determined the secondary structure content of MRJP1 *via* circular dichroism (CD) measurements to consist of 9.6% α-helices, 38.3% β-sheets and 20% β-turns. Very few studies report oligomer formation of MRJP1 and indicate a possible high stability against heat denaturation for MRJP1–3. MRJP1 is able to form oligomeric complexes of 280/90, 340 or 420 kDa (Šimúth, 2001; Tamura *et al.*, 2009*b*; Cruz *et al.*, 2011). In the oligomer of 280/90 kDa five non-covalently bonded MRJP1 molecules build a complex with one molecule of apisimin, a 5.5 kDa protein of RJ (Tamura *et al.*, 2009*a*,*b*; Cruz *et al.*, 2011). The 340 kDa oligomer consists of six MRJP1 molecules (Cruz *et al.*, 2011) and it is not known if apisimin is bound. These two different oligomerisation processes seem to be pH dependent with the pentamer formed at pH 6 and 7 and the hexamer at pH 8 (Cruz *et al.*, 2011). The oligomeric structure of 420 kDa consists also of MRJP1 in complex with apisimin, but the stoichiometry of this complex is not known (Bíliková *et al.*, 2002). Furthermore, it was shown that calcium ions lead to conformational changes in MRJP1 destroying the

oligomeric structure (Cruz *et al.*, 2011). Moreover, MRJP1 can bind calmodulin, a Ca^{2+}-binding protein that interacts and regulates various enzymes in the calcium-bound form (Calábria *et al.*, 2008) indicating again that Ca^{2+} might play a role in the regulation of the functions of the protein. Also for MRJP1 of *A. cerana* a 50 kDa monomeric and an oligomeric form of 300 kDa has been shown (Srisuparbh *et al.*, 2003). Currently, no oligomer formation has been shown for any of the other MRJPs with the exception of MRJP3 which was able to form dimers (Okamoto *et al.*, 2003).

The ability of MRJP1 to form diverse oligomers might be biologically essential as some functions of the protein seem to depend on the oligomeric or monomeric form (see Section VI). As all MRJPs share a high sequence identity, it may well be possible that context-dependent oligomer formation is a condition common to all MRJPs.

Concerning heat stability, neither 5 min boiling nor 30 min at 60°C could destroy specific cell culture activities of MRJP1 and 3, respectively (Watanabe *et al.*, 1998; Okamoto *et al.*, 2003). This was supported by Cruz *et al.* (2011), who did not detect signs of heat denaturation for MRJP1 while measuring CD spectra between 20 and 90°C. Analyses on the influence of storage temperature and time on MRJPs in RJ revealed that even after 1 year at room temperature MRJPs1–3 were still detectable in the RJ samples albeit in lower amounts than in fresh RJ (Li *et al.*, 2008). By contrast, one publication reported almost complete degradation of the monomeric form of MRJP1 after 7 days at 40°C whereas the oligomeric form was stable over this time period (Kamakura *et al.*, 2001*a*). These results suggest that additional studies are necessary to ascertain the stability of MRJPs and that the stability might be dependent on the monomeric or oligomeric form.

IV. GENOMIC STRUCTURE OF GENES AND RELATED PROTEINS

The haploid *Apis mellifera* genome contains a single copy of each *mrjp* gene on chromosome 11 with a highly conserved intron-exon structure with five introns in exactly the same position (Malecová *et al.*, 2003; Drapeau *et al.*, 2006). All nine *mrjp*s are located in a tandem array of approximately 60 kb, simultaneously harbouring a pseudogene *mrjp*-Ψ which is not transcribed (Drapeau *et al.*, 2006) (Pseudogenes are characterised by missing exons, large insertion-deletion polymorphisms or lack open reading frames.). Despite their close proximity, the different expression profiles of *mrjp*s (see Section II) suggest that they are not co-regulated (Drapeau *et al.*, 2006). 5′ upstream of the coding sequences there are binding sites for the transcription factors ultraspiracle (USP) and dead ringer (Dri) (Malecová *et al.*, 2003). USP belongs to a family of ligand-modulated nuclear hormone receptors that regulate homeostasis of reproduction, cell differentiation and development in *Drosophila melanogaster* (Segraves, 1991). The protein binds to two natural juvenile hormones (JHIII ester monoepoxide and bisepoxide) of *D. melanogaster* (Jones & Sharp, 1997). In *A. mellifera usp* is rapidly up-regulated by JH suggesting ligand-binding-dependent USP functions (Barchuk, Maleszka & Simões, 2004). Because JH titres are higher in early fifth instar queen larvae (Rachinsky *et al.*, 1990) it may well be that USP co-activates or -represses *mrjps* after JH binding. Given that *mrjps* are not co-expressed, a general regulation of all *mrjps* triggered by JH can be excluded.

