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Prediction of the post-translational modifications of adipokinetic hormone receptors from solitary to eusocial bees

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Abstract

Adipokinetic hormone receptor (AKHR) was regarded as the crucial regulator of lipid consuming, but now has been renewed as a pluripotent neuropeptide G protein-coupled receptor. It has been identified in all sequenced bee genomes from the solitary to the eusocial. In the current study, we try to clarify the transitions of AKHR on lipid utilization and other potential functions from solitary to eusocial bees. The results showed that the AKHRs were divided into different groups based on their social complexity approximately. Nevertheless, the critical motifs and tertiary structures were highly conserved. As to the post-translational modifications, the eusocial possessed more phosphorylation residues and modification patterns, which might be due to the necessity of more diverse functions. These results suggest that AKHRs are highly conserved on both primary motifs and tertiary structures, but more flexible on post-translational modifications so as to accommodate to more complicated eusocial life.

Introduction

The neuropeptide adipokinetic hormone (AKH) is one of the most studied neuropeptides, which has been verified to involve in many important physiological roles, such as mobilizing energy substrates during development, flight, starvation and other stress situations, stimulating heartbeat rate, inhibiting protein synthesis, extending life span and even in response to oxidative stress (Van der Horst, 2003; Isabel et al., 2005; Malik et al., 2012; Bednarova et al., 2013; Bednarova et al., 2015; Galikova et al., 2015; Kim & Neufeld, 2015; Zandawala et al., 2015). In general, AKHs are composed of 8 to 10 amino acid residues, with a pyroglutamate at the N_ terminus, an aliphatic amino acid residue in position 2, an FS, FT or FY sequence at positions 4 and 5, and either a W_amine or WGX_amine C_terminus (Gade & Auerswald, 2003). The physiological roles of AKH are realized by binding and activating its receptor.

The predicted gonadotropin releasing hormone receptor (GnRHR) in fruit fly and silkworm have been functionally identified as the AKH receptors (AKHRs), which belong to the rhodopsin-like G protein-coupled receptor (GPCR) family (Staubli et al., 2002; Lagerstrom & Schioth, 2008). As the largest transmembrane (TM) receptor family, all GPCRs share a common structural feature of 7-TM domain connected by alternating intracellular and extracellular loops, with an extracellular N_terminus and an intracellular C_terminus (Gether, 2000). Signal transduction from the extracellular to the intracellular is accomplished by ligand binding and G protein coupling (Caers et al., 2012). Since the split of proto- and deuterostomia at about 700 million years ago, the evolution of GnRHR was separated into two branches (Hauser & Grimmelikhuijzen, 2014). GnRHR was reserved in the deuterostomia lineage, while the ancestral receptor and its ligand genes were duplicated and diversified in the protostomian lineage, and resulted in three independent

hormonal systems signaling with AKH, corazonin and AKH/corazonin-related peptide, respectively (Hauser & Grimmelikhuijzen, 2014).

For survival during adverse periods, storage of glycogen and lipid in fat body is essential for the solitary bees, while the eusocial bees mainly consume nectar for flight and store large communal carbohydrates in the hive instead of individual energy reserve (Crailsheim, 1988; Panzenböck & Crailsheim, 1997; Votavova et al., 2015). Besides, the transition of nursing to foraging of honeybee workers is coupled with the stable lipid loss (Ament et al., 2011), which suggested that the regulation of AKHR might be more complicated in the eusocial than that in the solitary.

Recently, the genomic signatures of evolutionary transitions from solitary to group living were revealed through comparison of ten sequenced genomes of bee species with various social complexities. The eusocial evolution is associated with an increased capacity for gene regulation and a relaxed natural selection. As the "core set", AKH and AKHR were identified in all bee species (Kapheim et al., 2015). The mature AKH has been identified via mass spectrometry in the solitary bees (Lorenz et al., 2001). In honey bees (*Apis mellifera*), the transcription of AKH and AKHR has been detected in head and fat body, and the expression of AKHR was upregulated in response to the downregulation of both *vitellogenin* and *ultraspiracle* via RNA interference (Wang et al., 2012). The existence of additional TATA box in the promoter region of the *Akh* gene resulted in the diminished expression of AKH (Veenstra et al., 2012), which made it difficult to detect mature AKH via mass spectrometry (Boerjan et al., 2010; Sturm et al., 2016). What is more, the activation and desensitization mechanisms of AKHR become more attractive.

Apart from the regulation on transcription and translation, the transitions on signal pathways based on the post-translational modifications (PTMs) are also important for GPCR. In consideration of the various life styles and the potential pluripotency of AKHRs, the goal of the current study is to characterize the potential transitions of AKHRs in response to the increasing social complexity. The comparison of amino acid sequences, homology modelling, prediction of their ligand binding pockets and PTMs were taken into account. The increasing phosphorylation residues and modification patterns based on the conserved sequences and structures might be crucial.

Materials and methods

Sequences collection and phylogenetic analysis

With the amino acid sequence of AKHR in *Apis mellifera* as the query, the BLASTP (*https://blast.ncbi.nlm.nih.gov/Blast.cgi*) searches were carried out, and the amino acid sequences of GnRHIIR from ten bee species were obtained according to the similarity (Table 1). And then, these GnRHIIR sequences of other bee species have been taken as query to align with the AKHR sequence of *Apis mellifera* so as to confirm that they were homologous genes.

Multiple sequences alignment and phylogenetic analysis were conducted using *MEGA* version 6 software package by method reported previously with slight modifications (Tamura et al., 2013; Hauser & Grimmelikhuijzen, 2014). In detail, the available entire protein sequences were aligned with CLUSTALW, using the protein weight matrix of BLOSUM. The model selection analysis of neighbor-joining tree was performed using Maximum likelihood method. Based on the best-fit substitution model, phylogenetic analysis was calculated with 1000 bootstrap replicates. The evolutionary distances were computed using the JTT matrix-based method and in the units of the number of amino acid substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). All positions containing gaps and missing data were eliminated (Tamura et al., 2013). The AKHR of *Anopheles gambiae* (*A_gam*_AKHR, XP_001687839.2) was as outgroup.

Table 1. Sequences appeared in this study.

Names	Species	Accession number	Identities
*A_mel*_AKHR	*Apis mellifera*	NP_001035354	100%
*A_dor*_GnRHIIR	*Apis dorsata*	XP_006609474.1	97%
*A_flo*_GnRHIIR	*Apis florea*	XP_003690609.1	96%
*M_qua*_GnRHIIR	*Melipona quadrifasciata*	KOX71639.1	89%
*B_imp*_GnRHIIR	*Bombus impatiens*	XP_012249751.1	88%
*B_ter*_GnRHIIR	*Bombus terrestris*	XP_012166783.1	86%
*E_mex*_GnRHIIR	*Eufriesea mexicana*	XP_017764264.1	89%
*C_cal*_GnRHIIR	*Ceratina calcarata*	XP_017886236.1	87%
*D_nov*_GnRHIIR	*Dufourea novaeangliae*	XP_015436827.1	85%
*M_rot*_GnRHIIR	*Megachile rotundata*	XP_012146104.1	85%
*H_lab*_GnRHIIR	*Habropoda laboriosa*	XP_017789640.1	80%
*A_gam*_AKHR	*Anopheles gambiae*	ABD60146.1	62%

Homology modelling

Homology modelling is a powerful method for structure establishment of protein, especially for GPCRs, whose structures are difficult to be obtained due to the 7-transmembrane (TM) helices. To date, the crystal structure of insect GPCRs is still unavailable. Fortunately, the model of *A_gam*_AKHR has been characterized via homology modelling (Mugumbate et al., 2011). Based on the ligand-binding structure of *A_gam*_AKHR provided by Dr. Graham Jackson (University of Cape Town), the putative tertiary structures of bee AKHRs were established via SWISS-

MODEL and the structure alignment and calculation of ligand binding site were accomplished by Swiss-pdb Viewer 4.1.0 (Biasini et al., 2014). In consideration of the forces of hydrogen bonding, salt bridge and van der waals, the distance for ligand-binding residues prediction was restricted within 4 Å (Wang et al., 2009).

Prediction of post-translational modifications (PTMs)

The Glycosylation and myristoylation sites were predicted by Motif Scan (*http://myhits.isb-sib.ch/cgi-bin/motif_scan*). The serine, threonine and tyrosine residues of the intracellular domain, which were predicted to be phosphorylated by Protein kinase A (PKA), Protein kinase B (Akt/PKB), Protein kinase C (PKC), Insulin receptor (InsR) and unspecified kinases (UNSP), were calculated via the NetPhos 3.1 server (*http://www.cbs.dtu.dk/services/NetPhos/*) (Blom et al., 1999; Blom et al., 2004). The statistics were implemented via PASW statistics 18 software and the histograms were drawn by *SigmaPlot* version 12.5 software.