All MRJPs show a high identity (20–30%) on the amino acid level with Yellow proteins of *D. melanogaster* suggesting that they have a common evolutionary origin (Albert *et al.*, 1996; Kucharski *et al.*, 1998; Schmitzová *et al.*, 1998). Therefore, it is not surprising that the *mrjp* tandem array is flanked by five *yellow* genes and is directly located between *yellow-e3* and *yellow-h* (Fig. 2) (Drapeau *et al.*, 2006). In *D. melanogaster*, *yellow* was originally identified to be involved in cuticle melanic pigmentation (Nash, 1976). Today, three different hypotheses coexist to explain the exact role of the gene in the melanisation process (Ferguson *et al.*, 2011). Indeed we now know that Yellow proteins fulfil a multitude of functions in addition to pigmentation (Drapeau, 2003). Genes encoding Yellow or Yellow-like proteins are not limited to the genus *Drosophila* but can be found throughout arthropods including *Aedes aegypti*, *Anopheles gambiae*, *Tribolium castaneum*, *Bombyx mori*, *Heliconius* spp., *Nasonia vitripennis*, *Apis* spp., *Acyrthosiphon pisum* (summarised in Drapeau *et al.*, 2006; Ferguson *et al.*, 2011), *Bombus* spp. (Kupke *et al.*, 2012), *Megachile rotundata* and the seven ant species analysed herein (see Section V and genome references therein). In addition, at least one Yellow-like protein was identified in 45 bacterial species (Drapeau *et al.*, 2006; Ferguson *et al.*, 2011). Lately, Yellow protein has been associated with pigmentation of *Bombyx mori* larval head and tail spots (Ito *et al.*, 2010) and expression of some *yellow* genes correlated in *Heliconus* spp. with the development of wing patterns and pigmentation (Ferguson *et al.*, 2011). In addition to the role of *yellow* family gene expression in insect melanic pigmentation, *yellow* genes have also been found to be associated with behaviour and sex-specific reproductive maturation (Drapeau, 2003; Ferguson *et al.*, 2011). In spite of these multiple functions there is no specific functional conservation of the various *yellow* genes (Ferguson *et al.*, 2011). Both the number of *yellow* genes and their functions seem to vary within and among genera. In addition to the MRJPs, the *A. mellifera* genome comprises 10 *yellow* genes that also have diverse expression patterns. Whereas y*ellow-g* shows queen-specific ovary expression, *yellow-h* is expressed in developing queens. *Yellow-f* is expressed in the late embryonic stage while *yellow-e3*, which is claimed to be the progenitor of the *mrjp* family, shows expression in the hypopharyngeal gland (Drapeau *et al.*, 2006). Therefore, *Apis yellow* genes might be associated with development. Hojo *et al.* (2010) suggested that MRJPs may have regulatory functions that control development and behaviour in addition to their nutritional role in RJ (Albert *et al.*, 1999*a*). However, as *mrjps* evolved from *yellow* genes it remains to be clarified whether MRJPs are involved in development and behaviour as claimed for Yellow proteins

or if they developed additional new functions. Due to their similarity, MRJPs and Yellow proteins form a protein family termed the MRJP/Yellow family (Albert & Klaudiny, 2004). However, phylogenetic analyses show that Yellow proteins form a monophyletic group distant from MRJPs (Albert & Klaudiny, 2004) (Fig. 3). Other proteins belonging to the MRJP/Yellow family have also been identified in the saliva of the sand flies *Lutzomyia longipalpis* (Charlab *et al.*, 1999), *Phlebotomus papatasi* (Valenzuela *et al.*, 2001) and *Phlebotomus duboscqui* (Volf, Skarupová & Man, 2002) (Fig. 3). The saliva proteins of *L. longipalpis* are capable of binding biogenic amines particularly serotonin, catecholamines, and histamine (Xu *et al.*, 2011) and the homologue protein of *P. duboscqi* possesses lectin activity, i.e. it is a sugar-binding protein (Volf *et al.*, 2002). Although only few specific functions are known for proteins of the MRJP/Yellow family, e.g. the Yellow proteins of *D. melanogaster*, the variety of functions found in the family clearly suggest that MRJPs also may have manifold tasks in the honeybee.

V. EVOLUTION OF MRJPs

Whereas Drapeau *et al.* (2006) suggested that *mrjp* genes are specific and restricted to the genus *Apis*, subsequent genome sequencing projects revealed those genes to be common in various genera of the order Hymenoptera. Kupke *et al.* (2012) showed that *Bombus terrestris*, *Bombus ignitus* and *Bombus impatiens* possess only one copy of the *mrjp* gene which resides between *yellow-e3* and *yellow-h*. The *B. terrestris mrjp* gene comprises five introns in exactly the same positions as found in the honeybee *mrjp*s. Although the gene was expressed, MRJP is not a major protein of the hypopharyngeal gland of *B. terrestris* workers (Kupke *et al.*, 2012). The starkly different genome organisation between *Bombus* and *Apis* prompted us to address the organisation of the *mrjp* and *yellow* genes in other Hymenoptera using a phylogenetic approach.

Obviously the *mrjp*/*yellow* gene family is ancient, as Yellow-like proteins have been found in species including bacteria, fungi and insects (Drapeau *et al.*, 2006; Ferguson *et al.*, 2011). In spite of its phylogenetic age it is highly multifunctional, but nevertheless has been lost repeatedly in many phylogenetic lineages apparently losing and gaining functions. In *A. mellifera* rapid duplications of an ancestral *yellow* gene similar in structure to the extant *yellow-e3* have led to the expansion of the *mrjp* subfamily. Recent efforts in sequencing insect genomes confirmed the same organisational structure of *mrjp* and *yellow* genes for the dwarf honeybee *Apis florea*, except for the position of the pseudogene *mrjp*-Ψ (Fig. 2). A search in the currently available partial genome sequences of the Asian honeybee *A. cerana* and the giant honeybee *A. dorsata* revealed eight and four *mrjp* genes, respectively (Fig. 2) suggesting that the same cluster of *mrjp*/*yellow* genes is conserved throughout the genus *Apis*.