Results

Distinction of AKHRs from the solitary to the eusocial

Comparison of neuropeptides and the cognate receptors shows that AKH and AKHR belong to the "core set" which appear in all examined insects (Nygaard et al., 2011). To establish the clusters of AKHRs and GnRHIIRs, the phylogenetic analysis rooted by *A_gam*_AKHR was carried out (Figure 1). The results showed that they were divided into three principal clusters that were mainly corresponding to the advanced eusocial, the primitively eusocial and the solitary/subsocial groups. In spite of the advanced eusocial lifestyle of *Melipona quadrifasciata*, its GnRHIIR got together with the primitively eusocial bumblebees. Furthermore, the GnRHIIR of the solitary *Habropoda laboriosa* was classified uniquely, and there was a sub-branch between the solitary and the subsocial. Given the phylogenetic tree was based on the substitutions of amino acids, the critical sites and motifs of rhodopsin-like GPCR were detected in detail so as to identify the crucial features to support various social complexity.

Conserved motifs of AKHRs

As mentioned above, AKHR belongs to rhodopsin-like receptor family. The results derived from sequence alignment showed that all examined receptors shared a highly conserved sequence (Figure 2). Moreover, the conservation occurred in almost all domains except the N_terminus. Compared to the others, additional amino acids appeared in the N_terminus of *Bombus* GnRHIIRs and the ICL3 of *H_lab*_GnRHIIR. Since the rigorous similarity of primary sequences, the analysis of tertiary structures generated via homology modelling was carried out in succession.

Uniformed tertiary structures of AKHRs

Besides the highly conserved amino acid sequences, all of the eleven receptors shared a conserved structure (Figure 3A). Just as the *A_gam*_AKHR, the helices of bee AKHRs arranged by anticlockwise when viewed from the extracellular side. This is also a common feature of all rhodopsin-like GPCR members that had been reported previously. Since the longer sequences exhibited above, the AKHRs of *Bombus* had longer N_terminus than the others. Notably, all eleven bees shared the same AKH. As to the ligand binding pocket, the amino acid residues neighbored to the ligand were screened out. These residues distributed in all three ECLs and 6-TM helices (except the helix 4) and displayed high conservation (Figure 3B and 3C).

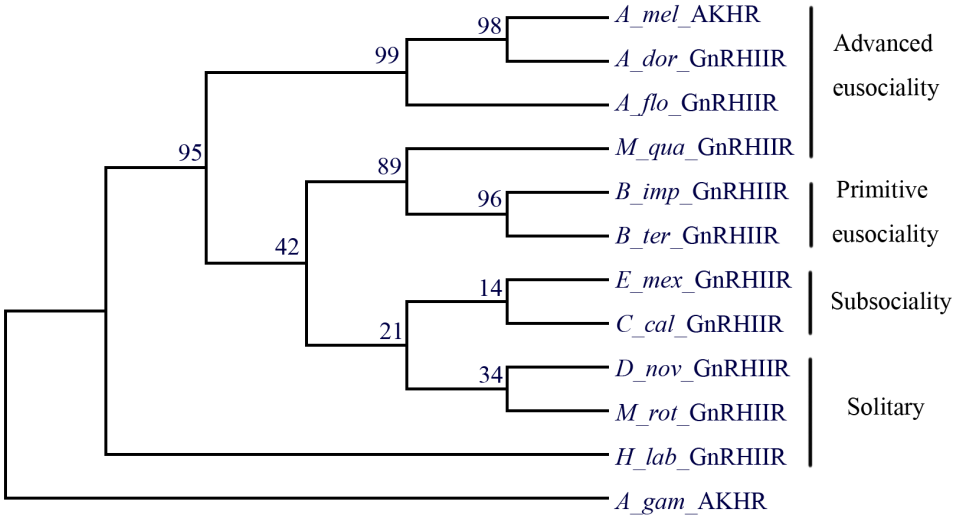

Fig 1. Phylogenetic tree of bees based on AKHRs. The amino acid sequences were grabbed from NCBI (National Center for Biotechnology Information) database, and the neighbor-joining tree were drawn by *MEGA* version 6 software. The percentage of replicate trees was shown next to the branches. The *A_gam*_AKHR was taken as outgroup to root the tree. The social complexity of species was labeled on the right.