A similar but apparently independent expansion of *major royal jelly protein-like* (*mrjpl*) genes (non-*Apis mrjp* genes) has occurred in the solitary parasitoid wasp *Nasonia vitripennis*. The genomes of the honeybee and *N. vitripennis* allowed the evolution of this gene family to be traced back to a common ancestor. However, in spite of the similarities it also became clear that multiple independent gene duplication events resulted in two functionally very diverse protein families (Werren *et al.*, 2010). With ten *mrjpl* genes, *N. vitripennis* has the largest number of *mrjpl* genes so far found in any insect. Five *mrjpl* genes are encoded by a tandem array on chromosome 3, flanked by members of the *yellow* gene family. This chromosome also harbours one additional *mrjpl* in a distal region. Three of the four remaining *mrjpls* are located on chromosome 4 and *mrjpl10* on scaffold 1011 (Fig. 2). Expression studies of the *mrjpl* genes showed diverse expression patterns across all developmental stages and tissues (Werren *et al.*, 2010).

An analysis of the *mrjpl* gene family in seven different ant species did not reveal any specific pattern of *mrjpl* copy numbers. The leaf cutter ant *Atta cephalotes* genome contained a total of eight *mrjpl* genes. However, five of these eight genes are putative pseudogenes (Suen *et al.*, 2011) and the position in the *yellow*-cluster is not confirmed for the remaining three copies (Fig. 2). This suggests that a high copy number of *mrjps* is an ancestral feature and that *Atta* is in the process of losing these genes (Suen *et al.*, 2011). Up to six possible *mrjpl* copies are present in the genome of the fungus-growing ant *Acromyrmex echinatior* but it is not clear how many are located between *yellow-e3* and *-h* and how many are functional or might be pseudogenes (Nygaard *et al.*, 2011). Two *mrjpl* genes can be found together on one scaffold, the four remaining ones are spread over several scaffolds (Fig. 2). The argentine ant *Linepithema humile* genome assembly revealed, with ten different *mrjpl* genes, the highest number in ants. Interestingly, the *L. humile* genome contains an independent radiation of *mrjpl* genes similar to those in *A. mellifera* and *N. vitripennis* (C.D. Smith *et al.*, 2011). These independent radiations in different hymenopteran lineages might indicate that the ancestral gene had a tendency to proliferate, allowing *mrjpls* to take on new functions and to respond quickly to selection under new environmental or social conditions (Werren *et al.*, 2010).

The loss of *mrjpl*s might be common among ants, as the genome sequences for the carpenter ant *Camponotus floridanus* and the jumping ant *Harpegnathos saltator* revealed only one and three *mrjpl* genes, respectively (Bonasio *et al.*, 2010). *C. floridanus* and *H. saltator* genomes contain a single conserved cluster of the *mrjpl*/*yellow* gene family which diverged from *A. mellifera* more recently than from *N. vitripennis*; only a single copy of *mrjpl* was found in this locus (Fig. 2) and *H. saltator* possesses two additional *mrjpl* genes elsewhere (Bonasio *et al.*, 2010). Not only has a decline in *mrjpl* copies been observed but also cases of total absence. Screening the genome of the fire ant *Solenopsis invicta* showed the presence of *yellow-e*, *-e3*, *-g* and *-g2* but the absence of *yellow-h* and any *mrjpl*s. Only five genes in the red harvester ant *Pogonomyrmex barbatus* share similarities to the *mrjp* and *mrjpl* genes of *A. mellifera* and *N. vitripennis* (C.R. Smith *et al.*, 2011). The fact

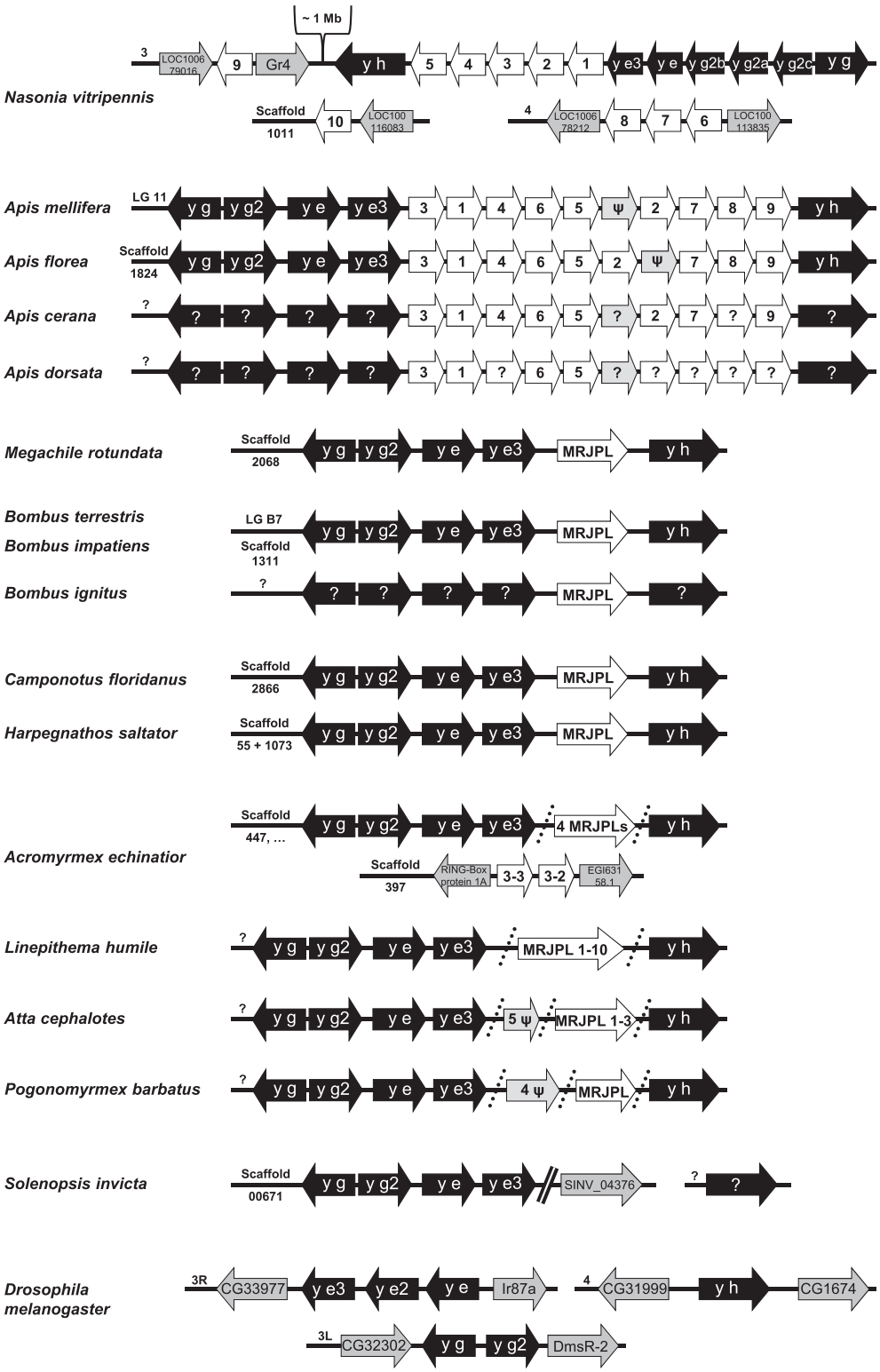

Fig. 2. Schematic overview of the organisational structure of the *major royal jelly protein/yellow* gene (*mrjp/yellow*) clusters across several Hymenoptera species and *Drosophila melanogaster*. The position of the clusters in the insect genomes is indicated by chromosome or scaffold numbers. Black arrows show *yellow* genes, white the *mrjp* or *mrjpl* genes, light-grey the *mrjp*-pseudo gene copies (ψ) and dark grey the flanking genes. Given that for some species only the draft genome was available, it was not possible to confirm that all *yellow* genes are on the same scaffold/chromosome (marked by dotted lines) or that all known gene copies (*yellow* and *mrjp/l*) are present, missing data are indicated by question marks.