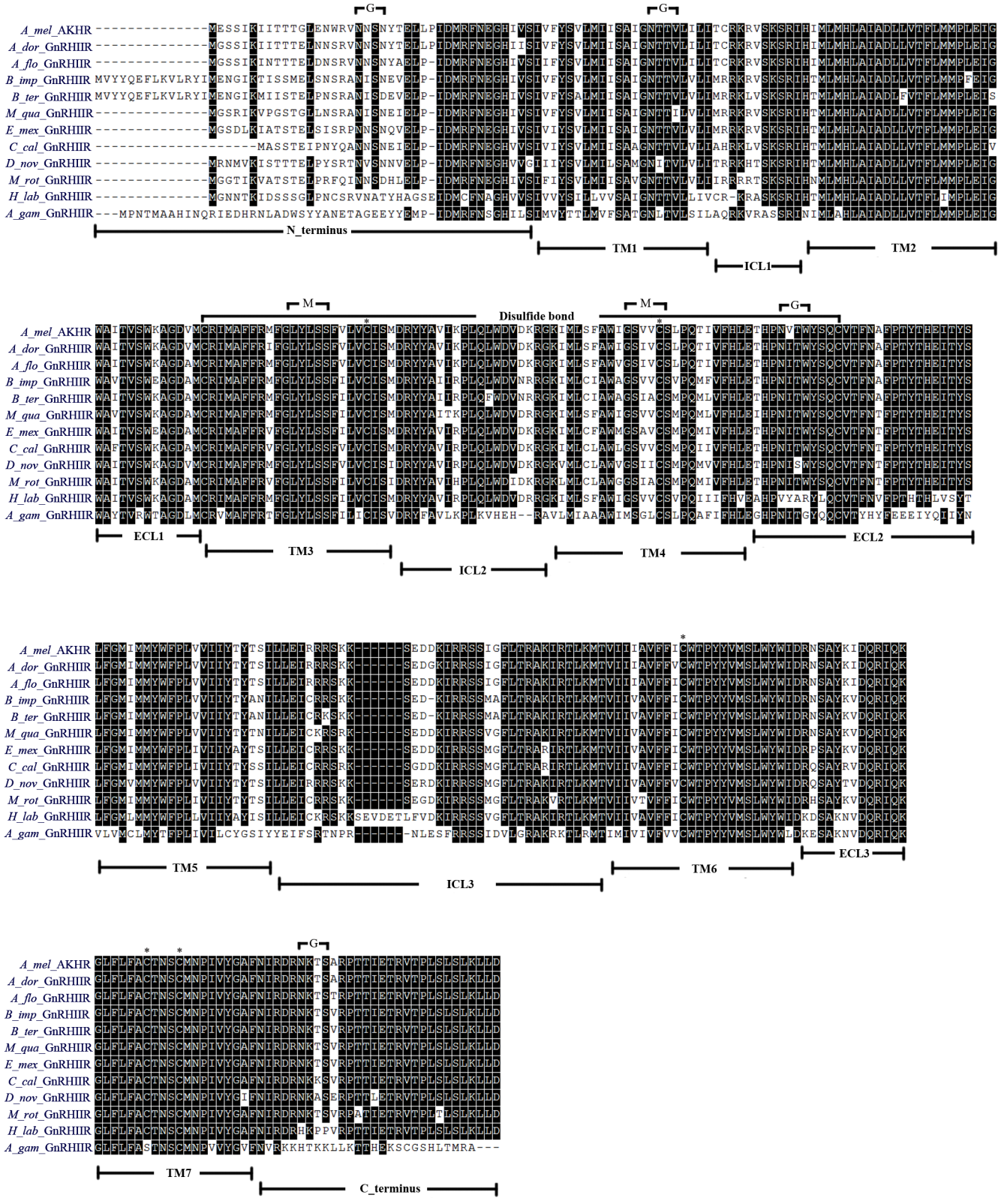

Fig 2. Sequence alignment and domains characterization. Based on the amino acid sequences obtained from NCBI database, the alignment was carried out by CLUSTALW and the identical residues were shaded. The names of each sequence were labeled on the left. A disulfide bond was identified between ECL1 and ECL2. The motifs of Asn_Glycosylation (G), myristoylation (M) and the conserved cysteine residues (*) of *A_mel*_AKHR were labeled on the top. The N_terminus, TM helices, ICLs, ECLs and C_terminus domains of AKHRs labeled at the bottom were characterized by TMHMM server (*http://www.cbs.dtu.dk/services/TMHMM-2.0/*).