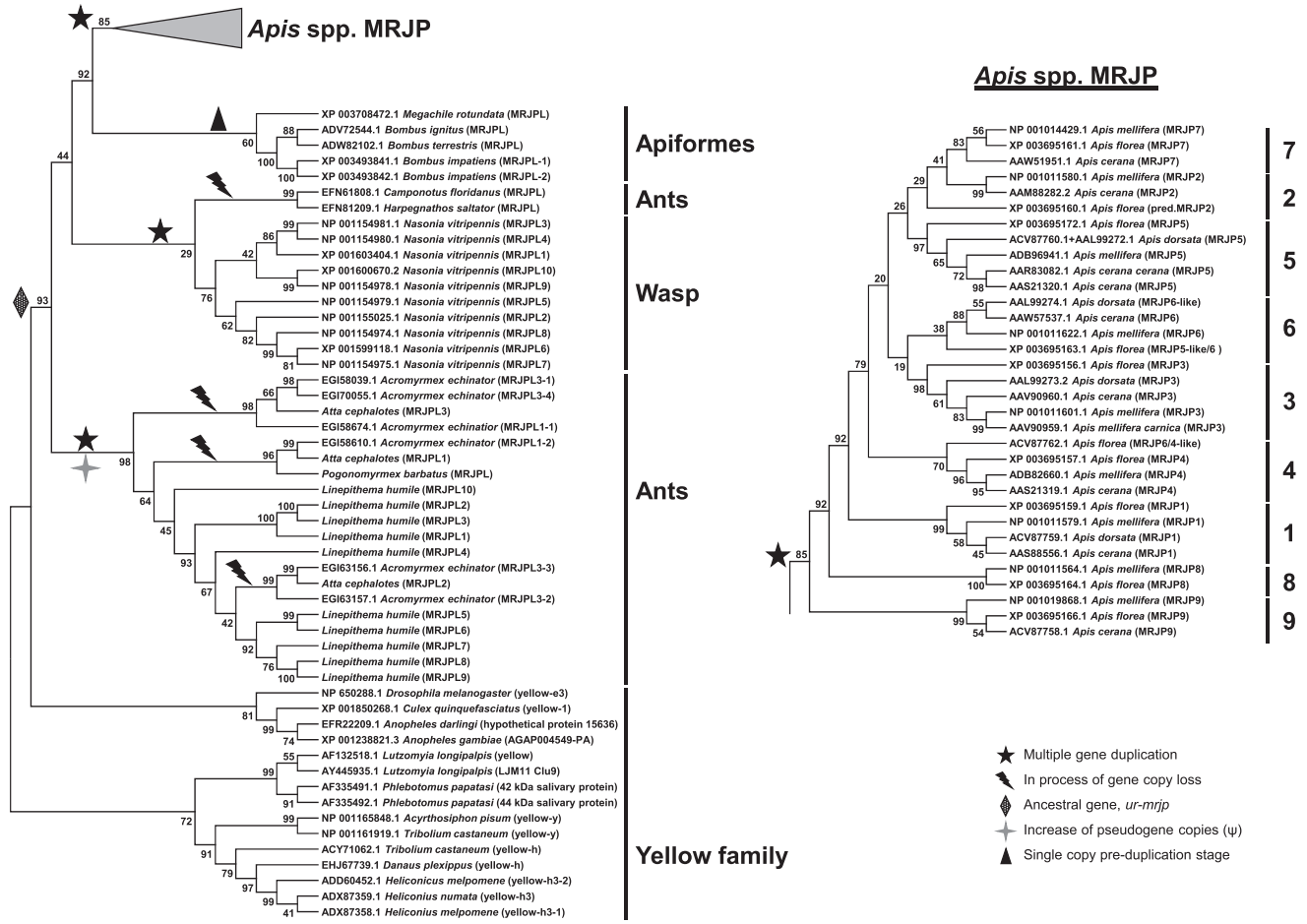

Fig. 3. Phylogeny of major royal jelly protein (MRJP), MRJP-like (MRJPL), salivary and yellow protein sequences of ants, bees, the wasp *Nasonia vitripennis* and non-hymenopteran insects. The maximum likelihood tree was reconstructed from aligned protein sequences, using the Jones-Taylor-Thornton (+G, +F) model, including bootstrap resampling (500 replications) to verify the topology of the inferred phylogenetic tree. Protein sequence alignment, model selection to determine the best model to reconstruct the phylogeny and tree reconstruction were performed in MEGA version 5 (Tamura *et al.*, 2011).

that four of these five detected *mrjpl* genes were fragmentary suggests that these genes may have been pseudogenised and have lost their function. Only one complete *mrjpl* copy was detected for *P. barbatus*, as known for *C. floridanus* and *H. saltator* (M. Helmkampf, personal communication). The yellow genes (*y-e*, *-e3*, *-g*, *-g2* and *-h*) associated with the conserved *mrjpl/yellow* gene cluster of *A. cephalotes*, *L. humile*, *P. barbatus* and *S. invicta* (except *y-h*) are similar to those of *Apis* and are presumably orthologous with most likely the same location and orientation on one single scaffold.

Kupke *et al.* (2012) reasoned that bumblebees might represent a transition species preserving a single-copy pre-duplication stage of honeybee MRJP evolution. This conclusion was drawn from a phylogenetic tree derived from only two genera within Apiformes (*Apis* and *Bombus*) and is therefore not supported by a sufficient breadth of data. The common eastern bumblebee *B. impatiens* and the buff-tailed bumblebee *B. terrestris* illustrate the same conserved cluster of typical surrounding *yellow* genes and one *mrjpl* copy as shown for some ant species (*C. floridanus* and *H. saltator*) (Kupke *et al.*, 2012) (Fig. 2). Expressed sequence tag (EST) library sequencing confirmed the presence and high homology of at least one *mrjpl* in the Asian bumblebee *B. ignitus* (Kupke *et al.*, 2012). Since there are indications of selection on the *mrjpl* gene compared to its neighbour *yellow-e3*, the *mrjpl* may evolve more rapidly, perhaps in an adaptive response to its function (Kupke *et al.*, 2012). As final example, the solitary alfalfa leafcutter bee *Megachile rotundata* has only one *mrjpl* copy, similarly to primitively eusocial bumblebees and highly eusocial ants. A comparison of all available hymenopteran genomes shows that the number of *mrjp/l* copies is not related to level of sociality (Fig. 2).

The *mrjp* and *mrjpl* gene sets of all taxa each form strongly supported clades within the monophyletic *mrjp/l* subfamily (Fig. 3). The topology strongly indicates that the radiation of the *mrjp/l* genes in each taxon occurred after the evolutionary split of their respective lineages. However, except for the production of RJ in *Apis*, the functions of these genes remain unknown but may nevertheless share similarities between *Apis*, non-*Apis* bees, ants and wasps and may have

evolved in response to similar selective pressures. Genome organisation and copy number is exceptionally variable and seems to be the result of very specific adaptations. A phylogenetic analysis of MRJPs shows three independent MRJPL and MRJP branches supported by high bootstrap values suggesting independent evolution of these proteins in *A. mellifera* and non-*Apis* bees, ants and *N. vitripennis* (Fig. 3).