More predicted phosphorylation patterns in AKHR of bees with higher social complexity

Given the importance of PTMs on receptor expression, structure stability and signal transduction, the motifs of glycosylation, myristoylation, palmitoylation and phosphorylation were detected.

The results displayed that there were four ASN_Glycosylation sites distributed in the N_terminus, TM1, ECL2 and C_terminus domains of *A_mel*_AKHR, *M_qua*_GnRHIIR, *B_imp*_GnRHIIR, *B_ter*_GnRHIIR, *E_mex*_GnRHIIR and *M_rot*_GnRHIIR (Figure 2). Compared to these six receptors, one more site appeared in the N_terminus domain of *A_dor*_GnRHIIR and *A_flo*_GnRHIIR, while the C_terminus one disappeared in *C_cal*_GnRHIIR and *D_nov*_GnRHIIR. There were also four glycosylation sites in the *H_lab*_AKHR, but three distributed in the N_terminus and one localized in the TM1 (Figure 4A, supplementary file 1).

As to the myristoylation sites, there were two common sites in TM3 and TM4 of all the receptors, and an additional site localized in the N_terminus of *M_qua*_AKHR and *H_lab*_AKHR (Figure 4B, supplementary file 1). For palmitoylation, the conserved cysteine site in the C_terminus of rhodopsin-like GPCRs has disappeared, but five more conserved cysteine residues in the TM3, TM4, TM6 and TM7 were found except the two for disulfide bond in ECL1 and ECL2 (Figure 2).

Phosphorylation is important for activity regulation of GPCR, especially the phosphorylation of intracellular domain which is related with receptor desensitization, arrestin recruitment and signal pathway switch (Tobin, 2008). Here, the residues of serine, threonine and tyrosine in the intracellular domains, which were documented as primary phosphorylation sites, were taken into account. In ICL1 domain, there were four residues in *D_nov*_GnRHIIR, three in *Apis_mel*_AKHR, *Apis_dor*_GnRHIIR, *Apis_flo*_GnRHIIR, *M_rot*_GnRHIIR, and two in the others. In ICL2, there were one tyrosine and one threonine residue in the *M_qua*_GnRHIIR but only one tyrosine residues

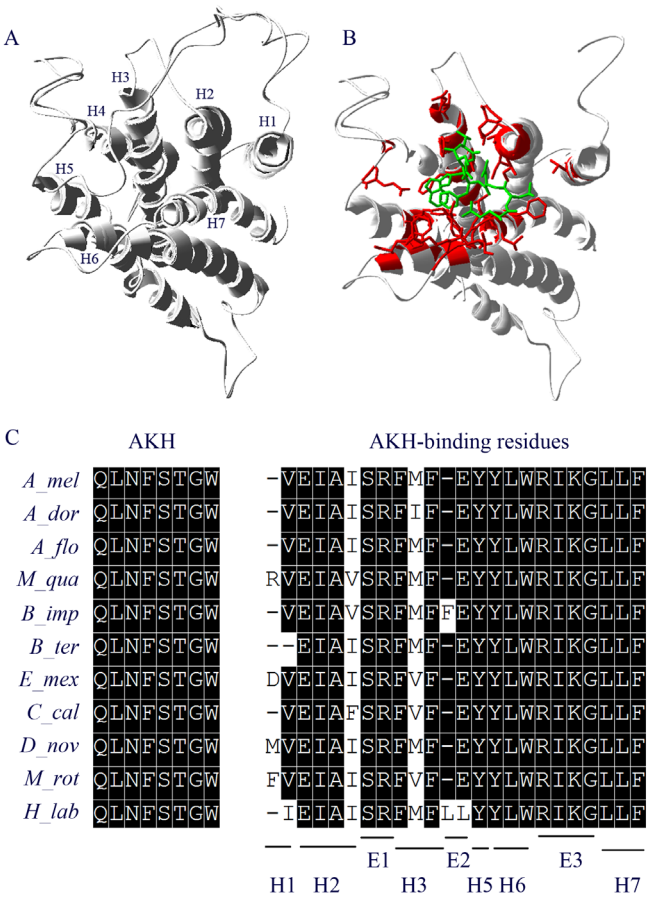

Fig 3. Structure and ligand-binding motifs of bee AKHRs. (A) Alignment of tertiary structures of AKHRs from *Apis mellifera*, *Apis dorsata*, *Apisflorea*, *Melipona quadrifasciata*, *Bombus impatiens*, *Bombus terrestris*, *Eufriesea mexicana, Ceratina calcarata, Dufourea novaeangliae*, *Megachile rotundata, Habropoda laboriosa*. The TM helices 1 to 7 (H1 to H7) were labeled sequentially. (B) AKH and the residues of binding pocket of *A_mel*_AKHR were labeled as green and red, respectively. (C) Sequence alignment of AKHs and the cognate binding pockets. The conserved sites (above 80%) were shaded. E: extracellular loop.

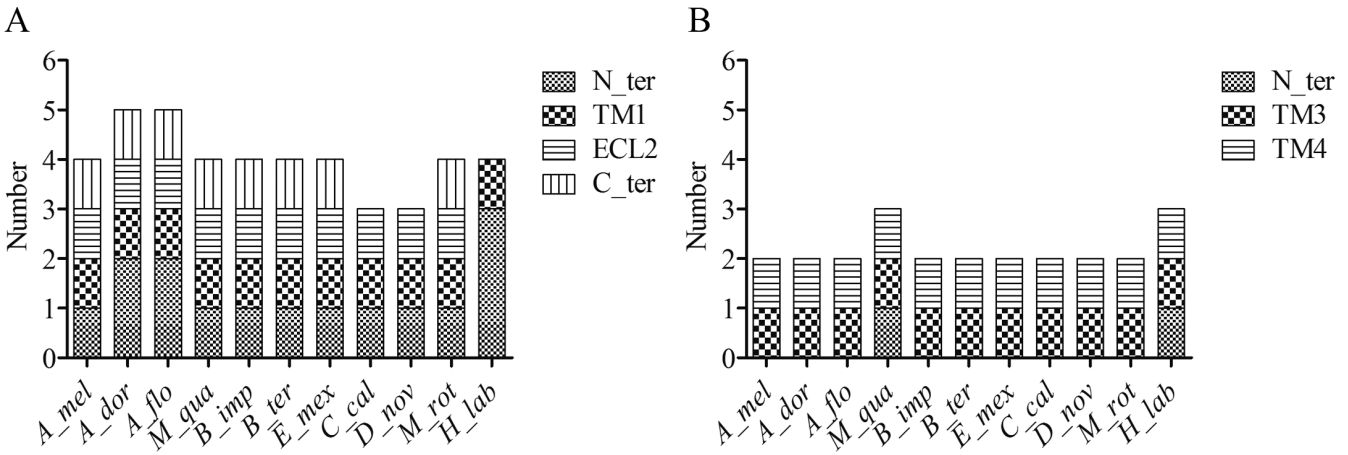

Fig 4. Location of predicted glycosylation and myristoylation sites. The number and distribution of glycosylation (A) and myristoylation (B) sites in the bee AKHRs were characterized. N_ter: N_terminus; C_ter: C_terminus.

in the others. For ICL3, there were five residues in GnRHIIR of *M_qua*, *E_mex*, *C_cal*, *D_nov*, *H_lab* and six in the others. As to the C_terminus, there were five sites in GnRHIIR of *H_lab* and *M_rot*, six in GnRHIIR of *D_nov* and *C_cal*, but seven in the others (Figure 5A, supplementary file 1). Furthermore, the statistics of potential kinase phosphorylation sites displayed similar patterns. Compared to the eusocial group, the solitary and subsocial group possessed more varieties in ICL1, but less in ICL2, ICL3 and C_terminus domains (Figure 5B, supplementary file 1).

Over all, the more social complexity, the more patterns on the potential phosphorylation modifications, especially in the C_terminus domain. For the kinase specific, PKC and unspecified kinase phosphorylation sites are the top two and localized in all the intracellular domains, while residues (tyrosine) phosphorylated by InsR just localized in ICL2 (Figure 5C, supplementary file 1).

Discussion

The current study demonstrated that the bee AKHRs shared a common primary structure which resulted in a uniform tertiary structure, but more varieties of kinase phosphorylation sites in the eusocial bees provided more complicated signal pathways in response to the eusocial life style.

Previously reports declared that the release of mature AKH in honeybee might be attenuate since the existence of a second TATA box in the promoter region of the gene and its significance might be largely lost (Veenstra et al., 2012). Recently, the neuropeptide RY amide in Drosophila has been thought to lose most of its physiological significance (Veenstra & Khammassi, 2017), while its receptor was still completely functional (Collin et al., 2011). Even though, the *Apis* AKH has been detected by mass spectrometry (Sturm et al., 2016), and the AKHR was still found to respond to the physiological change regardless of the carbohydrate or lipid metabolism (Woodard et al., 2011; Wang et al., 2012), which indicated that the activation and desensitization of AKHR and its physiological functions might be more complicated in response to the eusocial life.