Sharing one conserved cluster of *mrjpl/yellow* family genes within the insect families of ants and bees implies that either *mrjp/l* genes were selectively lost in both lineages during evolution, or that the duplications occurred at least three times independently in the honeybee, some ant and the wasp lineages - direct evidence in the form of a single-copy precursor gene is missing. It has been suggested that duplication and functional diversification of *mrjp*s correlates with the emergence of social behaviour in *A. mellifera* (Drapeau *et al.*, 2006). However, the fact that genomes of both solitary and highly eusocial Hymenoptera can comprise any number between 0 and 10 genes encoding *mrjp/l* genes, refutes this hypothesis clearly. Almost all Hymenoptera investigated contain the same array of *yellow* genes including *mrjp/l* gene(s) between *yellow-e3* and *yellow-h*, indicating that a single *mrjp* gene might be an ancestral feature, most likely originating from the duplication of *yellow-e3* as suggested by Drapeau *et al.* (2006). However, an origin from multiplication of *yellow-e3* cannot be excluded. Kupke *et al.* (2012) conclude that the ancestral *mrjp* (*ur-mrjp* gene) had some ancient pre-nutritive function, and that a nutritive function evolved later in the honeybee lineage. This is supported by the lack of the nitrogen-rich repetitive regions seen in *A. mellifera* MRJP3 and 5 in any of the 10 MRJPLs of *N. vitripennis* and *L. humile*. Furthermore, homology among all *Apis* MRJPs is much higher between than within the *Apis* species (Fig. 3). In the honeybees, MRJP1–7 appear to be derived from MRJP8 and 9, which share homologies with most of the MRJPL copies of the non-*Apis* Hymenoptera (Fig. 3).

VI. FUNCTIONAL STUDIES IN NON-*APIS* MODEL SYSTEMS

Most functional studies have been performed with MRJP1, the most abundant protein of RJ. Firstly, MRJP1 appears to have growth-factor-like activity: the protein (native and recombinant) enhances DNA synthesis, maintains proliferation and suppresses apoptosis of rat hepatocytes (Kamakura, Suenobu & Fukushima, 2001*b*; Kamakura & Sakaki, 2006). Furthermore, MRJP1 leads in these rat hepatocytes to increased phosphorylation of mitogen-activated protein (MAP) kinase and protein kinase B (Kamakura & Sakaki, 2006). In addition, MRJP1 stimulates the cell growth of human myeloid cells (Watanabe *et al.*, 1998) and of five different human lymphocytic cell lines (Watanabe *et al.*, 1996). The 290 kDa oligomer of MRJP1 and apisimin enhanced cell proliferation in human lymphoid cells (Tamura *et al.*, 2009*a*). Even recombinant MRJP1 of *Apis cerana* stimulated cell growth in a lepidopteran cell line (Shen *et al.*, 2010), further confirming the results of these studies.

In addition to growth factor functions, some MRJPs affect the production of various cytokines. The monomeric forms of MRJP1 (not the 420 kDa oligomer) and MRJP2 stimulate tumor necrosis factor (TNF)-α production by mouse macrophages (Šimúth *et al.*, 2004; Majtán *et al.*, 2006). MRJP3 suppresses interleukin (IL)-4 production of mouse spleen cells and down-regulates IL-2, IL-4 and interferon (IFN)-γ production of stimulated $CD4^+$ cells purified from mouse spleen cells (Okamoto *et al.*, 2003). Contrary to MRJP1 and 2, MRJP3 inhibits TNF-α production by stimulated macrophages and suppresses antibody response against ovalbumin antigen in mice, therefore, having immunosuppressive functions (Kohno *et al.*, 2004). Furthermore, MRJP1 and 2 are major allergens of RJ; they were shown to bind immunoglobulin E (IgE) of sera from patients with RJ allergy (Thien *et al.*, 1996; Rosmilah *et al.*, 2008). For MRJP1, IgE binding was shown to be dependent on glycosylation of the protein (Hayashi *et al.*, 2011). MRJP3 induces high amounts of anti-MRJP3 IgG1 antibodies in mice thereby exhibiting antigenicity (Okamoto *et al.*, 2003). All of these studies reveal that at least MRJP1–3 exhibit immunoregulatory effects *in vivo*.

Antibacterial effects against *Paenibacillus larvae* which causes a severe brood disease (American foulbrood) were shown for a glycosylated minor homolog of MRJP2 (MRJP2a); these effects are most likely due to glycosylation (Bíliková *et al.*, 2009). Furthermore, peptides comprising the last eight to nine amino acids of MRJP1 (named jelleins) that are tryptic digestion products found in RJ have antibacterial effects against some species of Gram-positive and Gram-negative bacteria (Fontana *et al.*, 2004).

Apart from the antibacterial effects against American foulbrood none of these known functions of MRJP1–3 address the role of the proteins in the honeybee.