As the members of the largest GPCR subfamily, the AKHRs of bees also share commonly conserved motifs, such as DRY in the beginning of ICL2, CWXP in the middle of TM6 and NPXXY in the end of TM7, and also a disulfide bond

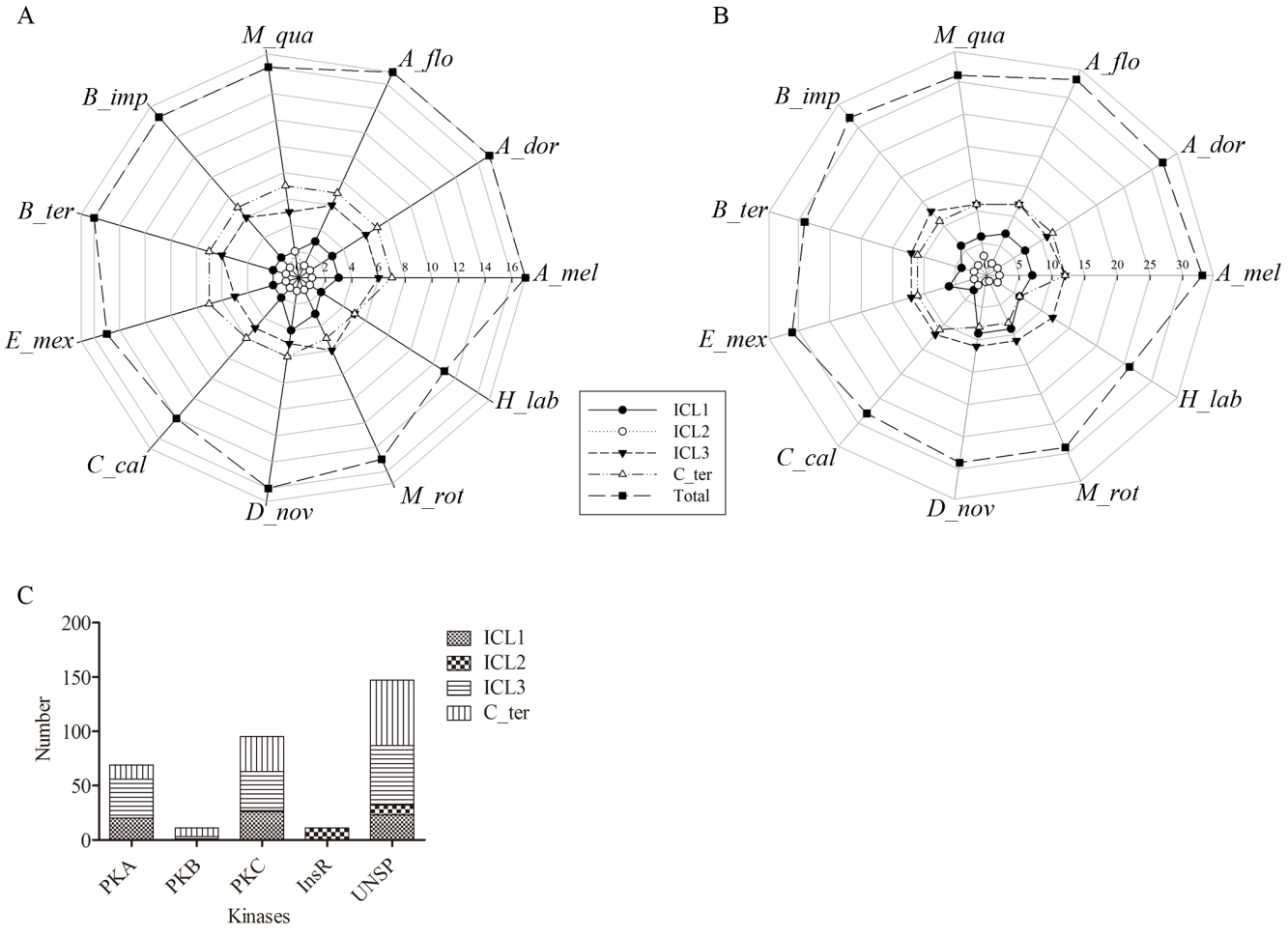

Fig 5. Phosphorylation features of AKHRs in the intracellular domains. Distribution of phosphorylation residues (Ser, Thr and Tyr) (A), varieties of potential protein kinase phosphorylation sites and their distribution (B and C) of bee AKHRs. PKA: Protein kinase A; PKB: Protein kinase B; PKC: Protein kinase C; InsR: Insulin receptor; UNSP: Unspecified kinase; ICL: Intracellular loop; C_ter: C_terminus.