VII. MRJPs AND QUEEN DIFFERENTIATION

The role of RJ for caste determination in the honeybee is the textbook classic paradigm for environmental caste differentiation in social insects. It was recently claimed that either MRJP1 or MRJP3 influence the fate of a female larva to develop into a queen (Kamakura, 2011; Huang *et al.*, 2012). Kamakura (2011) showed that feeding of the monomeric form of MRJP1, which he named Royalactin, to larvae shortened their developmental time, and increased body mass and ovary size at adult emergence as well as juvenile hormone (JH) titres in larvae. These effects were not observed with the oligomeric form of MRJP1 bound to apisimin (see Section III.3). Here, the monomeric MRJP1 acted on epidermal growth factor receptor (EGFR) signalling to induce queen differentiation in the larvae (Kamakura, 2011). Huang *et al.* (2012) based their study on the finding that feeding larvae with RJ caused different epigenetic modifications (Kucharski *et al.*, 2008). They fed worker

honeybees with histone deacetylation inhibitors which lead in general to higher amounts of acetylated lysines on histone proteins, eventually causing transcriptional activation. As a reaction to this manipulation, workers secreted a different isoform ratio of MRJP3 into RJ compared to controls (Huang *et al.*, 2012). Furthermore, feeding with the altered MRJP3-isoform RJ induced larval growth and led to increased synthesis of MRJP1–3 in these larvae (Huang *et al.*, 2012). In line with the current literature, the authors state that MRJP1 might be required to maintain basic growth for queen larvae, whereas the different MRJP3 isoforms may play a more important role in enhancing growth rate (Huang *et al.*, 2012).

The search for the 'queen determinator' is many decades old (Rembold, Lackner & Geistbeck, 1974) and other candidates have been suggested to induce queen differentiation. For instance, it has been shown that a higher sugar level and the correct ratio of fructose to glucose could increase the proportion of larvae developing into queens (Asencot & Lensky, 1976; Kaftanoglu, Linksvayer & Page, 2011). Spannhoff *et al.* (2011) showed that the unusual fatty acid 10-hydroxy-2-decenoic-acid (10-HDA), only found in RJ, harbours histone deacetylase inhibitor activity. They suggest that the caste switch in honeybees may be epigenetically regulated driven by the histone deacetylase inhibitor activity of 10-HDA leading to an increased transcription. Leimar *et al.* (2012) convincingly argue that a single determinator is unlikely to trigger caste determination in honeybees and indeed is not required from a perspective of evolutionary theory.

These selected examples show that the origin of the 'queen-inducing agent' is still under discussion. It may well be that MRJPs are somehow involved in queen differentiation, but given the theoretical and empirical evidence at this stage, it is very unlikely that one MRJP alone possesses queen-inducing capacity. Queen differentiation appears to remain a complex process.

VIII. FUTURE PERSPECTIVES

(1) How did the *mrjp/l* gene family evolve?

Since *mrjp/l* genes are only known from the Hymenoptera, this order remains key to the understanding their evolution. The Hymenoptera is not only one of the biggest orders within the animal kingdom, but also one of the most diverse with regard to life history. The multiple independent evolution of sociality in various taxa provides opportunities to study the significance of *mrjp/ls* for social evolution. Drapeau *et al.* (2006) proposed that the occurrence of MRJPs might be associated with the appearance of sociality in the genus *Apis*. Although this was plausible at that time, recent publications of several hymenopteran genomes revealed the occurrence of *mrjpl* genes also in solitary Hymenoptera like *Nasonia vitripennis*. Furthermore, *mrjp/l* gene copy number did not correlate with level of sociality and it now seems safe to reject their hypothesis. However, the available data on genome organisation are still very fragmentary and insufficient to allow reliable conclusions on gene evolution at present.

The phylogeny of the Hymenoptera has been extensively studied, with robust evolutionary trajectories clearly identifying multiple independent origins of eusociality (Goulet & Huber, 1993; Davis, Baldauf & Mayhew, 2010). Each of these trajectories can be used to test for a potential adaptive role of *mrjp/l*s in various taxa. With advances in cost-efficient genome technology it is inevitable that we will fill the remaining gaps in *mrjp/l* evolution: a large suite of hymenopteran species are currently on the list of the *i5k* initiative, that plans to sequence the genomes of 5000 insect and related arthropod species during the next 5 years (http://arthropodgenomes.org/wiki/i5K).

As stated earlier, *N. vitripennis* possesses 10 *mrjpl* genes, the largest number found to date. This remains the only sequenced parasitic hymenopteran and it will be highly informative to determine whether a high copy number of *mrjpl* genes is specific to parasitic Hymenoptera. It is therefore highly advantageous that several parasitic hymenopteran species are on the *i5k* list, including the parasitoid wasp *Aphelinus abdominalis*.

The seven ant species genomes sequenced to date do not show any obvious specific phylogenetic pattern in their *mrjpl* copy numbers which range from 0 to 10 genes. However, the number of ant genomes currently available is too low to allow general conclusions to be drawn. Four of the seven analysed species (*Acromyrmex echinator*, *Atta cephalotes*, *Pogonomyrmex barbatus* and *Solenopsis invicta*) belong to the same subfamily Myrmicinae. Two of these species have a high number of *mrjpl* pseudogenes (*P. barbatus* and *A. cephalotes*) whereas *S. invicta* has no *mrjpl* genes. Perhaps this indicates that a high copy number of *mrjpl* genes is the ancestral state and that the whole subfamily Myrmicinae is in the process of losing its *mrjpl*s. Within the phylogenetic tree (Fig. 3), seven MRJPLs of the Myrmicinae ants cluster together whereas three MRJPLs can be found within the ten MRJPs of *Linepithema humile* belonging to another ant subfamily. This might indicate that the MRJPLs of *L. humile* evolved independently in this subfamily originating from a common ancestor that is still present in some Myrmicinae. Again, sample sizes are small and sequencing additional species of the subfamily is required before we can either confirm or reject a monophyletic ancestry of *mrjp/l* copy number. Only a comprehensive study including other subfamilies within the ants will allow us to understand the evolution of *mrjp/l*s.

To address the evolution of the honeybee *mrjp*s, the Apidae will need to be comprehensively studied, screening for specific functional adaptations of the *mrjp/l*s and unravelling their phylogeny. The Apidae are particularly suitable for studies of *mrjp/l* evolution, because the taxon spans the full range of social organisation from solitary to eusocial, from annual to perennial, and from parasitic to social life history. Again, several species are included within the *i5k* initiative including the eusocial stingless bee *Melipona quadrifasciata*, the solitary south-eastern blueberry bee *Habropoda laboriosa* and the social parasitic cuckoo bumblebee *Bombus vestalis*. Hence, such

analyses will be possible in the near future. Last but not least, the social parasitic Cape honeybee *A. mellifera capensis* might be one of the most interesting bees to sequence in this context. These honeybees can have both a social and a parasitic life-history mode, unique within the genus *Apis* (Neumann & Moritz, 2002). The release of these genomes will greatly facilitate to the understanding of the evolution of *mrjp/ls* within the bees and definitely clarify the question whether one *mrjp/l* gene represents the ancestral state in this family and if the multiplication occurred only within the genus *Apis*.

(2) What are the functions of MRJP/Ls?

Whereas the phylogentic patterns of *mrjp/l* evolution can be traced using state-of-the-art routine genomic tools, questions relating to the functional adaptions of MRJP/Ls are more difficult to answer. Why do some members of the Apocrita have multiple copies of *mrjp/l* genes, such as the Argentine ant *Linepithema humile*, whilst other closely related species, like the fire ant *S. invicta*, survive perfectly well without them? Biochemical and molecular tools must be used in experimental approaches to dissect out the biochemical functions of these proteins.

One classical way to identify the function of a protein is to knockdown the corresponding gene. This has been done in the honeybee *via* RNA interference (RNAi); in embryos using double-stranded RNA (dsRNA) injection (Beye *et al.*, 2002) as well as in adult bees *via* injection or feeding of dsRNA and small interfering RNA (siRNA) (Amdam *et al.*, 2003; Jarosch *et al.*, 2011). The application of RNAi in embryos will show if the specific gene product has a basal function during development whereas the application in adult honeybees might expose functions in behaviour and during larval rearing if specifically applied to nurses. For example, we could test whether nurses can raise larvae if they are not able to produce a specific MRJP.

Alternatively, recombinant proteins can be produced to screen for specific interaction partners within the proteome of the honeybee. Identified interactions can be characterised and there is even the possibility to study the interaction in *in vitro* cultured honeybee embryonic cells (Bergem, Norberg & Aamodt, 2006). The stage seems to be set to unravel the functions of the MRJPs, not only in the honeybee.

IX. CONCLUSIONS

(1) Although much research has been carried out on the major royal jelly proteins (MRJPs) of *Apis mellifera* during the past four decades, our understanding of their functions is still limited.

(2) We can assume that the proteins have functions other than their nutritional value for young larvae, indeed even this nutritional value is still controversial when it comes to caste differentiation. The enormous diversity in *mrjp* expression patterns clearly indicates that MRJPs have additional functions in honeybees.

(3) The expression of *mrjps* in the brain and its co-regulation in response to juvenile hormone clearly indicates a fundamental effect of MRJPs on honeybee development and colonial organisation.

(4) Although some MRJPs act like growth factors, claims that one MRJP alone can induce queen development remain controversial and in conflict with other results. Since *mrjp/l* gene copy numbers vary dramatically within hymenopteran social insects, ranging from ten copies in *Linepithema humile* to probably not even one copy in *Solenopsis invicta*, other mechanisms than MRPJs must be involved in queen determination. Strikingly, the solitary parasitoid wasp *Nasonia vitripennis* has up to 10 copies of *mrjpl* genes again implying that the proteins must have a more general role that may contribute to but not determine caste in social insects.

(5) Since *mrjp/l* gene copy numbers are highly variable among both highly eusocial and solitary Hymenoptera the hypothesis of a correlation of MRJP/Ls with social behaviour can certainly be rejected.

X. ACKNOWLEDGEMENTS

We thank Martin Helmkampf (Arizona State University, Tempe, USA) for providing *mrjpl* sequences of *Atta cephalotes*, *Linepithema humile* and *Pogonomyrmex barbatus*. We also thank two anonymous reviewers for most helpful comments. A.B. and S.E. were supported by the project RoBeeTech (grant POSCCE 206/20.07.2010 SMIS code 618/12460 to Liviu A. Mărghitaş, Dan S. Dezmirean and R.F.A.M.) and the project BEE DOC funded by the EU FP 7 activity 2.1.3 KBBE-2009-1-3-03 grant 244956 CP-FP.

XI. REFERENCES

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(*Received 28 November 2012; revised 19 June 2013; accepted 20 June 2013; published online 15 July 2013*)