between ECL1 and ECL2. All these motifs are essential for the structure stability, ligands binding and signal transduction (Lagerstrom & Schioth, 2008).

The phylogeny of bees based on AKHRs was consistent with their social complexity (Kapheim et al., 2015), which might imply that there were some differences contributing to the eusocial evolution since the evolutionary distances were computed based on the units of the number of amino acid substitutions per site (Tamura et al., 2013). However, the sequence alignment results demonstrated that AKHRs of bees shared an overall common sequence similarity besides the motifs discussed above, but less conserved N-termini, in which no relevance of eusocial evolution had been detected. Since the highly similar sequences, the tertiary structures derived by homology modelling are also highly conserved. What is more, the predicted ligand binding pockets share a common composition of amino acids with slight differences.

Besides the conserved motifs and structures, PTMs is an important mechanism for GPCRs functional regulation, such as the glycosylation for subcellular location, the myristoylation for structure stability, the palmitoylation at the C_terminus for the dimerization and the phosphorylation of the intracellular domain for signal transduction (Goddard & Watts, 2012; Norskov-Lauritsen & Brauner-Osborne, 2015; Norskov-Lauritsen et al., 2015). The number and localization of predicted glycosylation and myristoylation sites are similar among all examined bee AKHRs with few exceptions, which suggests that the modifications are essential. However, compared to *Anopheles gambiae* AKHR, no conserved cysteine residues emerged in the C_terminus domains of bee AKHRs. Previously report demonstrated that the palmitoylation of μ-opioid receptor (OPRM1) could occurred in the carboxyl end of transmembrane helix 3 (TM3) and it was crucial for receptor homodimerization and G protein coupling (Zheng et al., 2012). Here, whether the five conserved cysteine residues found in the TM3, TM4, TM6 and TM7 will be palmitoylated and its function remain to be clarified.

Compared to the essential PTMs discussed above, phosphorylation is a more flexible regulatory process for GPCRs. The recruitment of specific protein kinase in defined phosphorylation manner may tailor the signal pathways to a particular physiological role, such as the PKA/PKC for desensitization, GRK for arrestin recruitment and signaling, Insulin receptor (InsR) for tyrosine phosphorylation of SH2 domain, Akt/PKB for internalization (Tobin, 2008). Characterization of phosphorylation and internalization of silkworm AKHR demonstrated that GRK2/5 and β-arrestin2 were involved and three threonine residues localized in the C_terminus were responsible for (Huang et al., 2011). In the intracellular domains of bee AKHRs, phosphorylation sites of PKA, PKB, PKC, InsR and unspecified kinases were predicted which indicated that the receptor composed possibility of several signaling responses to defined physiological role. Overall, the eusocial species contained more phosphorylation sites than those in the solitary, especially in the most studied ICL3 and C_terminus domain. Recently, a "magic flute" model was confirmed that the arrestin could recognize various phosphorylation patterns of GPCRs through its phosphate-binding concave surface so as to contribute to the functional diversity of receptors (Yang et al., 2015). That reminds us that the increasing of phosphorylation sites might result in more various regulation of AKHRs so as to be suitable for the eusocial life style.

Conclusion

Previous publications have documented that AKH and AKHR appeared in all sequenced bee genomes and the lipid-dependence was various from the solitary to eusocial bees. Given that all the bees share the same AKH, some critical changes of structures or PTMs might occur in the AKHRs in response to the major transitions of social complexity. The results suggest that the bee AKHRs share highly conserved tertiary structures and ligand binding pockets. For the PTMs, the increasing phosphorylation residues and patterns might provide more various signal pathways with the enhancing of social complexity. It is anticipated that the potential functions and the relevant mechanisms of phosphorylation modifications will attract the most attention.

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Supplementary file

The predicted results of post-translational modifications of AKHRs are provided online as supplementary file.

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http://periodicos.uefs.br/index.php/sociobiology/rt/suppFiles/2098/0

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